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INVESTIGATION OF RESISTANCE MECHANISMS AND THEIR TRANSMISSION IN EMERGING PATHOGENS APPLYING THE «ONE HEALTH» APPROACH

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"What you do makes a difference, and you have to decide what kind of difference you want to make." – Jane Goodall

"Every great dream begins with a dreamer. Always remember, you have within you the strength, the patience, and the passion to reach for the stars to change the world." - Harriet Tubman

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Acronyms

Abbreviation	description
A	
A. fumigatus	*Aspergillus fumigatus*
ABC	Adenosine triphosphate-Binding Cassette
ABPA	Allergic Bronchopulmonary Aspergillosis
AmB	Amphotericin B
AMC	Antimicrobial Consumption
AMR	Antimicrobial Resistance
ANI	Average Nucleotide Identity
ARAF	Azole-resistant *A. fumigatus*
ARB	Antibiotic Resistant Bacteria
ARG	Antibiotic Resistance Genes
ATU	Area of Technical Uncertainty
B	
BAL	Bronchoalveolar Lavage
BPPL	Bacterial Priority Pathogens List
C	
CBP	Clinical Breakpoints
CCPA	Chronic Cavitary Pulmonary Aspergillosis
CFPA	Chronic Fibrosing Pulmonary Aspergillosis
cKP	Classical *Klebsiella pneumoniae*
CLSI	Clinical and Laboratory Standards Institute
COPD	Chronic Obstructive Pulmonary Disease
CP	Carbapenemase
CPA	Chronic Pulmonary Aspergillosis
CPKP	Carbapenemase-producing *K. pneumoniae*
CSF	Cerebrospinal Fluid
Cyp51	14-alfa-sterol-demethylase
Cyp51A	14-alpha-sterol-demethylase A
Cyp51B	14-alpha-sterol-demethylase B
D	
DBO	diazabicyclooctanone
DDD	Defined Daily Doses
DHFR	Dihydrofolate Reductase
DHPS	Dihydropteroate Synthase

DIDs	Defined Daily Doses per 1,000 inhabitants per day
DMI	Demethylation Inhibitor
DNA	Deoxyribonucleic Acid
E	
E. coli	*Escherichia coli*
EARS-BE	European (EU) Antimicrobial Resistance Surveillance Network for Belgium
EARS-Net	European Antimicrobial Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control
ECHA	European Chemicals Agency
ECOFFs	Epidemiological Cut-off values
EEA	European Environment Agency
EFSA	European Food Safety Authority
EMA	European Medicines Agency
EORTC/MSGERC	European Organization for Research and Treatment of Cancer and Mycosis Study Group Education and Research Consortium
ESAC-Net	European Surveillance of Antimicrobial Consumption Network
ESCMID-ECMM-ERS	European Society for Clinical Microbiology and Infectious Diseases, the European Confederation of Medical Mycology and the European Respiratory Society
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EUVSEC	EU Surveillance *Salmonella*/ *E. coli*
F	
FAO	Food and Agriculture Organization of the United Nations
FASFC	Federal Agency for Safety of the Food Chain
FPPL	Fungal Priority Pathogens List
H	
HGT	Horizontal Gene Transfer
hvKp	Hypervirulent *Klebsiella pneumoniae*
I	
IA	Invasive Aspergillosis
ICE	Integrated and Conjugative Elements
IFD	Invasive Fungal Disease
IPA	Invasive Pulmonary Aspergillosis
IS	Insertion Sequences
ISA	Isavuconazole
ITC	Itraconazole

J	
JIACRA	joint inter-agency report on integrated analysis of antimicrobial agent consumption and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals
JRC	Joint Research Centre
K	
K. pneumoniae	*Klebsiella pneumoniae*
KpSC	*Klebsiella pneumoniae* species complex
L	
L-AmB	Liposomal Amphotericin B
LCR	Ligase chain reaction
LPS	Lipopolysaccharide
M	
MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry
MAT	Mating Types
MC	Malt Chloramphenicol
MC+T	Malt Chloramphenicol + Tebuconazole
McC	McConkey
McC + CTX	McConkey + cefotaxim
mcr	Mobile Colistin Resistance
MDR	Multidrug Resistant
MGE	Mobile Genetic Elements
MIC	Minimal Inhibitory Concentration
MRGN	Multi-Resistant Gram-Negative bacilli
MSI	Mass Spectrometry Identification platform
N	
NA	Nutrient Agar
nif	Nitrogen-fixing operon
NIHDI	National Institute for Health and Disability Insurance
non-WT	Non-wildtype
NSIH	National Surveillance of Infections in Healthcare settings
NSIH-AMR	National Surveillance of AMR
O	
OHHLEP	One Health High Level Expert Panel
P	

PBP	Penicillin-Binding Protein
PCR	Polymerase Chain Reaction
pEtN	Phosphoethanolamine
POS	Posaconazole
PPS	Point-Prevalence Studies
R	
R&D	Research and Development
S	
SAIA	Subacute Invasive Aspergillosis
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SBI	Sterol-Biosynthesis Inhibiting
SNP	Single Nucleotide Polymorphisms
ss	Sensu stricto
T	
TB	Tuberculosis
Teb	Tebuconazole
Tn	Transposons
TR	Tandem repeat
V	
VOR	Voriconazole
W	
WGS	Whole Genome Sequencing
WHO	World Health Organization
WT	Wild-type
X	
XDR	Extremely Drug-Resistant

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Summary

This thesis aimed to explore antimicrobial resistance in Belgium in two critical clinical pathogens, *Klebsiella pneumoniae* and *Aspergillus fumigatus*, using a One Health approach. The One Health perspective emphasizes the interconnections between human, animal, and environmental health to address global health challenges like antimicrobial resistance (AMR). AMR occurs when microorganisms acquire resistance through genetic mutations or horizontal gene transfer, posing a global health crisis. *K. pneumoniae* is a leading cause of AMR-related deaths and has been identified as a priority pathogen by the world health organization (WHO) due to its resistance to carbapenems and 3rd-generation cephalosporins. Similarly, *A. fumigatus* is a critical fungal pathogen, with antifungal resistance exacerbated by cross-use of antifungals in healthcare and agriculture. Both pathogens highlight the necessity of a One Health approach to comprehensively manage AMR, ensuring health interventions in one domain (human, animal, or environmental) do not undermine others. This thesis underscores the urgent need for global strategies to combat the growing threat of AMR in these pathogens.

The study is divided into five parts. The first section aimed at identifying the prevalence of *K. pneumoniae* in the food chain and in wastewater. Following this, the occurrence of antibiotic resistance in *K. pneumoniae* across the food chain, diseased animals, wastewater, and the clinical setting was investigated. Here, we mainly focused on the presence of *K. pneumoniae* resistant to third generation cephalosporins (3GC) and carbapenems. Whole genome sequencing was then conducted on a subset of *K. pneumoniae* isolates from all sectors to explore the resistance mechanisms and the potential transmission and genetic relatedness between sectors in the One Health framework. In the second study we describe a rare resistance mechanism that was linked to ertapenem resistance in a *K. pneumoniae* strain from a companion animal and elucidated the resistance mechanism using both short and long read sequencing techniques. For the third study, the prevalence of azole-resistant *Aspergillus fumigatus* in various environmental settings (agriculture, horticulture, and composting facilities) was analyzed to identify potential hotspots for azole resistance selection. Additionally, the occurrence of azole resistance in *A. fumigatus* from veterinary clinical samples, including food producing animals, pets and zoo animals, was examined in the fourth study. Lastly, a study was conducted to investigate the link between the environment and a high-risk Humboldt penguin population in a zoological setting.

K. pneumoniae was detected in 18.6% of meat samples at the distribution level compared to 1.7% in healthy food producing animals. In 35.6% of wastewater samples *K. pneumoniae* was detected. Antimicrobial susceptibility testing (AST) revealed moderate to high resistance rates to azithromycin, high to extremely high resistance rates to ciprofloxacin and cefotaxime and low to moderate colistin resistance in human clinical and wastewater isolates. High rates of ertapenem resistance were observed

in isolates resistant to third generation cephalosporins and carbapenems. Whole-genome sequencing identified significant genetic diversity among 286 isolates, with 141 distinct sequence types, including high-risk MDR clonal groups like CG258, CG15, CG147, and CG307. Transmission pathways were observed both within and across sectors, with notable clusters involving *K. pneumoniae* ST147 from the food chain at distribution level and wastewater, suggesting contamination during meat processing. In the second study, the *K. pneumoniae* strain (ST11) was isolated from a companion animal and showed resistance to critically important antibiotics, including, azithromycin, cefotaxime, ciprofloxacin, gentamicin and ertapenem. The strain carried three plasmids, including the IncFIB(K) plasmid harboring multiple antibiotic resistance genes (ARG's), such as $bla_{\text{SCO-1}}$, and the IncR plasmid with the $bla_{\text{DHA-1}}$ gene. A mutation in outer membrane protein *OmpK37* was observed. These genetic elements contributed to its ertapenem resistance. In the third study, environmental samples were collected in Belgium, including agriculture, horticulture, and composting facilities, to detect *A. fumigatus* and assess its azole resistance. Composting facilities showed a high prevalence of airborne *A. fumigatus* spores, though none were found in mature compost samples. In total, two colonies from composting facilities showed resistance to medical azoles. In horticulture, 4 azole resistant *A. fumigatus* (ARAf) colonies were isolated. Agriculture was confirmed as a coldspot. Across all samples, a prevalence of ARAf of 2.62% was detected, with TR34/L98H and TR46/Y121F/T289A mutations present in the resistant isolates. In study 4 and 5, azole resistance was assessed in veterinary clinical isolates. In study 4, isolates from food producing animals, pets, wild birds and the zoo were included. Azole resistance testing revealed five resistant isolates (3.3%), including two from pigeons, two from cows, and one from a cat. Three isolates exhibited pan-azole resistance, with the TR34/L98H mutation in the *cyp51A* gene found in 80% of the resistant cases. In study 5, environmental sampling inside a penguin enclosure revealed (non-significant) seasonal fluctuations in *A. fumigatus* contamination in the air. Between 2017 and 2022, 189 Humboldt penguins died, with 51 cases confirmed as pulmonary aspergillosis. Resistance was observed in 17.14% of clinical and 11.76% of environmental strains, mainly associated with the TR34/L98H mutation in the *cyp51A* gene. Microsatellite genotyping showed no identical genotypes between veterinary isolates, but closely related genotypes were found between some environmental and veterinary strains, suggesting possible transmission.

The study emphasizes the importance of the One Health approach in tackling AMR, as it demonstrates the interconnectedness of human, animal, and environmental health. Clonal transmission of *K. pneumoniae* within and across sectors highlights the need for coordinated surveillance and intervention strategies to mitigate the spread of resistant strains. Furthermore, we confirmed that the environment is the main driver behind azole resistance selection in *A. fumigatus* and found the TR34/L98H to be the most prevalent mutation. Further research is necessary to identify hotspots in Belgium. The implementation of standardization in surveillance methods is crucial.

Résumé

Cette thèse a pour objectif d'explorer la résistance aux antimicrobiens (RAM) en Belgique chez deux pathogènes critiques, *Klebsiella pneumoniae* et *Aspergillus fumigatus*, en adoptant une approche One Health. Cette perspective met l'accent sur les interconnexions entre la santé humaine, animale et environnementale pour relever des défis mondiaux tels que la résistance aux antimicrobiens. La RAM survient lorsque des micro-organismes acquièrent une résistance par des mutations génétiques ou transferts horizontaux de gènes, constituant ainsi une crise sanitaire mondiale. *K. pneumoniae* est une cause majeure de décès liés à la RAM et a été identifié comme un pathogène prioritaire par l'Organisation Mondiale de la Santé en raison de sa résistance aux carbapénèmes et aux céphalosporines de troisième génération. De même, *A. fumigatus* est un pathogène fongique critique dont la résistance aux antifongiques est exacerbée par l'utilisation croisée de ces derniers en médecine et en agriculture. Ces deux pathogènes illustrent la nécessité d'une approche *One Health* pour gérer la RAM de manière globale, garantissant que les interventions de santé dans un domaine ne compromettent pas les autres. Cette thèse met en lumière l'urgence de stratégies mondiales pour combattre la menace croissante de la RAM chez ces pathogènes.

L'étude se compose de cinq études. La première partie vise à déterminer la prévalence de *K. pneumoniae* dans la chaîne alimentaire et dans les eaux usées. Ensuite, l'occurrence de la RAM chez *K. pneumoniae* dans la chaîne alimentaire, les animaux malades, les eaux usées et le cadre clinique a été examinée, avec un accent particulier sur la résistance aux céphalosporines de troisième génération (3GC) et aux carbapénèmes. Un séquençage génomique complet a ensuite été réalisé sur une sélection d'isolats de *K. pneumoniae* pour explorer les mécanismes de résistance, les transmissions potentielles et la parenté génétique. La deuxième étude décrit un mécanisme de résistance rare à l'ertapénème dans une souche de *K. pneumoniae* provenant d'un animal de compagnie, élucidé à l'aide de techniques de séquençage court et long. Dans la troisième étude, la prévalence d'*A. fumigatus* résistant aux azoles a été analysée dans divers milieux environnementaux (agriculture, horticulture et installations de compostage) pour identifier des foyers potentiels de sélection de la résistance. Dans la quatrième étude, la résistance aux azoles d'*A. fumigatus* provenant d'échantillons vétérinaires a été examinée. Enfin, une cinquième étude a exploré le lien entre l'environnement et une population de manchots de Humboldt en captivité dans un zoo.

K. pneumoniae a été détecté dans 18,6 % des échantillons du secteur de la distribution, contre 1,7 % dans la production primaire. Dans 35,6 % des échantillons d'eaux usées, *K. pneumoniae* était présent. Les tests de sensibilité antimicrobienne ont révélé des taux de résistance modérés à élevés à l'azithromycine, des taux de résistance élevés à extrêmement élevés à la ciprofloxacine et au céfotaxime, et une résistance faible à modérée à la colistine dans les isolats cliniques humains et les eaux usées. Des taux élevés de résistance à l'ertapénème ont été observés chez les isolats résistants aux 3GC et aux carbapénèmes. Le séquençage complet du génome a révélé une diversité génétique significative parmi

286 isolats, identifiant 141 types de séquences distincts, incluant des groupes clonaux à haut risque tels que CG258, CG15, CG147 et CG307. Des voies de transmission ont été observées au sein et entre les secteurs, avec des clusters notables impliquant *K. pneumoniae* ST147 provenant de la chaîne alimentaire et des eaux usées, suggérant une contamination lors de la transformation de la viande. Dans la deuxième étude, la souche de *K. pneumoniae* (ST11) isolée d'un animal de compagnie a montré une résistance à plusieurs antibiotiques, dont l'azithromycine, la céfotaxime, la ciprofloxacine, la gentamicine et l'ertapénème. Cette souche portait trois plasmides, dont le plasmide IncFIB(K) contenant le bla_{SCO-1}, et le plasmide IncR avec le gène bla_{DHA-1}. Une mutation dans la protéine de membrane externe *OmpK37* a été identifiée, contribuant à son profil de résistance. Dans la troisième étude, des échantillons environnementaux ont été prélevés dans l'agriculture, l'horticulture et les installations de compostage pour détecter *A. fumigatus* et évaluer sa résistance aux azoles. Les installations de compostage ont montré une prévalence élevée de spores d'*A. fumigatus* dans l'air, bien qu'aucune n'ait été détectée dans les échantillons de compost. Au total, deux colonies provenant des installations de compostage ont montré une résistance aux azoles. En horticulture, quatre colonies résistantes aux azoles ont été isolées, tandis que l'agriculture a été confirmée comme un *coldspot*. Sur tous les échantillons, une prévalence de 2,62 % d'*A. fumigatus* résistant aux azoles (ARAf) a été détectée, avec des mutations TR34/L98H et TR46/Y121F/T289A présentes. Dans les études 4 et 5, la résistance aux azoles a été évaluée dans les isolats cliniques vétérinaires. Dans l'étude 4, des isolats provenant d'animaux de production, d'animaux de compagnie, d'oiseaux sauvages et de zoos ont été inclus. Les tests de résistance aux azoles ont révélé cinq isolats résistants (3,3 %). Trois isolats présentaient une résistance pan-azole, avec la mutation TR34/L98H étant retrouvée dans 80 % des cas de résistance. Dans l'étude 5, l'échantillonnage environnemental à l'intérieur d'un enclos de manchots a révélé des fluctuations saisonnières (non significatives) dans la contamination de l'air par *A. fumigatus*. Entre 2017 et 2022, 189 manchots de Humboldt sont morts, avec 51 cas confirmés d'aspergillose pulmonaire. Une résistance a été observée dans 17,14 % des souches cliniques et 11,76 % des souches environnementales, principalement associée à la mutation TR34/L98H dans le gène *cyp51A*. Le génotypage microsatellite n'a révélé aucun génotype identique entre les isolats vétérinaires, mais des génotypes étroitement liés ont été identifiés entre certaines souches environnementales et vétérinaires.

Cette étude souligne l'importance de l'approche *One Health* dans la lutte contre la RAM, en démontrant l'interconnexion entre la santé humaine, animale et environnementale. La transmission clonale de *K. pneumoniae* au sein et entre les secteurs met en évidence la nécessité de stratégies de surveillance et d'intervention coordonnées pour atténuer la propagation des souches résistantes. De plus, nous avons confirmé que l'environnement est le principal moteur de la sélection de la résistance aux azoles chez *A. fumigatus*, avec la mutation TR34/L98H étant la plus fréquente. Des recherches supplémentaires sont nécessaires pour identifier les *hotspots* en Belgique. La mise en œuvre de la standardisation des méthodes de surveillance est cruciale.

Samenvatting

Dit doctoraatsproject had als doel om antimicrobiële resistentie (AMR) in België te onderzoeken bij twee kritieke pathogenen, *Klebsiella pneumoniae* en *Aspergillus fumigatus*, met behulp van de One Health-benadering. Het One Health-perspectief benadrukt de onderlinge verbindingen tussen menselijke, dierlijke en milieugezondheid om wereldwijde gezondheidsuitdagingen zoals AMR aan te pakken. AMR ontstaat wanneer micro-organismen resistentie verwerven door genetische mutaties of horizontale genoverdracht, wat een wereldwijde gezondheidscrisis veroorzaakt. *K. pneumoniae* is een belangrijke oorzaak van AMR-gerelateerde sterfgevallen en is door de WHO geïdentificeerd als een prioritair pathogeen vanwege de resistentie tegen carbapenems en derde generatie cefalosporinen. Evenzo is *A. fumigatus* een kritisch schimmelpathogeen, waarbij antischimmelresistentie verergert door het kruisgebruik van antischimmelmiddelen in de gezondheidszorg en de landbouw. Beide pathogenen benadrukken de noodzaak van een One Health-benadering om AMR uitgebreid te beheersen, waarbij wordt gegarandeerd dat gezondheidsinterventies in het ene domein (mens, dier of milieu) de andere niet ondermijnen. Deze thesis onderstreept de dringende noodzaak van mondiale strategieën om de groeiende dreiging van AMR bij deze pathogenen te bestrijden.

Deze thesis is verdeeld in vijf studies. De eerste studie was gericht op het identificeren van de prevalentie van *K. pneumoniae* in de voedselketen en in afvalwater. Vervolgens werd het voorkomen van antibioticaresistentie in *K. pneumoniae* in de voedselketen, zieke dieren, afvalwater en de klinische setting onderzocht. Hier lag de focus voornamelijk op de aanwezigheid van *K. pneumoniae* die resistent is tegen derde generatie cefalosporinen (3GC) en carbapenems. Vervolgens werd op een subset van *K. pneumoniae*-isolaten whole genome sequencing uitgevoerd om de resistentiemechanismen en mogelijke overdracht tussen sectoren te onderzoeken. In de tweede studie beschrijven we een zeldzaam resistentiemechanisme dat verband houdt met ertapenemresistentie in een *K. pneumoniae*-stam van een gezelschapsdier en verduidelijkten we het mechanisme met behulp van zowel korte als lange sequentietechnieken. In de derde studie werd de prevalentie van azool-resistente *A. fumigatus* in verschillende omgevingsinstellingen (landbouw, tuinbouw en composteerinstallaties) geanalyseerd om mogelijke hotspots voor de selectie van azoolresistentie te identificeren. Daarnaast werd het voorkomen van azoolresistentie in *A. fumigatus* uit veterinaire monsters, waaronder productiedieren, gezelschapsdieren en dieren uit de dierentuin, onderzocht in de vierde studie. Ten slotte werd een studie uitgevoerd om de link tussen het milieu en een Humboldt-pinguïnpopulatie in een dierentuin te onderzoeken.

K. pneumoniae werd gedetecteerd in 18,6% van de monsters op distributieniveau vergeleken met 1,7% in de primaire productie. In 35,6% van de afvalwatermonsters werd *K. pneumoniae* gedetecteerd. Antimicrobiële gevoeligheidstests toonden matige tot hoge resistentieniveaus tegen azitromycine, hoge tot extreem hoge resistentieniveaus tegen ciprofloxacine en cefotaxime en lage tot matige colistine-resistentie in klinische en afvalwaterisolaten. Hoge percentages ertapenemresistentie

werden waargenomen in isolaten die resistent zijn tegen 3GC en carbapenems. Whole-genome sequencing identificeerde enorme genetische diversiteit onder 286 isolaten, met 141 verschillende sequentietypes, waaronder risicovolle clonale groepen zoals CG258, CG15, CG147 en CG307. Transmissie werd waargenomen binnen en tussen sectoren, met een opvallende cluster met *K. pneumoniae* ST147 uit de voedselketen en afvalwater, wat suggereert dat er besmetting is opgetreden tijdens vleesverwerking. In de tweede studie werd de *K. pneumoniae*-stam (ST11) geïsoleerd uit een gezelschapsdier en toonde resistentie tegen verschillende antibiotica, waaronder azithromycine, cefotaxime, ciprofloxacine, gentamicine en ertapenem. De stam droeg drie plasmiden, waaronder het IncFIB(K)-plasmide met $bla_{\text{SCO-1}}$, en het IncR-plasmide met het $bla_{\text{DHA-1}}$-gen. Een mutatie in het buitenmembraaneiwit *OmpK37* werd waargenomen. Dit alles droeg bij aan zijn resistentieprofiel. In de derde studie werden omgevingsmonsters verzameld in België, waaronder landbouw, tuinbouw en composteerinstallaties, om *A. fumigatus* te detecteren en de azoolresistentie te beoordelen. Een hoge prevalentie van *A. fumigatus*-sporen in composteerinstallaties werd genoteerd, hoewel er geen in compostmonsters werden gevonden. In totaal waren twee kolonies resistent. In de tuinbouw werden 4 azool-resistente kolonies geïsoleerd. Landbouw werd bevestigd als een *coldspot*. In alle monsters werd een prevalentie van ARAf van 2,62% gedetecteerd, met TR34/L98H- en TR46/Y121F/T289A-mutaties in de resistente isolaten. In studie 4 en 5 werd azoolresistentie beoordeeld in veterinaire klinische isolaten. In studie 4 werden isolaten uit productiedieren, gezelschapsdieren, wilde vogels en de dierentuin opgenomen. Azoolresistentietests onthulden vijf resistente isolaten (3,3%), waaronder twee van duiven, twee van koeien en één van een kat. Drie isolaten vertoonden pan-azoolresistentie, met de TR34/L98H-mutatie in het cyp51A-gen in 80% van de resistente gevallen. In studie 5 toonden omgevingsmonsters in een pinguïnverblijf (niet-significante) seizoensgebonden schommelingen in *A. fumigatus*-besmetting in de lucht. Tussen 2017 en 2022 stierven 189 Humboldt-pinguïns, waarbij 51 gevallen werden bevestigd als pulmonale aspergillose. Resistentie werd waargenomen in 17,14% van de klinische en 11,76% van de omgevingsstammen, voornamelijk geassocieerd met de TR34/L98H-mutatie in het *cyp51A*-gen. Microsatelliet-genotypering toonde geen identieke genotypen tussen veterinaire isolaten, maar nauw verwante genotypen werden gevonden tussen enkele omgevings- en veterinaire stammen, wat mogelijk wijst op overdracht.

De studie benadrukt het belang van de One Health-benadering bij het aanpakken van AMR, aangezien het de onderlinge verbondenheid van menselijke, dierlijke en milieugezondheid aantoont. Clonale overdracht van *K. pneumoniae* binnen en tussen sectoren onderstreept de noodzaak van gecoördineerde bewakings- en interventiestrategieën om de verspreiding van resistente stammen te beperken. Bovendien hebben we bevestigd dat het milieu de belangrijkste drijfveer is achter de selectie van azoolresistentie in *A. fumigatus* en vonden we dat de TR34/L98H-mutatie de meest voorkomende was. Verder onderzoek is nodig om hotspots in België te identificeren. De implementatie van standaardisatie in bewakingsmethoden is cruciaal.

General preamble

Research on the critical pathogens *Klebsiella pneumoniae* and *Aspergillus fumigatus* within the One Health sector is vital due to the interconnected nature of human, animal, and environmental health in the development and spread of antimicrobial resistance (AMR). These pathogens pose a significant global health threat and studying them within this comprehensive framework provides several key benefits. Furthermore, data on the prevalence of these pathogens in veterinary health, the food chain and environmental health is lacking in Belgium.

Firstly, both pathogens are known for their increasing resistance to critical drugs. *K. pneumoniae* is highly resistant to carbapenems and third-generation cephalosporins, while *A. fumigatus* has developed resistance to azole antifungals, which are crucial for treating *A. fumigatus*. This resistance complicates treatment options, making infections harder to manage, and heightens the urgency to develop new strategies for combating these pathogens. Secondly, these pathogens affect not only human health but also animals and the environment. *K. pneumoniae* can be found in hospitals, animal farms, and wastewater, while *A. fumigatus* is widely present in the environment, with hotspots in green waste management environments and plant bulb waste. Research within a One Health approach allows us to understand how these different sectors contribute to the evolution and transmission of AMR. By looking at the broader context, we can identify key points of intervention that might be missed in siloed approaches. Another critical aspect is understanding the transmission pathways between sectors. *K. pneumoniae* may spread from livestock to humans via the food chain or through environmental contamination, such as wastewater, while *A. fumigatus* can develop resistance due to the widespread use of antifungals in agriculture and infect the susceptible population. This cross-sectoral research helps to map out how these pathogens move between humans, animals, and the environment, revealing opportunities for more effective containment strategies, protecting both public and animal health.

In the first main part of the thesis, research on the prevalence of (antimicrobial-resistant) *K. pneumoniae* in the different sectors of the One Health framework was performed. Using whole genome sequencing (WGS), we elucidated the resistance mechanisms responsible for phenotypical resistance, and we elucidated clusters within and across sectors of the One Health framework. In the second part of the thesis, we focused on identifying the prevalence of azole-resistant *A. fumigatus* in the environmental and animal sector. Sequencing was performed to elucidate the resistance mechanisms, which proved to be mainly from the environmental route of resistance selection. We identified some shortcomings in our research and included these in the project proposal for the upcoming National Action Plan on Antimicrobial Resistance (NAP AMR 2025-2029).

In summary, studying *A. fumigatus* and *K. pneumoniae* within the One Health framework is crucial for developing comprehensive solutions to combat AMR. This approach not only addresses the immediate health risks but also helps prevent future outbreaks of resistant infections by recognizing the interconnectedness of human, animal, and environmental health.

CHAPTER 1 – INTRODUCTION

1 *One Health in antimicrobial resistance in bacteria and fungi*

This thesis aimed at filling some knowledge gaps about the occurrence of antimicrobial resistance in two critical pathogens in Belgium: the bacterium *Klebsiella pneumoniae* and the fungus *Aspergillus fumigatus*. This research was tackled using the One Health perspective.

One Health is a comprehensive, integrative approach aimed at sustainably balancing and optimizing the health of people, animals, and ecosystems (Figure 1). It acknowledges the interdependence of the health of humans, domestic and wild animals, plants, and the wider environment (The Food and Agriculture Organization of the United Nations (FAO) et al., 2021). This approach encourages collaboration across various sectors and disciplines to address health challenges such as infectious diseases, antimicrobial resistance (AMR), and food safety, while promoting ecosystem integrity. By linking humans, animals, and the environment, One Health addresses the full spectrum of disease control, from prevention to management, enhancing global health security. It operates at multiple levels - community, national, regional, and global - relying on effective governance, communication, and coordination (The Food and Agriculture Organization of the United Nations (FAO) et al., 2021) .The United Nations Quadripartite and regional entities like the European Union advocate for the One Health approach, emphasizing the interconnectedness of human, animal, and environmental health in managing the global AMR crisis (FAO et al., 2022; The Food and Agriculture Organization of the United Nations (FAO) et al., 2021).

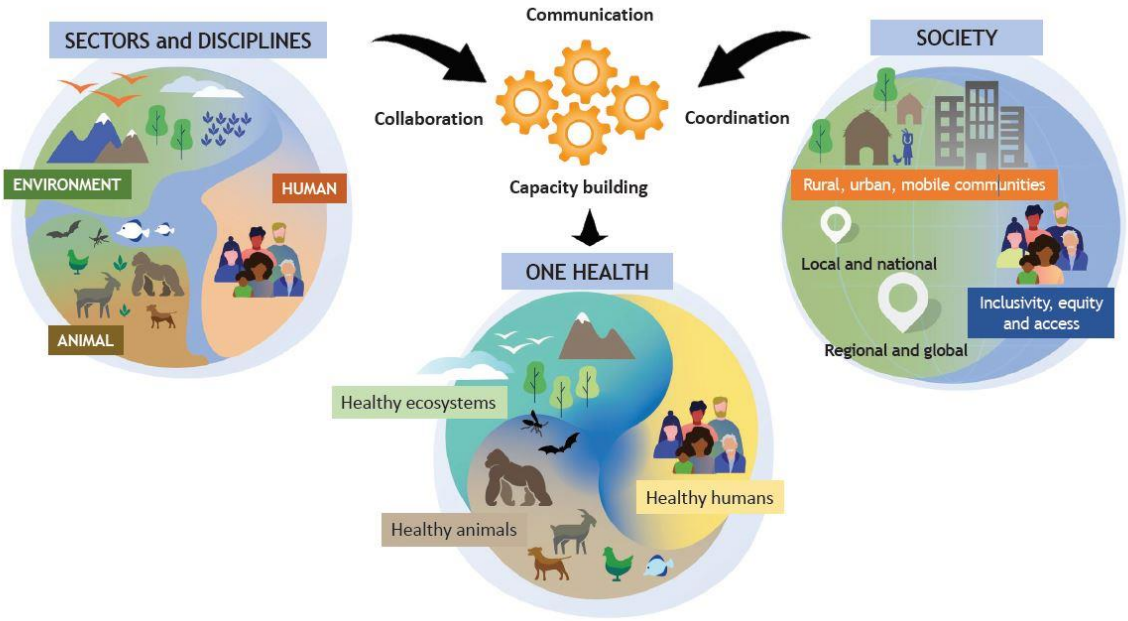


Figure 1 - The One Health definition by the OHHLEP integrates the health of humans, animals, plants and the wider environment. The approach includes multiple sectors, disciplines and communities to tackle threats to health and ecosystems (The Food and Agriculture Organization of the United Nations (FAO) et al., 2021).

Antimicrobial resistance can occur when microorganisms acquire genetic information, either by mutation, recombination or horizontal gene transfer (HGT) of antibiotic resistance genes (ARGs) (Djordjevic et al., 2024). AMR is a leading cause of death globally, especially in low-resource settings, with an estimated 4.95 million deaths associated with bacterial AMR in 2019 (Murray et al., 2022). The six leading pathogens responsible for deaths associated with antimicrobial resistance in 2019 were *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*. These pathogens were attributed to 929,000 (660,000–1,270,000) deaths directly due to AMR and 3.57 million (2.62–4.78 million) deaths associated with AMR (Murray et al., 2022).

In 2017, the World Health Organization (WHO) published its first Bacterial Priority Pathogens list (BPPL) (Figure 2) of antibiotic-resistant bacteria to guide research and development (R&D) of new antibiotics (Tacconelli et al., 2018). This was updated in 2024 where gram-negative bacterial pathogens remain a critical priority (World Health Organization, 2024). The pathogens were grouped according to the species and the type of resistance and then stratified in three priority tiers: critical, high and medium (World Health Organization, 2024). Carbapenem-resistant and 3rd generation cephalosporin-resistant *Enterobacterales*, including *Klebsiella pneumoniae* (*K. pneumoniae*) were taken up into the critical group. In 2017, 3rd generation cephalosporin-resistant *K. pneumoniae* and carbapenem-resistant *K. pneumoniae* were ranked as third and fifth, respectively, on the BPPL (Tacconelli et al., 2018). In the recent version of 2024, CRKP was ranked first and *K. pneumoniae* resistant to third generation cephalosporins (3GCs) as sixth in the BPPL (World Health Organization, 2024).

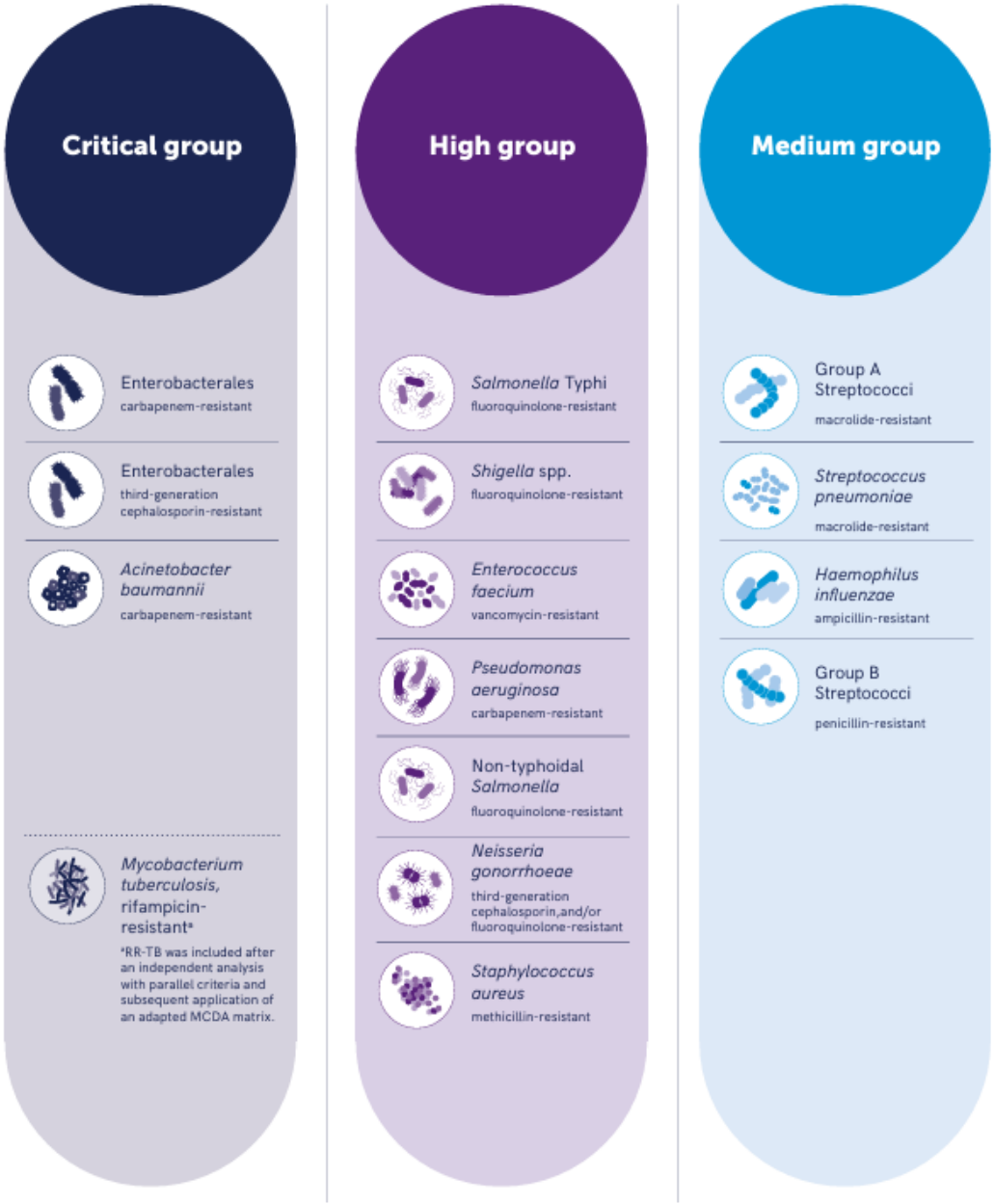


Figure 2 - WHO Bacterial Priority Pathogens list, 2024 (World Health Organization, 2024)

Antifungal drugs are vital for treating infections in humans and animals, and fungicides protect agricultural crops. However, the limited variety of antifungal agents leads to their cross-use in agriculture and health, fostering the development of resistance. Resistant strains in the environment exhibit resistance to the same antifungals used in medical treatments, complicating effective clinical care. This interconnection underscores the necessity of a One Health approach to address fungal diseases and antifungal resistance, ensuring that treatment and protection in one area do not compromise the health of plants, animals, or humans in other areas (Woods et al., 2023).

In 2022, the WHO published the fungal priority pathogens list (FPPL) (Figure 3) to guide R&D and public health action (World Health Organization, 2022). Here, priorities were based on public health importance and antifungal resistance. Of the 19 fungal pathogens included, *Aspergillus fumigatus* was included in the critical group (World Health Organization, 2022).

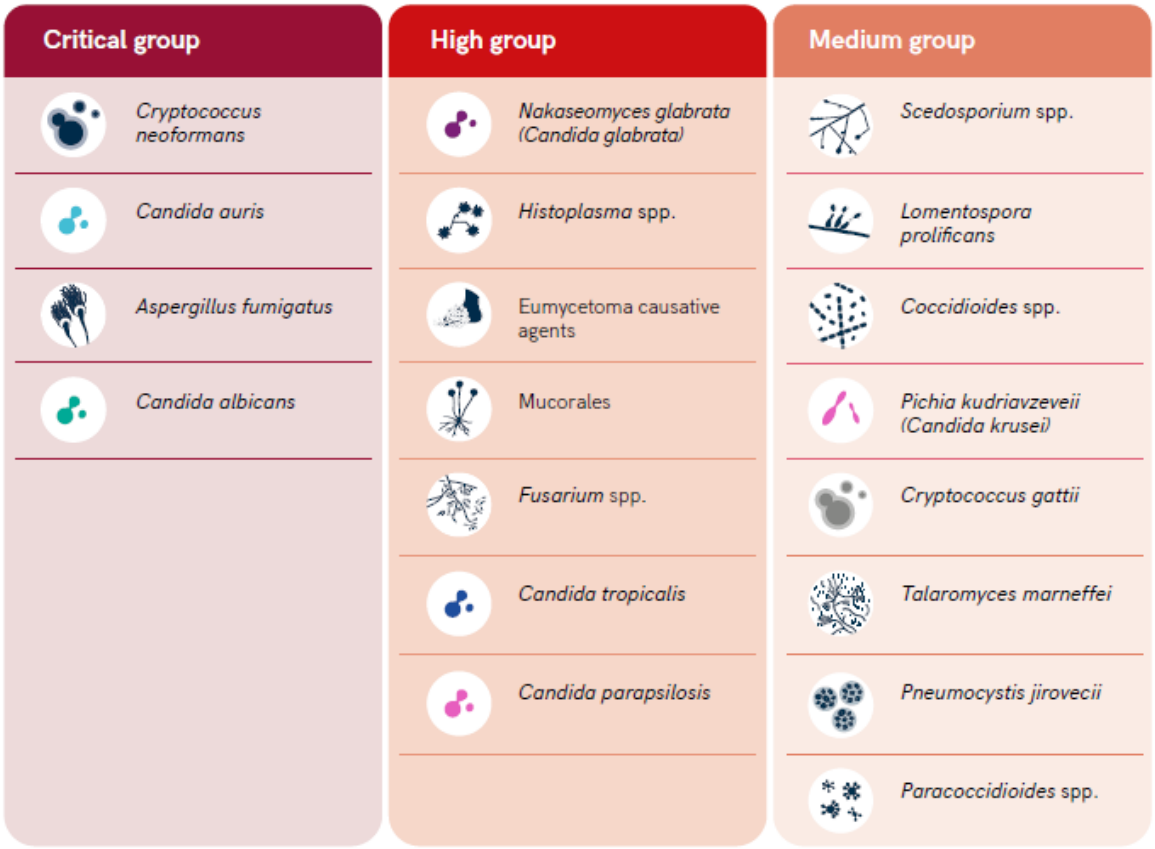


Figure 3 - WHO Fungal Priority Pathogens List (World Health Organization, 2022)

Research on the critical pathogens *A. fumigatus* and *K. pneumoniae* within the One Health framework is essential due to their AMR threat and their impact on human, animal, and environmental health. Both pathogens are developing resistance to key drugs - *K. pneumoniae* to carbapenems and third-generation cephalosporins, and *A. fumigatus* to azole antifungals - complicating treatment. The One Health approach helps to understand how AMR develops and spreads across sectors, such as hospitals, animal farms, and environmental settings, and identifies transmission pathways between humans, animals, and the environment. This comprehensive view is crucial for coordinated strategies to mitigate the spread of AMR and protect public health.

2 Klebsiella pneumoniae

Klebsiella pneumoniae is a member of the Enterobacteriaceae family, which also includes notable genera such as *Salmonella* spp. and *Escherichia* spp. (Adeolu et al., 2016). *K. pneumoniae* has been recognized as a disease-causing bacterium since it was first identified by Carl Friedländer in 1882 as a cause of pneumonia, and it continues to be one of the most prevalent nosocomial pathogens worldwide (Pendleton et al., 2013). *K. pneumoniae* is a Gram-negative, rod-shaped, facultative anaerobic, non-motile and encapsulated bacterium (Guo et al., 2012). It is widely considered as an opportunistic pathogen, however, *K. pneumoniae* can act as a commensal and be carried asymptomatically in the intestinal tract, skin, nose, and throat of healthy individuals. It can cause a range of infections in hospitalized patients. Moreover it can survive in myriad ecological niches, both host-associated and free living (Wyres, Lam, et al., 2020). These include environmental niches, including water, soil, and plant matter, animals such as insects, birds, reptiles and many different mammals (Holt et al., 2015; Podschun & Ullmann, 1998; Wyres, Lam, et al., 2020).

Relatively little is known about the broader population of *K. pneumoniae*, and data on its transmission, pathogenicity, and the global evolution and spread of multidrug-resistant (MDR) clones remain scarce. *K. pneumoniae* is recognized as both a source and reservoir of ARGs, with many major AMR gene families first identified in this bacterium before being detected in other Gram-negative bacteria (Chaves et al., 2001; Nordmann et al., 2009, 2011; Sirot et al., 1988). Therefore, expanding our understanding of the extensive population framework of *K. pneumoniae*, beyond a few well-known clones, is crucial. This knowledge is considered fundamental for supporting efforts to control the significant threat this bacterium poses to human health (Holt et al., 2015).

2.1 Cellular composition

The bacterial cell envelope, which includes the membranes and structures surrounding the cytoplasm, is far more complex than a simple membrane. Unlike cells in higher organisms, bacteria must navigate unpredictable, dilute, and often hostile environments. To thrive, they have developed a sophisticated cell envelope that not only protects them but also regulates the selective passage of nutrients and waste. Over a century ago, Christian Gram developed a staining technique that categorized nearly all bacteria into two major groups: Gram-positive, which retain the stain, and Gram-negative, which do not. This distinction is based on fundamental structural differences in their cell envelopes. The bacterial envelope consists of three main layers: the outer membrane (OM), the peptidoglycan cell wall, and the cytoplasmic or inner membrane (IM). These layers enclose an aqueous compartment known as the periplasm (Figure 4) (Silhavy et al., 2010).

The outer membrane (OM) is a defining characteristic of Gram-negative bacteria, absent in Gram-positive bacteria. Unlike typical biological membranes, the OM is not a phospholipid bilayer; it contains phospholipids only in its inner leaflet, while its outer leaflet is made of glycolipids, primarily lipopolysaccharide (LPS). LPS is notorious for causing endotoxic shock in septicemia from Gram-negative infections. The peptidoglycan layer, composed of repeating units of N-acetylglucosamine-N-acetylmuramic acid cross-linked by pentapeptide side chains, provides structural rigidity and determines cell shape. The OM is anchored to this peptidoglycan layer by Braun's lipoprotein (Lpp). Between the OM and the inner membrane (IM) lies the periplasm, a densely packed, viscous compartment containing various proteins and enzymes, helping to compartmentalize harmful substances. The IM is a typical phospholipid bilayer (Silhavy et al., 2010).

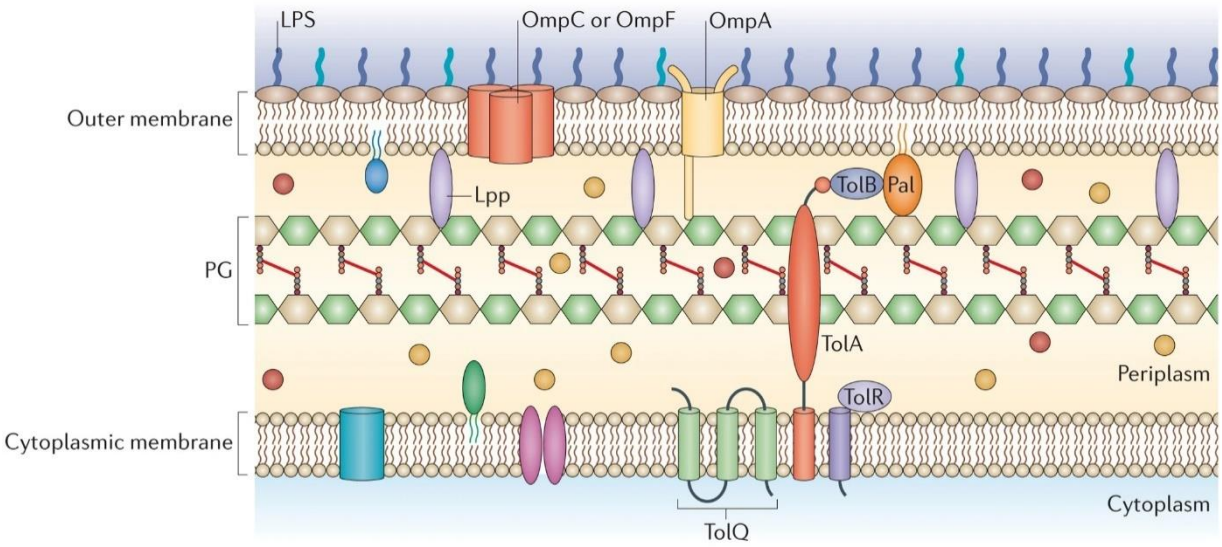


Figure 4 – The composition of the cell membrane of Gram-negative bacteria. The cell envelope of Gram-negative bacteria is composed of two membranes: the cytoplasmic membrane and the outer membrane. The cytoplasmic membrane consists of a phospholipid bilayer, while the outer membrane features an inner leaflet of phospholipids and an outer leaflet of lipopolysaccharide (LPS), which itself is made up of lipid A, a core oligosaccharide, and an O antigen. Between these two membranes lies the periplasmic space, housing the peptidoglycan (PG) layer and various periplasmic proteins. The PG layer consists of long polymers of repeating N-acetylglucosamine–N-acetylmuramic acid (NAG–NAM) disaccharides, linked by both traditional 4–3 (D-Ala–meso-diaminopimelic acid (mDAP)) crosslinks and non-traditional 3–3 (mDAP–mDAP) crosslinks. Envelope proteins can be soluble (periplasmic), transmembrane, or anchored to the membrane via covalently attached lipid appendages (lipoproteins). The stability of the envelope is maintained by several crosslinking mechanisms: covalent crosslinking of Braun's lipoprotein (Lpp) in the outer membrane to the PG layer, non-covalent interactions between the PG and the porin outer-membrane protein A (OmpA), and non-covalent interactions involving the Tol–Pal complex, which spans the envelope from the cytoplasmic membrane to the outer membrane. (Schwechheimer & Kuehn, 2015)

2.2 Population genomics

The genus *Klebsiella* consists of Gram-negative, encapsulated, nonmotile, rod-shaped bacteria that are oxidase-negative. It falls under the order *Enterobacterales*, which includes a diverse range of biochemically distinct genera, such as the model organism *Escherichia coli* and significant human pathogens like *Salmonella* spp., *Yersinia* spp., *Serratia* spp., *Enterobacter* spp., *Citrobacter* spp., *Kluyvera* spp., *Leclercia* spp., *Raoultella* spp., and *Cronobacter* spp. (Dong et al., 2022). The *Klebsiella* genus encompasses a broad variety of species, including members of the *K. pneumoniae* species complex (KpSC) and other *Klebsiella* species (e.g., *K. indica*, *K. terrigena*, *K. spallanzanii*, *K. huaxiensis*, *K. oxytoca*, *K. grimontii*, *K. pasteurii*, and *K. michiganensis*), which share only about 90% nucleotide identity with KpSC (Dong et al., 2022).

Whole-genome sequencing (WGS) has revealed that a significant portion of isolates identified as *K. pneumoniae* through biochemical or proteomics assays in clinical and research labs actually belong to closely related species, sharing 95–96% average nucleotide identity (ANI) with *K. pneumoniae* (Gorrie et al., 2017; Holt et al., 2015; Long, Linson, et al., 2017; Rodrigues et al., 2018). This group is collectively known as the *K. pneumoniae* species complex (KpSC), with its members sharing only 90% ANI with other *Klebsiella* species (Fig. 1). Within the KpSC, *K. pneumoniae* sensu stricto (ss) is the most prevalent in clinical collections, typically representing about 85% of isolates identified as *K. pneumoniae* (Gorrie et al., 2018; Heinz, Ejaz, et al., 2019; Henson et al., 2017; Holt et al., 2015; Long, Olsen, et al., 2017; Wyres, Lam, et al., 2020). *K. pneumoniae* is considered the type species due to its clear clinical significance (Wyres, Lam, et al., 2020).

Members of the KpSC were first distinguished based on *gyrA* sequences and designated as phylogroups of *K. pneumoniae* (KpI, KpII, KpIII). WGS later confirmed that a difference of ANI ≥3% genome-wide was sufficient to discriminate new species (Bialek-Davenet et al., 2014a; Holt et al., 2015), and has facilitated the identification of additional member species (Figure 5): *Klebsiella pneumoniae* (Kp1), *Klebsiella quasipneumoniae* subsp. *quasipneumoniae* (Kp2) (Brisse et al., 2014), *Klebsiella quasipneumoniae* subsp. *similipneumoniae* (Kp4) (Brisse et al., 2014), *Klebsiella variicola* subsp. *variicola* (Kp3) (Rosenblueth et al., 2004), *Klebsiella variicola* subsp. *tropica* (Kp5) (Rodrigues et al., 2019), *Klebsiella quasivariicola* (Kp6) (Long, Linson, et al., 2017) and *Klebsiella africana* (Kp7) (Rodrigues et al., 2019). The niche specificity of these species remains poorly understood, although *Klebsiella variicola* is notably plant-associated and typically possesses a nitrogen-fixing operon (nif) and cellulases, which are absent in the other species (Blin et al., 2017; Holt et al., 2015; Rosenblueth et al., 2004). All species within this group have been isolated from the human gut, and all, except *K. variicola* subsp. *tropica*, have been found in human infections (Rodrigues et al., 2019; Wyres, Lam, et al., 2020). *K. variicola* and *K. quasipneumoniae* are relatively common causes of nosocomial infections,

occurring at 10–20% of the frequency of *K. pneumoniae* sensu stricto (Holt et al., 2015; Long, Olsen, et al., 2017; Potter et al., 2018). These species can acquire antimicrobial resistance genes and plasmids from *K. pneumoniae* sensu stricto (Brinkac et al., 2019; Mathers et al., 2019) and have been implicated in nosocomial outbreaks (Mathers et al., 2019; Rodríguez-Medina et al., 2019). Both have also been reported as causes of community-acquired liver abscesses in humans (Breurec et al., 2016; Rodríguez-Medina et al., 2019) and have been isolated from dogs (Brisse & Duijkeren, 2005), cows (Giannattasio-Ferraz et al., 2022), ducks (Ma et al., 2020) and a horse (Mondo et al., 2021).

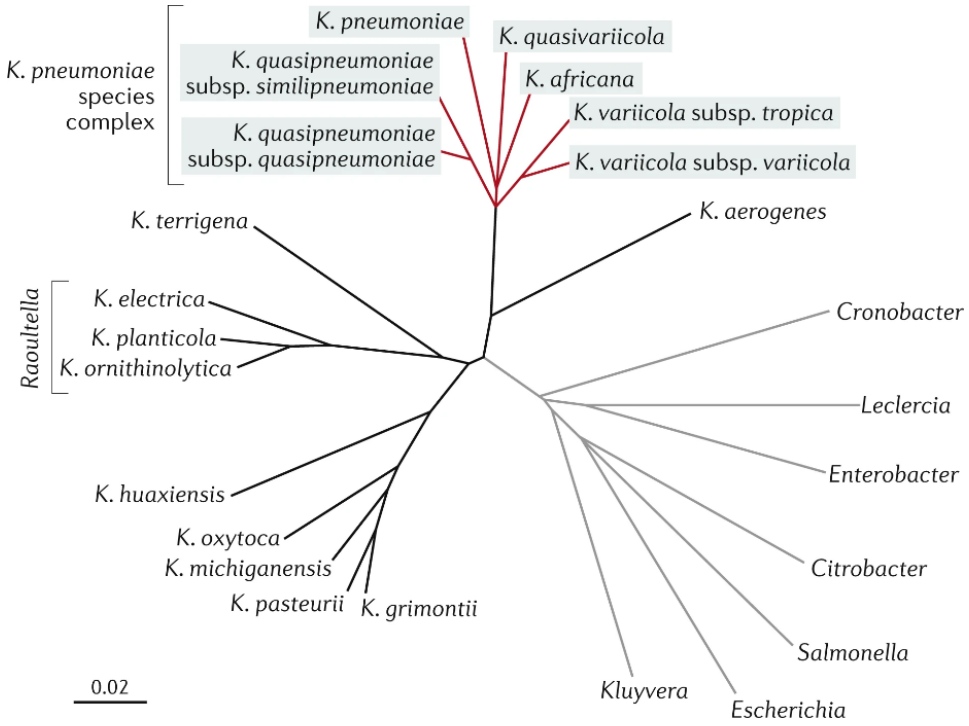


Figure 5 - Taxonomic position of *Klebsiella pneumoniae*. Whole-genome-based tree showing the phylogenetic relationships between *K. pneumoniae*, its close relatives in the KpSC (red branches), other members of the *Klebsiella* genus (black branches) and the Enterobacteriaceae family (grey branches). The tree was inferred from mash distances of representative whole genome sequences. Scale bar is the estimated average nucleotide divergence. Note *K. electrica*, *K. planticola* and *K. ornithinolytica* have been assigned the genus name *Raoultella* based on *gyrB* sequences, but this is under debate as it renders *Klebsiella* non-monophyletic. (Wyres, Lam, et al., 2020).

Multilocus sequence typing (MLST) is a nucleotide sequence-based method which is adequate for characterizing the genetic relationships among bacterial isolates (Diancourt et al., 2005a; Enright & Spratt, 1999). In *K. pneumoniae*, the MLST scheme is based on seven housekeeping genes (*rpoB, gapA, mdh, pgi, infB, phoE*, and *tonB).* This allows the use of unambiguous data and implementation of multiuser international databases. Dialek-Davenet et al. developed a core-genome multilocus sequence typing (cgMLST) scheme, which includes 694 highly conserved genes, included in the freely accessible BIGSdb-Kp database (Bialek-Davenet et al., 2014a) (accessible via https://bigsdb.pasteur.fr/*Klebsiella/*).

Population genomics of *K. pneumoniae* shows that hundreds of different lineages exist (Wyres, Lam, et al., 2020). Some of these lineages correspond closely to the clonal groups (CGs), which are defined by the degree of similarity in the allelic profiles across the 694 highly conserved core genes used in the cgMLST scheme (Bialek-Davenet et al., 2014a). This 694-gene cgMLST scheme is defined by the combination of the 7 MLST genes, 53 ribosomal MLST genes, and 634 strict cgMLST genes (Bialek-Davenet et al., 2014a). CGs are defined as groups of cgMLST profiles having <100 allelic mismatches (i.e., 14.4% of the 694 alleles) with at least 1 other member of the group. A total of 14 CGs are currently described (Bialek-Davenet et al., 2014a). A subset of these CGs disproportionately contribute to the global disease burden, which we term 'global problem clones'. This subset includes CG15, CG101, CG147, CG258 and CG307. They are each widely geographically distributed and commonly implicated in MDR healthcare-associated infections (HAIs) and/or outbreaks. Collectively, these are referred to as the 'global MDR clones' (Navon-Venezia et al., 2017; Wyres, Lam, et al., 2020; Wyres & Holt, 2016).

Within a bacterial species, a subset of genes is conserved across all members, known as the core genome. In *Klebsiella pneumoniae*, the core genome, defined as present in over 95% of isolates, comprises approximately 2,000 genes (Holt et al., 2015). Genes that differ among isolates form the accessory genome, which includes both chromosomally encoded genes and those found on plasmids. Since *K. pneumoniae* genomes typically contain 5,000–6,000 genes, the majority of the genome is accessory in nature. The pangenome represents the total set of genes present in the species, including both core and accessory genes. Some species exhibit open pangenomes with significant variability (such as *K. pneumoniae*), while others have closed pangenomes with minimal gene content differences.

Accessory genes play roles in specific processes, such as nitrogen fixation (Fouts et al., 2008), or encode virulence factors and antibiotic resistance mechanisms (Bi et al., 2015). These genes are often acquired through horizontal gene transfer, evidenced by the presence of mobile genetic elements and genomic islands in many isolates. Genomic islands may contribute to adaptation at infection or colonization sites (R. M. Martin & Bachman, 2018). A study analyzing 328 *Klebsiella* isolates identified nearly 30,000 unique protein-coding sequences, defining the known *Klebsiella* pangenome (Holt et al., 2015).

2.3 Ecology and lifestyle

Globally, the majority of *K. pneumoniae* infections are opportunistic healthcare-associated infections (HAIs), often referred to as "classical" *K. pneumoniae* (cKp) infections. These commonly manifest as pneumonia, urinary tract infections, and wound infections, which can progress to bacteremia (Podschun & Ullmann, 1998). Vulnerable groups, including neonates, the elderly, individuals with medical devices, and immunocompromised patients, are particularly at risk. In these groups, infections are often due to overgrowth and lack of immunological control of commensal *K. pneumoniae* strains.

Intestinal carriage is a significant risk factor for *K. pneumoniae* HAIs, with colonization linked to a fourfold increase in infection risk among intensive care and oncology patients (Gorrie et al., 2017; R. M. Martin et al., 2016). Genomic studies suggest that gut-colonizing strains are the most common source of *K. pneumoniae* infections in healthcare settings (Gorrie et al., 2017; R. M. Martin et al., 2016), and high bacterial loads in fecal samples have been associated with an increased risk of carbapenem-resistant *K. pneumoniae* (CRKp) bacteremia in long-term care facilities (Shimasaki et al., 2019).

Of particular clinical concern is the rise of multidrug-resistant (MDR) and especially CRKp strains. A recent meta-analysis estimated a 42% mortality rate for CRKp-associated HAIs, compared to 21% for carbapenem-susceptible strains (Xu et al., 2017). Treatment options for MDR and CRKp infections are limited, leading to a resurgence in the use of colistin. Other treatment options include the use of tigecycline, fosfomycin and temocillin. Moreover, ceftolozane–tazobactam, meropenem–vaborbactam, imipenem–cilastatin/relebactam and ceftazidime–avibactam are antimicrobials that combine β-lactams with β-lactamase inhibitors and are potent alternatives (Bassetti et al., 2019; Karampatakis et al., 2023). Additionally, research is ongoing for developing a vaccine against *K. pneumoniae.* A tetravalent bioconjugate vaccine (Kleb4V) is currently being assessed in a phase 1/2 clinical trial (NCT04959344) (Frost et al., 2023).

Outside hospital settings, *K. pneumoniae* can act as a true pathogen, causing severe community-acquired infections (CAIs) in otherwise healthy individuals without typical HAI risk factors. These CAI strains are generally susceptible to antibiotics. Common CAIs include endophthalmitis, pneumonia, necrotizing fasciitis, non-hepatic abscesses, meningitis, and pyogenic liver abscesses in the absence of biliary tract disease (Wyres, Lam, et al., 2020). These infections, often caused by hypervirulent *K. pneumoniae* strains (hvKp), are characterized by unusual or multiple infection sites and are frequently accompanied by bacteremia and/or metastatic spread. Host risk factors for *K. pneumoniae* CAIs include alcoholism (associated with pneumonia) and diabetes (linked to pyogenic liver abscesses), alongside numerous pathogen-related risk factors. The hvKp strains are associated with the acquisition of mobile elements and/or large virulence plasmids encoding virulence determinants (M. J. Martin et al., 2023; Wyres, Lam, et al., 2020). The most common hvKp clonal group is CG23, followed by CG65 (including ST65 and ST375) and CG86 (Wyres, Lam, et al., 2020).

K. pneumoniae infections have been described in livestock and in companion animals, causing mainly urinary, enteric, mammary, reproductive, and respiratory disorders. Other clinical manifestations include abscesses, otitis, hepatitis, conjunctivitis, pyodermitis, sepsis, and encephalitis (K. Harada et al., 2016; M. G. Ribeiro et al., 2022; Z. Zhang et al., 2022). Since the use of carbapenems has never been licensed for food-producing animals in any country worldwide (WHO, 2015), their residues in foods of animal origin is not permitted (Bonardi & Pitino, 2019). However, there are limited

reports regarding the occurrence of ESBL/AmpC producing *K. pneumoniae* and CRKP in food products or in food-producing animals. For example, *K. pneumoniae* carrying $bla_{\text{OXA-48}}$ and $bla_{\text{CTX-M-15}}$ were isolated from raw milk in Lebanon (Diab et al., 2017), *K. pneumoniae* carrying $bla_{\text{OXA-48}}$, bla_{KPC} and bla_{NDM} were detected in poultry (Hamza et al., 2016) and *K. pneumoniae* producing $bla_{\text{KPC-2}}$ was isolated from pigs in China (W. Zhang et al., 2019). Reports on the occurrence of carbapenemase-producing *K. pneumoniae* from companion animals also exist: $bla_{\text{OXA-48}}$ from dogs in Germany (Stolle et al., 2013), ESBL-producing *K. pneumoniae* isolated from dogs in Portugal

2.4 Pathogenicity

The interaction between *K. pneumoniae* and human hosts is complex and dynamic, with the bacterium capable of functioning as a commensal, an opportunistic pathogen, or a true pathogen. Commensal colonization in the gut and respiratory tract is common, though prevalence varies by age, geographic location, and healthcare exposure. In the community, gut colonization is estimated at ~ 4–6% in the USA and Australia (Conlan et al., 2012; Gorrie et al., 2017), but rises to ~16% in Norway (Raffelsberger et al., 2021) and to ~25% among individuals with recent healthcare contact in the USA, Australia, and England (Gorrie et al., 2017; Ludden et al., 2020; R. M. Martin et al., 2016). In contrast, higher community carriage rates for KpSC are reported in Asia, ranging from 7.8% to 87% (Chung et al., 2012; Y.-T. Lin et al., 2012; X. Zhang et al., 2018). Gut colonization can persist for over 12 months, though the exact duration is not well understood (Löhr et al., 2013; Mo et al., 2020).

Numerous genetic factors contribute to the ability of *K. pneumoniae* strains to cause disease in humans, such as underlying mechanisms (diabetes, COPD, chronic alcoholism) (Paczosa & Mecsas, 2016) and host immune evasion strategies (Bengoechea & Sa Pessoa, 2019). All *K. pneumoniae* strains possess a set of core chromosomally encoded pathogenicity factors essential for initiating opportunistic infections in mammalian hosts. These include the *ent* locus for the biosynthesis of the siderophore enterobactin (Ent), the *fim* and *mrk* loci for the production of type 1 and type 3 fimbriae, respectively, as well as the variable loci responsible for the biosynthesis of capsular polysaccharide (K antigen) and lipopolysaccharide (O antigen) (Bialek-Davenet et al., 2014a; Follador et al., 2016; Holt et al., 2015; Wyres et al., 2016) (Figure 6). Enterobactin (or an alternative acquired siderophore) is crucial for growth in most environments (Bachman et al., 2012), while the other factors facilitate early infection stages and help the bacterium evade host defense mechanisms (Bengoechea & Sa Pessoa, 2019; Paczosa & Mecsas, 2016).

Large-scale genomic comparisons have uncovered significant allelic and gene-content diversity within the capsule (K antigen) and LPS (O antigen) biosynthesis loci, which account for roughly 10% of the *K. pneumoniae* pan-genome (Holt et al., 2015). The capsule is produced via a Wzy-dependent

pathway, with conserved genes (*wzi*, *wza*, *wzb*, *wzc*, *wzx*, and *wzy*) present in most K-antigen biosynthesis loci (Pan et al., 2015). However, the genes responsible for capsule-specific sugar synthesis are highly diverse, variably present, and frequently rearranged within the population. So far, over 180 distinct K-loci combinations have been predicted from whole genome data, each thought to encode a unique capsule type (Follador et al., 2016; Lam et al., 2022, p. 2; Wyres et al., 2016; Wyres, Nguyen, et al., 2020). However, only 77 have been differentiated using traditional serological typing (Ørskov & Fife-Asbury, 1977). Capsule types K1 and K2 are notably associated with invasive disease and are highly conserved in hypervirulent clones (K1 in CG23 and K2 in others) (Bialek-Davenet et al., 2014a; Lee et al., 2016; Russo & Marr, 2019; Wyres, Wick, et al., 2019).

The relative virulence of most capsule types in *K. pneumoniae* remains poorly understood, and non-hypervirulent clones, including global MDR clones, display significant K-locus diversity (Bowers et al., 2015; Wyres, Wick, et al., 2019). An exception is CG307, which consistently carries the KL102 locus and an additional, structurally distinct capsule synthesis locus that is rare in the broader *K. pneumoniae* population (Wyres, Hawkey, et al., 2019).

Gene-content variation has also been used to define 12 distinct O-loci. Unlike the K-loci, the genes that determine the nine recognized LPS O serotypes and over five subtypes, are found both at the O-locus and in other genome regions (Clarke et al., 2018). Clinical isolates of *K. pneumoniae* most commonly feature O1 and O2 serotypes (Follador et al., 2016). Currently, there is no conclusive evidence linking K-locus or O-locus variation to niche or host specialization, as observed in other bacterial species (Mostowy & Holt, 2018).

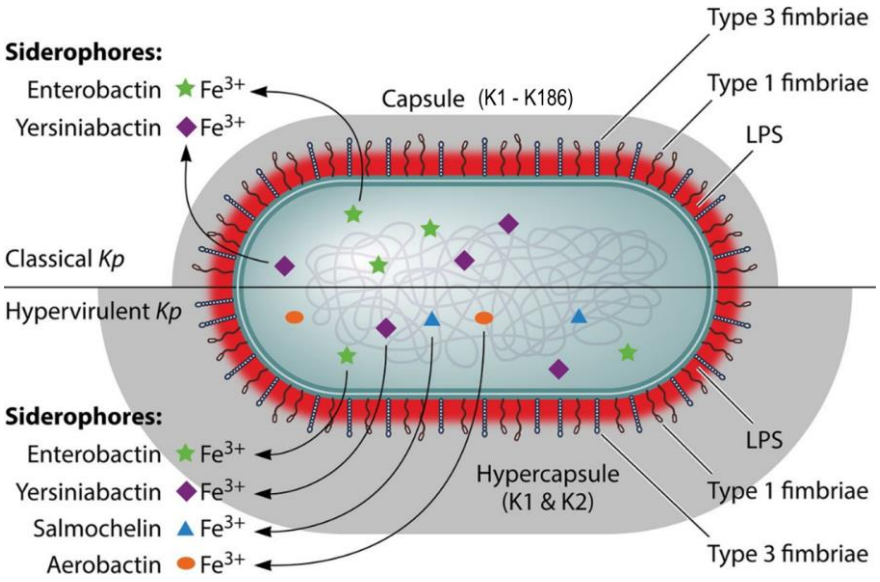


Figure 6 – The virulence factors in classical and hypervirulent *K. pneumoniae*. *K. pneumoniae strains*, both classical (cKp) and hypervirulent (HvKp), possess four well-characterized virulence factors: the capsule, lipopolysaccharide (LPS), fimbriae (type 1 and type 3), and siderophores. The capsule is an extracellular polysaccharide matrix that encases the bacteria, with classical strains producing capsules of serotypes K1 to K186, where K1 and K2 are linked to increased pathogenicity. HvKp strains produce a hypercapsule, which significantly increases capsular material, predominantly of the K1

serotype, with the remainder being K2. LPS, a key component of the outer membrane, is found in both classical and HvKp strains, with O-antigen serotypes ranging from O1 to O12. Both strain types also produce membrane-bound adhesive structures known as type 1 and type 3 fimbriae and secrete iron-scavenging siderophores. Enterobactin is nearly universally produced, while yersiniabactin is present in about half of classical strains and nearly all HvKp strains. Salmochelin and aerobactin are rarely found in classical strains but are typically secreted by HvKp strains, with aerobactin being the most abundantly expressed siderophore. Adapted from (Paczosa & Mecsas, 2016).

2.5 Epidemiology

2.5.1 Human clinical isolates

Studies on the epidemiology of *K. pneumoniae* are mainly focused on CRKp and *K. pneumoniae* resistant to 3GCs. The emergence of hypervirulent clones, and the combination of hvKp with AMR genes has equally gained interest. As described before, hundreds of distinct *K. pneumoniae* lineages exist, with some of them causing localized problems (Gorrie et al., 2018; Henson et al., 2017), while others are globally widespread. Local outbreaks likely represent sporadic emergence in a given time and place, and the factors influencing their frequency, likelihood and duration of persistence are unknown (Ellington et al., 2019). Many *K. pneumoniae* strains remain localized issues, causing limited infections beyond their origin. However, a subset of highly resistant lineages, such as those resistant to 3GCs and/or carbapenems, have emerged as global threats. These include the widely studied CG258, as well as CG15, CG20, CG29, CG37, CG147, CG101, and CG307 (Figure 7). Though not genetically related, these lineages are geographically widespread and common causes of MDR HAIs and outbreaks (Navon-Venezia et al., 2017; Wyres, Hawkey, et al., 2019; Wyres & Holt, 2016). Notably, while many studies attribute most 3GC-resistant *K. pneumoniae* and/or CRKp infections to a few dominant clones (such as ST11, ST15, ST101, and ST258/ST512 (Figure 7), which accounted for 57% of CRKp infections in the Europe-wide EuSCAPE study) (David et al., 2019), sporadic MDR strains or local problem clones still contribute significantly to the infection burden in many regions, as seen in a recent UK survey where they comprised over 33% of CRKp strains (Turton et al., 2018).

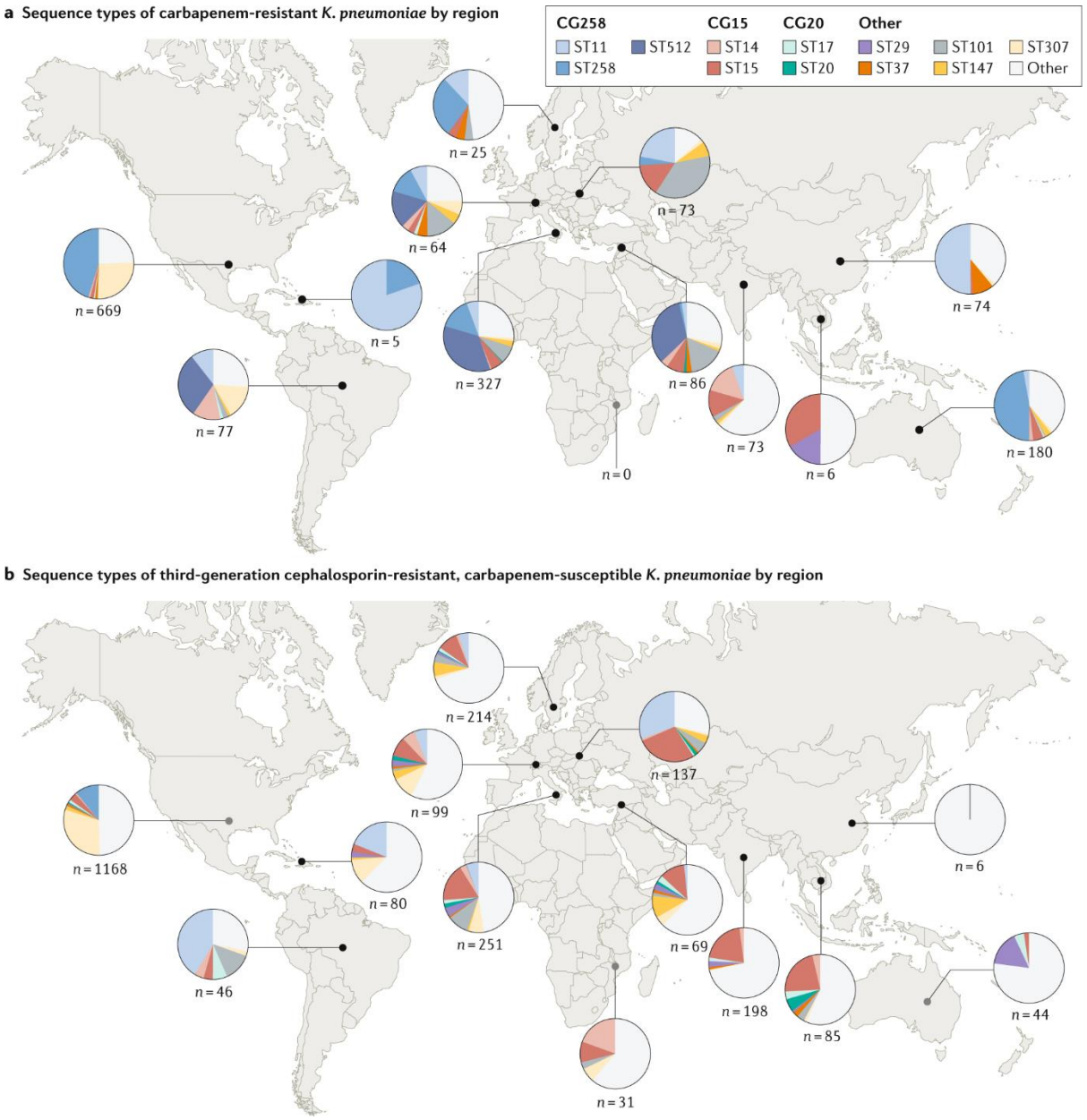


Figure 7 - Geographical distribution of *K. pneumoniae* clones harboring resistance to carbapenems and third-generation cephalosporins. Regional frequencies of sequence types (STs) for carbapenem-resistant *K. pneumoniae* (CRKp) (part **a**) and third-generation cephalosporin (3GC) -resistant, carbapenem-susceptible *K. pneumoniae* (part **b**) are depicted. These data come from recent studies where isolates were selected independently of ST or local transmission events or outbreaks and are organized by regions as defined by the United Nations Statistics Division. Black markers represent data from multiple healthcare institutions, while grey markers denote data from single institutions. ST258 and its derivative, ST512, are predominant among CRKp in the Americas and southern Europe. ST11, a diverse clade from which ST258 originated through recombination, accounts for approximately 12% of CRKp cases across Europe. ST11 is the leading cause of CRKp infections in China. Recently, CG307 has started to replace ST258 and ST512 as the dominant CRKp lineage in the Americas and southern Europe, and it has become the leading CRKp lineage in South Africa. However, outside these regions, CG307 is usually carbapenem-susceptible and 3GC-resistant due to the blaCTX-M-15 plasmid. CG15 (including ST15, ST14, and related types) is a prominent 3GC-resistant clone across various regions and has been linked to numerous reports of disseminated carbapenem-resistant variants (Wyres, Lam, et al., 2020).

The EuSCAPE study elucidated the European-wide population structure and the epidemiology of carbapenem-non-susceptible *K. pneumoniae* strains, analysing the genomes of 1,717 *K. pneumoniae* isolates. The genomes were analysed for the presence of β-lactam resistance determinants that could be linked to carbapenem resistance and divided the collection in five groups. The first group contained one or more of any carbapenemase gene (*bla*$_{KPC}$ like, *bla*$_{NDM}$ like, *bla*$_{VIM}$ like, bla$_{OXA-48}$ like, *bla*$_{IMP}$ like, *bla*$_{GES}$ like, *bla*$_{GIM}$ like, *bla*$_{AIM}$ like, *bla*$_{BIC}$ like, *bla*$_{DIM}$ like, *bla*$_{IMI}$ like, *bla*$_{NMC-A}$ like, *bla*$_{SIM}$ like, *bla*$_{SPM}$ like, *bla*$_{LMB}$ like, *bla*$_{FRI}$ like, *bla*$_{SME}$ like, *bla*$_{KHM}$ like, *bla*$_{BKC}$ like, *bla*$_{OXA-23}$ like, bla$_{OXA-51}$ like and *bla*$_{OXA-58}$ like), regardless of other mechanisms; group two contained any ESBL gene and/or an AmpC gene, in combination with porin defects; group three contained any ESBL gene and/or an AmpC gene, without presence of any obvious porin defects; group four did not show presence of any ESBL or AmpC gene but porin defects were present; and in group five, all of the above determinants were absent. Figure 8 displays the geographical distribution of carbapenem resistance mechanisms among isolates submitted during the EuSCAPE survey (n = 1,717) (David et al., 2019).

In contrast, HvKp infections globally are dominated by a consistent subset of lineages. The most prevalent is CG23, followed by CG65 (including ST65 and ST375) and CG86, with CG25, CG66, and CG380 playing lesser roles (Lee et al., 2016; J.-C. Lin et al., 2014; Shi et al., 2018; Siu et al., 2011). Reflecting the high incidence of hypervirulent disease in the Asia-Pacific region, CG23 was recently identified among the top two most common clones associated with blood stream infections (BSIs) in China, Vietnam, and Laos, accounting for over 10% of isolates (Wyres, Nguyen, et al., 2020; Y. Zhang et al., 2016). Outside the Asia-Pacific region, data on ST23's prevalence are limited, as most studies focus on MDR strains, and ST23 isolates are typically drug-susceptible (Lam, Wyres, Duchêne, et al., 2018; Wyres, Wick, et al., 2019). However, existing evidence indicates that ST23 represents 2% or less of clinical isolates in other regions (Heinz, Brindle, et al., 2019; Henson et al., 2017; Wyres, Nguyen, et al., 2020), despite its circulation among humans for over a century (Lam, Wyres, Duchêne, et al., 2018), far longer than well-known MDR clones like ST258 and ST307, which only emerged in the mid-1990s (Wyres, Lam, et al., 2020).

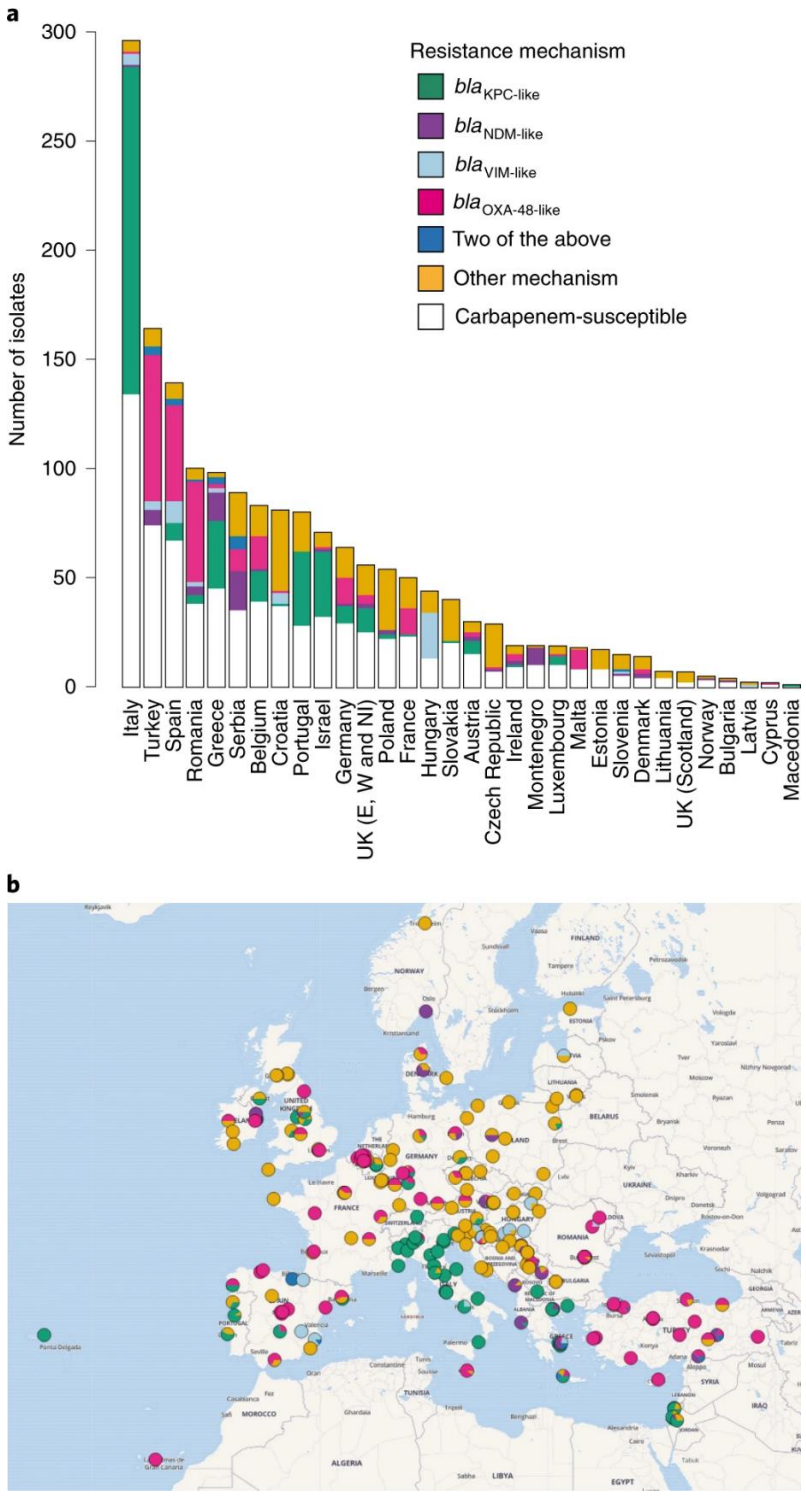


Figure 8 - Geographical distribution of carbapenem resistance mechanisms among isolates submitted during the EuSCAPE survey. a. All of the isolates submitted by participating countries and analysed in this study, partitioned into: those that possess one or more of four major carbapenemase genes (*bla*$_{KPC}$ like, *bla*$_{OXA-48}$ like, *bla*$_{NDM}$ like and *bla*$_{VIM}$-like), regardless of whether they were submitted as carbapenem-non-susceptible or -susceptible; those that lack any of the four genes and were submitted as carbapenem-non-susceptible ('Other mechanism'); and those submitted as carbapenem-susceptible. E=England; W=Wales; NI=Northern Ireland. **b**. Pie charts showing the proportions of different resistance mechanisms in isolates submitted as carbapenem-non-susceptible by participating hospitals. (David et al., 2019)

EARS-Net, managed by the European Centre for Disease Prevention and Control (ECDC), is the leading epidemiological surveillance system for AMR across the European Union (ECDC, 2017). It plays a vital role in monitoring and reporting on the prevalence and spread of AMR in European countries. Each year, EARS-Net collects and analyzes data on AMR in key pathogens isolated from clinical invasive samples, such as blood and cerebrospinal fluid (CSF). The pathogens monitored include *E. coli, K. pneumoniae, P. aeruginosa, Acinetobacter* species, *S. pneumoniae, S. aureus, E. faecalis,* and *E. faecium*. EARS-BE, the Belgian counterpart to EARS-Net, has a broader scope by including antimicrobial susceptibility testing (AST) on urinary isolates in addition to those from blood and cerebrospinal fluid (CSF) samples (Vilain et al., 2024). In Belgium, *K. pneumoniae* accounted for 8% of hospital-acquired bloodstream infections (HABSIs) in 2020, making it the second most common Enterobacterales species. Between 2011 and 2021, the prevalence of CRKP in clinical isolates from invasive (blood or CSF) samples from the EARS-BE surveillance increased from 0.4% to 1.4% (BELMAP editorial, 2023). This rate is about twice as high as in neighboring countries (0.73% in 2021) but remains significantly lower than the EU average of 11.6% (BELMAP editorial, 2023).

2.5.2 Non-human environments

Research suggests that *K. pneumoniae* can persist in a broad range of reservoirs including the hospital environment (Clarivet et al., 2016; Mathers et al., 2019; Paskova et al., 2018), retail meat (Belmar Campos et al., 2014; Cardozo et al., 2021; Davis et al., 2015; Huizinga et al., 2019; Rodrigues et al., 2022), food-producing animals (Bobbadi et al., 2020; Bonardi & Pitino, 2019; Daehre, Projahn, Semmler, et al., 2018; Köck et al., 2018), companion animals (Collineau et al., 2023; Debergh et al., 2022), vegetables and the farm environment (Ben Said et al., 2015), surface water (Zurfluh et al., 2013) and wastewater (Galler et al., 2014; Heljanko et al., 2024; Tiwari et al., 2022).

K. pneumoniae was included in the European Antimicrobial Resistance Surveillance Network in Veterinary Medicine (EARS-Vet) to monitor AMR in sick animals (Mader et al., 2022). While data on lineage distributions in non-human reservoirs are limited, some reports indicate overlaps between lineages isolated from clinical and other sources. For instance, the globally problematic clone ST11 has been found in a dog (Debergh et al., 2022) and in poultry (R. Zhang et al., 2019b), ST101 in pigs (De Koster et al., 2022), ST307 in a tortoise (Foster et al., 2020), and ST23 in non-human primates (Anzai et al., 2017). Previous attempts to identify markers distinguishing human clinical isolates from those in plants, animals, or the environment have not been successful (Holt et al., 2015; Struve & Krogfelt, 2004)

There are notably high levels of ESBL-producing *K. pneumoniae* in broiler poultry in Spain and Germany (Cortés et al., 2010; Daehre, Projahn, Semmler, et al., 2018). These bacteria have also been isolated from retail poultry meat in Germany, suggesting that poor hygiene or accidental cross-

contamination in slaughterhouses may facilitate their spread through food products (Gelbíčová et al., 2019). This poses a risk of colonization or infection in humans through contact or consumption of contaminated food. A study by Meijs et al. (2021) found a 9.8% carriage rate of ESBL-producing *E. coli* /*K. pneumoniae* (results were not stratified per species) among veterinary healthcare workers in the Netherlands, highlighting occupational exposure to animals (especially companion animals such as dogs, cats and rabbits) as a potential source of these bacteria in the general population (Meijs et al., 2021; Savin, Bierbaum, Mutters, et al., 2022). In the general population in the Netherlands, a prevalence of carriage of ESBL-enterobacteriaceae was 4.5% (van den Bunt et al., 2019) and ranged from 1.4% to 10.9% among research centers in a livestock dense area (Wielders et al., 2017). In contrast, Raffelsberger et al. report an ESBL-*E. coli* gastrointestinal carriage prevalence of 3.3% and 0.08% for *K. pneumoniae* in Norway (Raffelsberger et al., 2021). Farm and slaughterhouse workers are also at an increased risk of colonization due to exposure to contaminated animals, air, dust, and process waters (Dohmen et al., 2017). Additionally, process waters from poultry and pig slaughterhouses, including scalding water, as well as municipal wastewater, have been identified as key hotspots for the spread of ESBL-producing *K. pneumoniae* into the environment (Savin, Bierbaum, Blau, et al., 2020; Savin, Bierbaum, Hammerl, et al., 2020b, 2020a; Savin, Bierbaum, Schmithausen, et al., 2022). Similarly, carbapenemase-producing *K. pneumoniae* (CPKP) have been detected in municipal wastewater in the Netherlands (Blaak et al., 2021) and slaughterhouse and municipal wastewater in Germany (Savin, Bierbaum, Mutters, et al., 2022).

Wastewater, especially those receiving hospital effluents, can represent a hotspot for high-risk bacterial pathogens, which could be discharged into surface water due to inadequate wastewater treatment (Savin, Bierbaum, Schmithausen, et al., 2022). Furthermore, the presence of ESBL-producing *K. pneumoniae* and CRKp in municipal WWTPs suggests potential dissemination within the general population and underscores the impact of clinical wastewater on municipal sewer systems. Multiple studies have documented high levels of CP-producing *Klebsiella* species in clinical wastewater that is subsequently discharged into municipal systems (Kehl et al., 2022; Kizny Gordon et al., 2017; Müller et al., 2018; Surleac et al., 2020). The detection of these bacteria in wastewater effluent indicates that conventional biological treatment methods are inadequate for fully eliminating microbial loads, emphasizing the detrimental effects of improperly treated wastewater on aquatic environments. Similar observations of ESBL and carbapenemase (CP)-producing *K. pneumoniae* in Austrian and Swiss rivers, particularly in urbanized areas, further illustrate the impact of human pollution on water systems (Bleichenbacher et al., 2020; Lepuschitz et al., 2019; Savin, Bierbaum, Mutters, et al., 2022).

2.6 Antimicrobial consumption

2.6.1 Human

The European Surveillance of Antimicrobial Consumption Network (ESAC-Net), organized by the ECDC, monitors antimicrobial consumption (AMC) across Europe using standardized protocols. Data from Belgium is provided by the National Institute for Health and Disability Insurance (NIHDI), covering community and hospital settings. AMC is measured in Defined Daily Doses (DDDs) per 1000 inhabitants per day (DID), and hospital data is estimated to account for reporting delays and corrections (BELMAP editorial, 2023).

Belgium also has a more detailed national surveillance system, BeH-SAC, focused on hospitals, which allows for benchmarking between hospitals and measures AMC in DDDs per 1000 patient days and per 1000 admissions. However, both ESAC-Net and BeH-SAC only account for reimbursed antimicrobial use, potentially underestimating consumption, especially for drugs with stricter reimbursement criteria like fluoroquinolones and newer antibiotics like cefiderocol. To address this, IQVIA (https://www.iqvia.com/) sales data are used to estimate the total volume of fluoroquinolones consumed (BELMAP editorial, 2023). In addition to these systems, point-prevalence studies (PPS) are conducted regularly in hospitals and long-term care facilities to assess the prevalence of antimicrobial use, with data collected on a single day by local collectors. These studies provide observed prevalence rates with 95% confidence intervals, contributing further insights into antimicrobial usage. Detailed methodologies for these surveillance efforts are available in national reports (BELMAP editorial, 2023; Lucy Catteau et al., 2023; Vandael et al., 2021).

In 2022, Belgium saw a significant 18.8% increase in systemic antibiotic consumption in the community setting compared to 2021 (Figure 9). This rise follows a decade-long trend of decreasing antibiotic use, which included a sharp decline during the COVID-19 pandemic in 2020. The increase in 2022 is largely attributed to the relaxation of COVID-19 measures, leading to a return to normal healthcare and social activities. Despite this increase, antibiotic consumption in 2022 remained slightly below pre-pandemic levels in 2019, though still above the EU average (BELMAP editorial, 2023).

All major antibiotic subgroups saw a mild to moderate rise in consumption between 2021 and 2022, except for tetracyclines, which decreased slightly. However, compared to 2019, there was a noticeable decline across most subgroups, with macrolides and fluoroquinolones experiencing the largest reductions. Notably, after stricter reimbursement criteria for fluoroquinolones were introduced in 2018, their consumption had dropped significantly, but 2022 marked the first year of increased non-reimbursed fluoroquinolone use, with total consumption estimated to have risen by 12.6% from 2021 (BELMAP editorial, 2023).

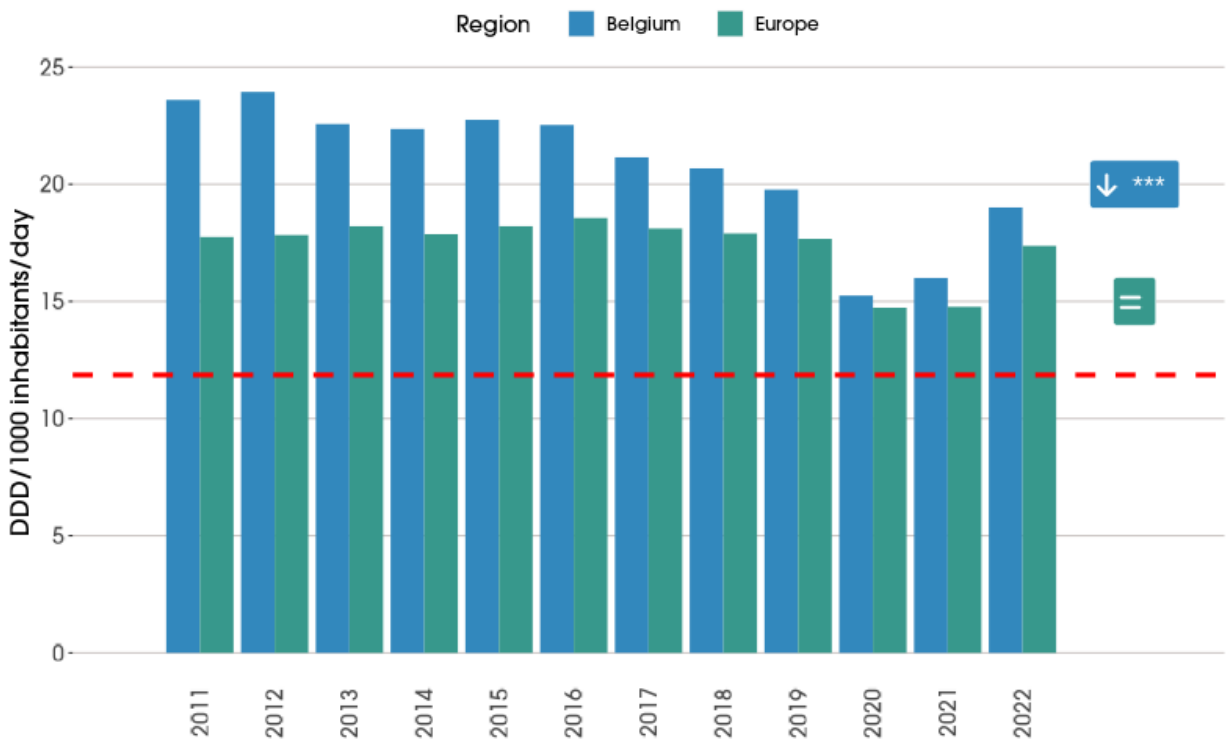


Figure 9 - Consumption of antibacterials for systemic use (ATC group J01) in the community in Belgium (blue), and EU/ EEA (green), expressed as Defined Daily Doses per 1000 inhabitants and per day (DID). Dashed red line represents national target reduction by 2024 to 40% of 2019 consumption. Results are indicated as *** for results with p-values p<0.001. "↓" represents a decreasing trend and "=" indicates no increasing or decreasing trend, without large variation (range less than 25% of the mean). Data source: ESAC-Net. (BELMAP editorial, 2023).

2.6.2 Intersectorial

The BELMAP report emphasizes the "One Health" approach, highlighting the need for cross-sector cooperation to combat AMR. To compare AMC across human and veterinary sectors, a common metric - antibiotic consumption (mg) per estimated biomass (kg) - was used (Figure 10). The report follows the methodology of JIACRA (joint inter-agency report on integrated analysis of antimicrobial agent consumption and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals) reports with adjustments specific to Belgian data (BELMAP editorial, 2023).

Human AMC data, categorized by ambulatory and hospital sectors, was retrieved from The European Surveillance System (TESSy) database and converted into mg of active product using WHO standards. For animals, AMC data was already available in mg/kg biomass from the BelVet-SAC reports. In 2021, Belgium reported the sale of 83 tonnes of antimicrobials for humans and 172 tonnes for animals, with corresponding biomass estimates of 720 and 2,114 thousand tonnes, respectively. The data shows significant reductions in AMC over time in both humans and animals, with a strong

correlation between the two sectors, suggesting effective stewardship efforts. However, Belgium's data deviates from European trends in two keyways: first, Belgium consistently reports higher human AMC than animal AMC, unlike Europe, where human AMC surpassed animal AMC only from 2016. Second, hospital AMC in Belgium accounted for a higher proportion (20.9%) of total human AMC in 2021, compared to the European average of 10%. Additionally, while ambulatory AMC has decreased, hospital AMC has remained steady, increasing its share of total human consumption from 15.3% in 2012 to 20.9% in 2021 (BELMAP editorial, 2023).

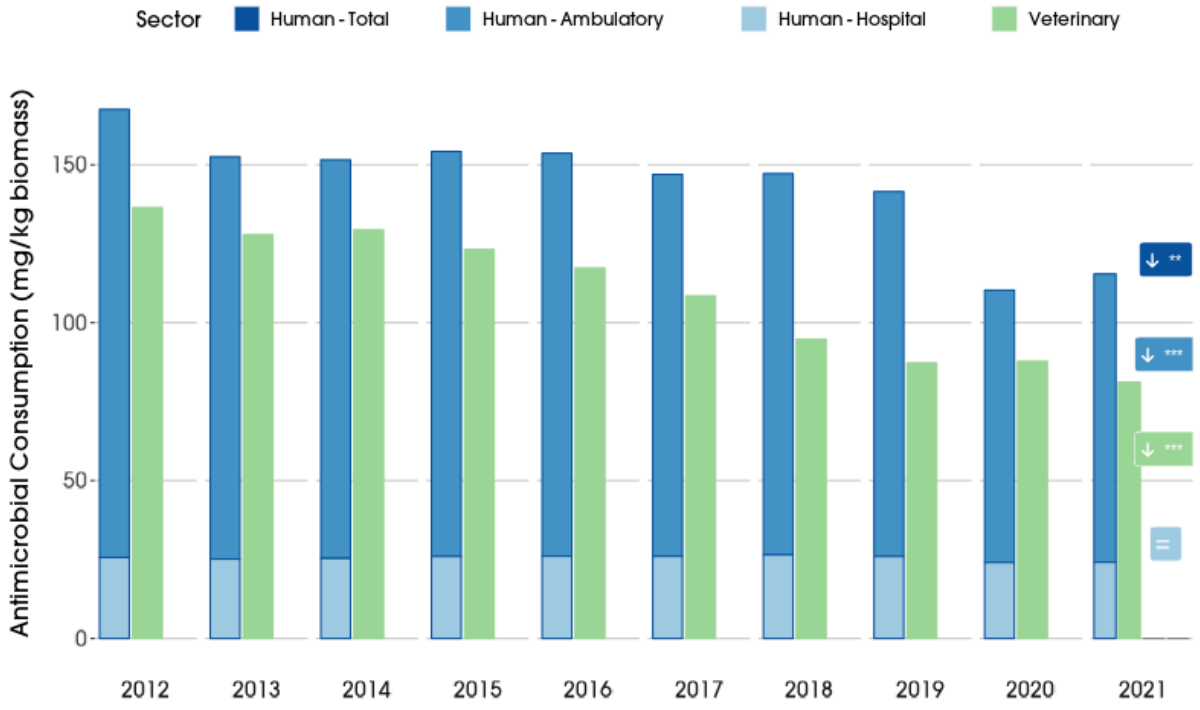


Figure 10 - Population-weighted mean of the total consumption of antimicrobials in humans (blue shades) and food-producing animals (green), for the period 2011 – 2021. Results are indicated as ** and *** for results with p-values 0.01<p<0.001 and p<0.001, respectively. "↓" represents a decreasing trend and "=" indicates no increasing or decreasing trend, without large variation (range less than 25% of the mean) Source: Human data: Reimbursed consumption ESAC-Net, Veterinary: Total sales BelVet-SAC report (BELMAP editorial, 2023).

2.7 Antibiotics

Antibiotics were originally characterized as naturally occurring chemical compounds produced by microorganisms, capable of inhibiting or killing bacteria and other microorganisms. However, many antibiotics identified to date are not purely natural but are instead synthetic or semi-synthetic derivatives (Quinn et al., 2011; Walsh, 2000). Davies and Davies (2010) expanded the definition of antibiotics to include "any class of organic molecule that inhibits or kills microbes by specific interactions with bacterial targets, regardless of the compound's source or class" (Davies & Davies, 2010).

The concept of antibiotics first emerged in 1928 when Alexander Fleming discovered penicillin. He observed that a *Penicillium* spp. produced a substance that could inhibit bacterial growth (Fleming, 1929). Penicillin was not introduced into human medicine until World War II (Gaynes, 2017). However, it wasn't the first antibiotic used in clinical practice; sulfonamides had already been in use since the mid-1930s (Figure 11) (Gaynes, 2017). Antibiotics are categorized based on their effects as bactericidal (bacteria-killing) or bacteriostatic (bacteria growth-inhibiting) agents and are typically classified by their mechanism of action (Gothwal & Shashidhar, 2015).

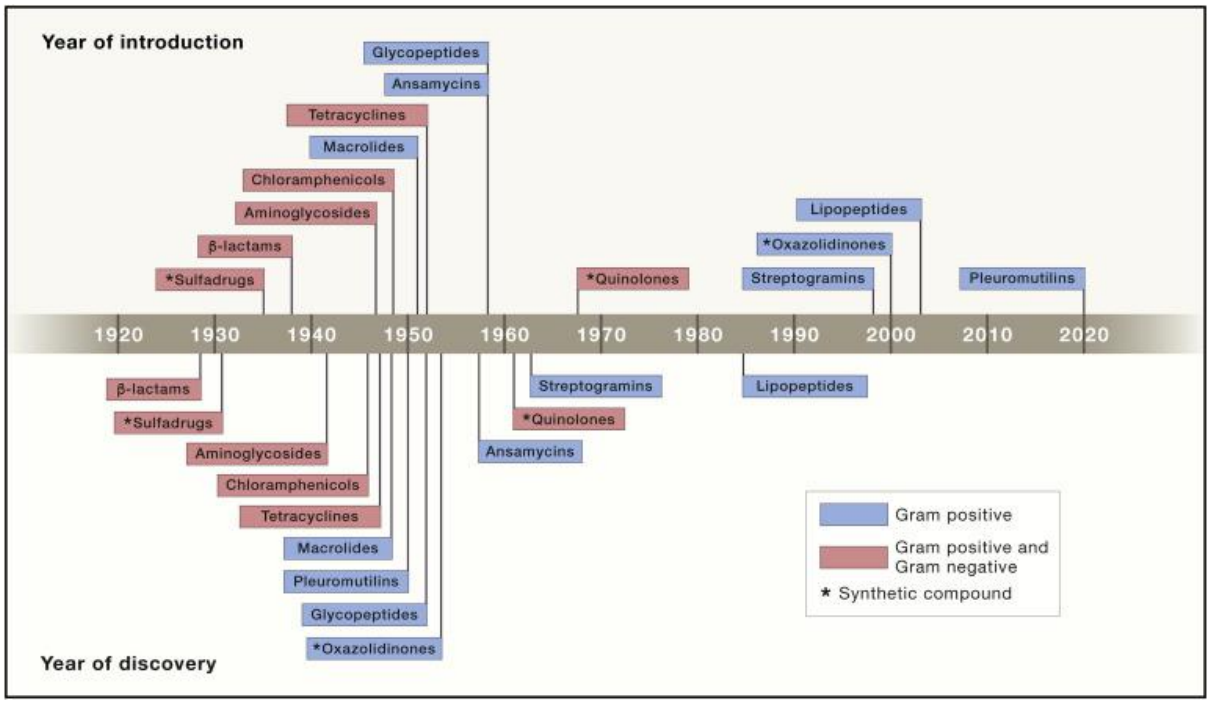


Figure 11 - Timeline of the year of antibiotic discovery (bottom) and the year of the introduction of the first member of the antibiotic class in clinical practice (top). Broad-spectrum antibiotics are displayed in red. * synthetic compound. (K. Lewis, 2020)

2.8 Mechanisms of action of antibiotics

Antibiotics target several key bacterial processes, including: (i) cell wall synthesis, (ii) the folate synthesis pathway, (iii) protein synthesis, (iv) cell membrane structure, and (v) DNA replication and repair (Figure 12). For comprehensive reviews on the mechanisms of action for various antibiotic classes, refer to Walsh (2000), Chellat et al. (2016), Kapoor et al. (2017), and Baquero and Levin (2021).

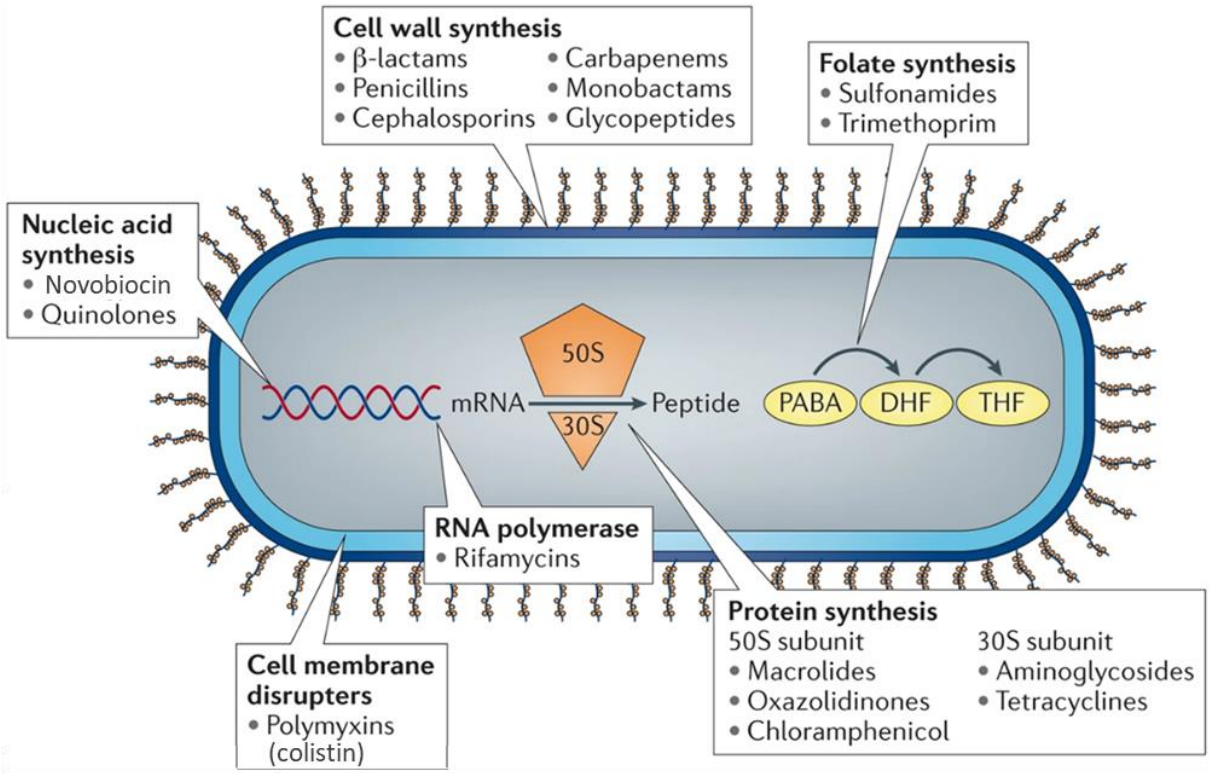


Figure 12 - Sites of antibacterial action and key antibiotic classes/molecules. Antibiotics can be classified by their mechanism of action. PABA, para-aminobenzoic acid; DHF, dihydrofolic acid; THF, tetrahydrofolic acid; Modified from (Brown, 2015).

2.8.1 Cell wall synthesis inhibitors

β-lactam antibiotics, such as penicillins and cephalosporins, are prominent examples of antibiotics that target bacterial cell wall synthesis. These antibiotics inhibit transpeptidases by binding to their active sites, thereby preventing the normal crosslinking of peptide chains within the peptidoglycan layer. This disruption weakens the cell wall, making it more susceptible to lysis or osmotic pressure changes. Glycopeptides, such as vancomycin, also target peptidoglycan but through a different mechanism: they bind directly to the peptide substrates, obstructing their interaction with enzymes involved in peptidoglycan crosslinking, such as transpeptidases or transglycosylases (Quinn et al., 2011; Walsh, 2000).

2.8.2 Folate synthesis pathway

Sulfonamides and trimethoprim inhibit the bacterial folic acid synthesis pathway. Sulfonamides block dihydropteroate synthase (DHPS), which prevents the formation of dihydrofolic acid (Baquero & Levin, 2021; Quinn et al., 2011; Sköld, 2001). Trimethoprim inhibits dihydrofolate reductase (DHFR), blocking the reduction of dihydrofolic acid to tetrahydrofolic acid, another essential step in folic acid metabolism (Baquero & Levin, 2021; Quinn et al., 2011; Sköld, 2001).

2.8.3 Protein synthesis

Several antibiotics target bacterial protein synthesis, by either affecting the 30S or 50S ribosomal subunit. Macrolides, such as erythromycin, bind to the large 50S ribosomal subunit, promoting the dissociation of peptidyl-tRNA from the ribosome during elongation, thus halting peptide progression. Macrolides may also prevent the formation of the 50S subunit by binding to its precursors (Chellat et al., 2016; Quinn et al., 2011). Tetracyclines act on the 30S ribosomal subunit, preventing aminoacyl-tRNA from associating with the ribosome (Grossman, 2016). Aminoglycosides also bind to the 30S subunit, but they cause misreading during translation, leading to errors in protein synthesis (Chellat et al., 2016; Quinn et al., 2011).

2.8.4 Cell membrane structure

Polymyxins, particularly polymyxin B and polymyxin E (colistin), disrupt the bacterial cell membrane by displacing magnesium and calcium ions. They achieve this by binding to phosphate residues in the lipopolysaccharide (LPS) layer, which destabilizes the membrane, increasing its permeability (Andrade et al., 2020; Baron et al., 2016).

2.8.5 DNA replication and repair

Fluoroquinolones disrupt DNA replication and repair by targeting type II topoisomerases, such as DNA gyrase and type IV topoisomerase, which are critical for chromosomal supercoiling, DNA synthesis, transcription, and cell division. These antibiotics form complexes with the enzymes and DNA, blocking the re-ligation of DNA strands, ultimately leading to cell death (Correia et al., 2017; Quinn et al., 2011).

2.9 Antimicrobial resistance

Antibiotics have been a groundbreaking advancement in medicine since their introduction in clinical use for ~80 years, saving countless lives and marking a pivotal moment in human history. However, their widespread use has led to the rapid emergence of resistant bacterial strains, raising concerns about a potential return to the pre-antibiotic era (Davies & Davies, 2010). Despite this, antibiotic resistance is not a new phenomenon but a natural one, predating the introduction of antibiotics in human medicine (D'Costa et al., 2011; Perry et al., 2016). Evidence of ancient resistance is found in permafrost, which has been frozen for millions of years (Wagner et al., 2014). Studies have shown that antibiotic resistance genes can be extracted from ancient DNA, including 5,000-year-old samples and microorganisms isolated for millions of years (Perron et al., 2015). These findings indicate that resistance mechanisms were already present in ancient microbial populations, with some even mobilized to plasmids and transposons long before the modern era (Perry et al., 2016).

A variety of mechanisms can be responsible for bacterial antibiotic resistance. Bacteria can be intrinsically resistant to specific antibiotics, whereby the cell can use genes it already possesses to survive antibiotic exposure. For example, *K. pneumoniae* is intrinsically resistant to ampicillin due to the presence of the $bla_{\text{SHV-1}}$ gene in their chromosome. On the other hand, bacteria, and especially *K. pneumoniae,* have the potential to acquire resistance, whereby whereby mutations, or gain of genetic material via horizontal gene transfer (HGT), provides new capacities that mediate survival (Darby et al., 2023).

2.9.1 Antimicrobial resistance mechanisms

Antibiotic resistance occurs when bacteria undergo changes that render antibiotics ineffective. Bacteria have developed several mechanisms to resist antibiotics (Figure 13). The four main strategies are: (i) reduction in antibiotic penetration, such as by altering the cell membrane to decrease permeability or actively expelling the antibiotic via efflux pumps; (ii) modification of the antibiotic, through chemical alterations or inactivation; (iii) alterations in target sites, which may involve target protection, mutations at the target site, overproduction of the target, enzymatic modifications, or the replacement/bypass of the target site; and (iv) utilization of alternative metabolic pathways (Darby et al., 2023).

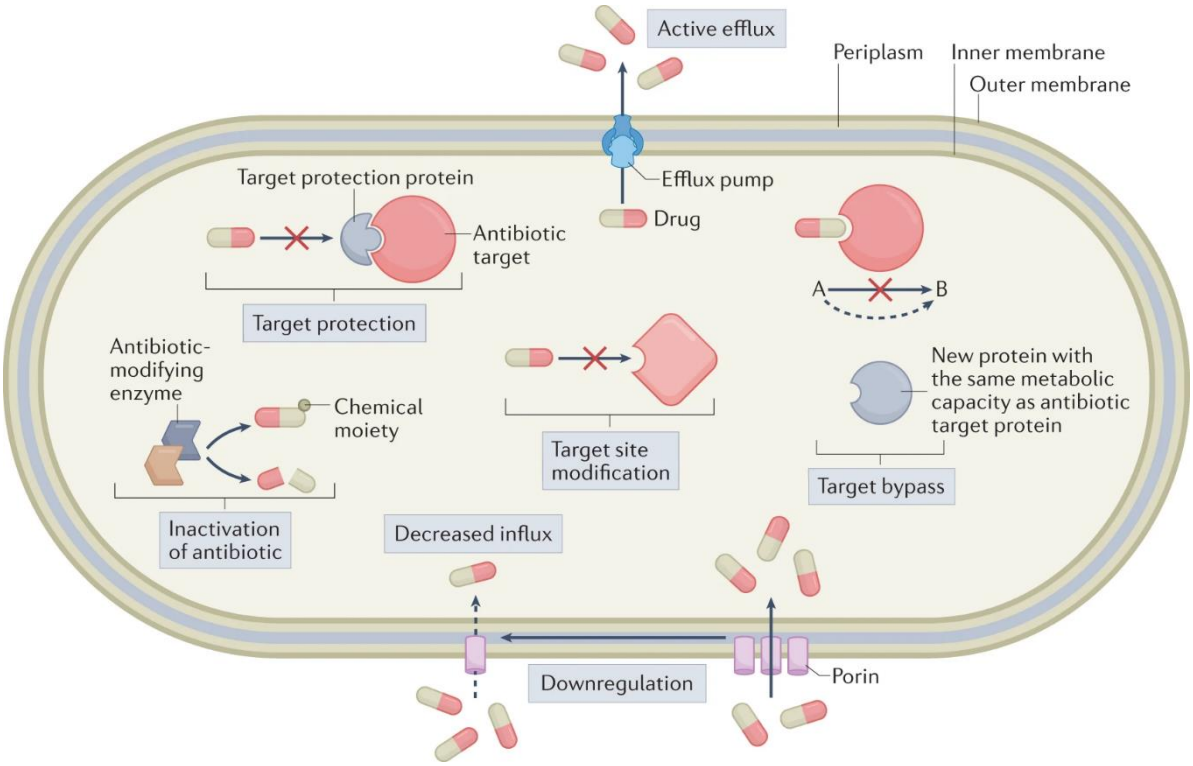


Figure 13 - Overview of the molecular mechanisms of antibiotic resistance. Antibiotic inactivation is mediated by enzymes that either degrade or chemically modify the antibiotic molecule. Enzymatic degradation typically involves the hydrolysis of a functional group on the antibiotic, rendering it ineffective. Antibiotic-modifying enzymes add chemical groups to the antibiotic, preventing it from binding to its target. Target site alteration occurs when the antibiotic's target is modified to reduce its binding affinity, which can result from mutations in the gene encoding the target protein or enzymatic modification of the binding site. In target bypass, the original target's function is taken over by a new protein that the antibiotic cannot inhibit, rendering the original target redundant and the antibiotic ineffective. Decreased antibiotic influx is achieved through alterations in membrane structure, such as the downregulation of porins, which are transmembrane proteins responsible for passive transport of compounds, including antibiotics, into the bacterial cell. Active efflux is facilitated by transmembrane efflux pumps that export antibiotics out of the bacterial cell, reducing their intracellular concentration. Target protection involves the binding of a target protection protein to the antibiotic target, thereby shielding it from antibiotic-mediated inhibition. (Darby et al., 2023).

2.9.1.1 Reduction in antibiotic penetration

Efflux pumps, which are transmembrane proteins, transport a wide range of toxic compounds, including antibiotics, out of bacterial cells in an energy-dependent manner. Although all bacteria possess multiple efflux pumps, they play a particularly critical role in mediating AMR in Gram-negative bacteria. These pumps work in concert with the bacteria's impermeable double membrane, making these pathogens intrinsically resistant to many antibiotics. The effectiveness of different efflux systems varies depending on the specific drug, with some providing high levels of resistance and others offering lower levels. However, efflux serves as a fundamental "platform" mechanism that enhances the efficacy of most other resistance mechanisms. (Darby et al., 2023). In *K. pneumoniae*, the efflux pumps *AcrAB* and *oqxAB* are most frequently observed (Bharatham et al., 2021; Y. Li et al., 2023).

Antimicrobial resistance in *K. pneumoniae* can also occur due to the reduced entry of antimicrobial agents into bacteria by reducing the outer membrane pore protein (Y. Li et al., 2023). Outer membrane proteins (OMPs) or porins are trimeric transmembrane proteins that are abundantly expressed on the outer membranes of Gram negative bacteria (Y. Li et al., 2023). In *K. pneumoniae*, *OmpK35*/*OmpK36* are the two major nonspecific porins associated with AMR (Y. Li et al., 2023). Carbapenem resistance has been described in *K. pneumoniae* due to the presence of an AmpC gene and *OmpK37* porin deficiencies (Debergh et al., 2022).

2.9.1.2 Antibiotic modification

Antibiotic resistance can arise through the modification of antibiotics by inactivating enzymes. These enzymes can neutralize antibiotics either by hydrolysis or by transferring chemical groups onto the antibiotic molecule. For example, β-lactamases, a class of hydrolytic enzymes produced by resistant bacteria, deactivate the β-lactam ring in penicillins and cephalosporins. β-Lactamases have evolved in nature in response to the production of β-lactam antibiotics by microorganisms and have been studied since the 1940s (Darby et al., 2023). The hydrolysis neutralizes the acylating group, which is essential for targeting the active site serine in peptidoglycan synthetase, thereby rendering the antibiotic ineffective (Darby et al., 2023). The list of characterized β-lactamases continues to increase; to date, the Beta Lactamase DataBase records over 8,000 distinct β-lactamases (Naas et al., 2017). *K. pneumoniae* is intrinsically resistant to numerous β-lactamase genes attributed to the prevalence of bla_{SHV} β-lactamase in the chromosome, and ampicillin resistance is a defining trait of the organism (Y. Li et al., 2023). β-lactamase genes, grouped into ESBLs, AmpC and carbapenemases, are frequently found in *K. pneumoniae* (Wyres, Lam, et al., 2020; Wyres & Holt, 2018).

Another significant example of antibiotic inactivation involves tetracycline-inactivating enzymes, which catalyze the oxidation of tetracyclines. The *tet(X)* enzyme family is the most well-known of these, having been identified across various bacterial classes. These enzymes can spread horizontally via transposable elements, imparting high-level resistance to tetracyclines (Darby et al., 2023).

Antibiotics can also be rendered ineffective through the addition of chemical groups by drug-modifying enzymes. Such enzymes have been identified for various antibiotics, including aminoglycosides, macrolides, rifamycins, streptogramins, lincosamides, and phenicols. For instance, aminoglycosides can be modified by acetyltransferases, phosphotransferases, or nucleotidyltransferases, which alter the hydroxyl or amino groups of the drug, significantly reducing its affinity for the target (Ramirez & Tolmasky, 2010). These aminoglycoside-modifying enzymes are often encoded on mobile

genetic elements, as well as on chromosomes, and are found in both Gram-positive and Gram-negative bacteria (Darby et al., 2023).

2.9.1.3 Target site alterations

The selective toxicity of most antibiotics relies on their high specificity for crucial bacterial targets. Antibiotics typically bind to a primary target with high affinity, inhibiting essential cellular functions and leading to bacterial growth inhibition or death. Resistance can arise when the structure of this primary target is altered or protected, reducing the efficiency of antibiotic binding. For example, quinolones inhibit topoisomerase enzymes by binding near their active site, but amino acid substitutions in these proteins can reduce binding and confer resistance. Similarly, β-lactam antibiotics target penicillin-binding proteins (PBPs), and mutations in genes encoding PBPs, such as PBP3 in *E. coli*, are linked to resistance. Resistance can also result from random point mutations, which accumulate over time under drug pressure, or from high-frequency mutations and recombination events (Darby et al., 2023).

Modification of the LPS layer on the outer membrane is a key mechanism of the complex mechanism leading to colistin resistance in Gram-negative bacteria. Colistin's efficacy largely depends on its ability to target LPS, leading to membrane disruption and cell death (Elias et al., 2021; Sabnis et al., 2021). Resistance arises when LPS is modified with chemical groups that alter its charge, thereby reducing the drug's binding affinity. This modification is primarily achieved by the transfer of phosphoethanolamine (pEtN) from phosphatidylethanolamine to LPS, mediated by pEtN transferase enzymes. The activity of these enzymes is regulated by chromosomally encoded systems such as *PmrAB*, *PhoPQ* or *mgrB* in many Gram-negative species, including *K. pneumoniae*, with resistant mutants being vertically inherited within strains (Darby et al., 2023; Huang et al., 2020; Shein et al., 2022).

In 2015, a novel family of mobilizable pEtN transferases, termed *mcr* (mobile colistin resistance), was discovered. This finding was particularly alarming due to the critical role of colistin in treating multidrug-resistant infections and the potential for *mcr*-mediated resistance to spread rapidly across bacterial populations. Subsequent studies have identified a growing family of *mcr* genes in various bacterial species worldwide (Hamel et al., 2021). These *mcr* enzymes confer resistance in a manner similar to chromosomally encoded systems, posing a significant threat to public health. To date, several mobile colistin resistance genes (*mcr-1* to *mcr-10*) have been identified (Darby et al., 2023; Ling et al., 2020; X. Wang et al., 2018; Y. Wang et al., 2020; Y.-Q. Yang et al., 2018). In *K. pneumoniae*, several *mcr* genes have been described: *mcr-1* (Ling et al., 2020; S. Ribeiro et al., 2021) *mcr-3* (Ling et al., 2020), *mcr-7* (Ling et al., 2020), *mcr-8* (X. Wang et al., 2018), *mcr-9* (Y. Wang et al., 2020).

2.9.1.4 Utilization of alternative metabolic pathways (target bypass)

Sulfonamides are antibiotics that target the bacterial folic acid pathway. To counteract this, bacteria can produce enzymes with reduced affinity for these drugs. Specifically, the *sul1*, *sul2*, and *sul3* genes, found mainly on mobile genetic elements, encode drug-resistant DHPS. For trimethoprim, which inhibits DHFR, resistance is often due to genes such as *dfrA* and *dfrB* located in integrons, or through enzyme overproduction resulting from chromosomal mutations. Resistance to trimethoprim-sulfamethoxazole can develop if bacteria acquire or overproduce novel DHFR and DHPS variants. This adaptation reduces drug binding and maintains folic acid production, allowing the bacteria to survive (Darby et al., 2023).

2.9.1.5 β -lactams antibiotics and β -lactamases

In this section, we delve deeper into β-lactam antibiotics. Resistance to 3GCs and/or carbapenems in *K. pneumoniae* poses a significant global health threat. As a result, these antibiotics are classified within the critical priority group of the WHO's BPPL (World Health Organization, 2024).

β-lactams are the most widely used class of antibiotics. Since the discovery of benzylpenicillin in the 1920s, thousands of new penicillin derivatives and related β-lactam classes, including cephalosporins, cephamycins, monobactams, and carbapenems, have been developed. Each new class has been designed either to broaden the spectrum of activity against additional bacterial species or to counteract specific resistance mechanisms that have emerged in targeted bacterial populations. Resistance to β-lactams is primarily due to the production of β-lactamase enzymes by bacteria, which hydrolyze the β-lactam ring, rendering the drug inactive. The latest strategy to overcome this resistance involves the development of novel broad-spectrum β-lactamase inhibitors that are effective against a wide range of problematic β-lactamases, including cephalosporinases and serine-based carbapenemases, which severely restrict therapeutic options (Bush & Bradford, 2016). β-lactam antibiotics are classified in four main groups: penicillins, cephalosporins, carbapenems and monobactams (Figure 14) (Bush & Bradford, 2016).

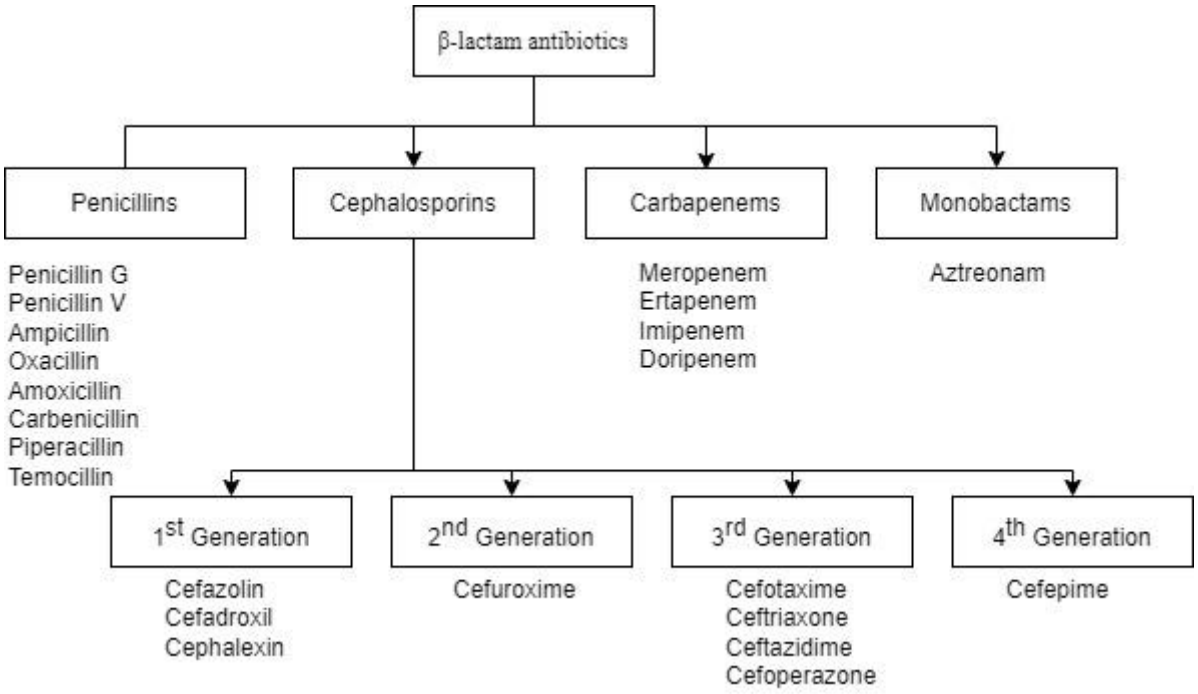


Figure 14 - Classification of β-lactam antibiotics. Based on (Bush & Bradford, 2016).

Mode of action of β-lactam antibiotics

β-lactam antibiotics are bactericidal agents that disrupt bacterial cell wall formation by covalently binding to essential penicillin-binding proteins (PBPs), which are enzymes involved in the final stages of peptidoglycan cross-linking in both Gram-negative and Gram-positive bacteria. Each bacterial species possesses its own unique set of PBPs, typically ranging from three to eight enzymes per species. The mechanism by which β-lactam antibiotics inhibit peptidoglycan transpeptidation was first detailed by Tipper and Strominger in 1965, who highlighted the structural similarity between penicillin G and the terminal D-Ala-D-Ala dipeptide of the nascent peptidoglycan in dividing bacterial cells (Tipper & Strominger, 1965). This process involves the binding of penicillin or another β-lactam antibiotic to an active site serine residue present in all functional PBPs. (Bush & Bradford, 2016).

β-lactamases

The WHO has identified carbapenem-resistant and 3rd generation cephalosporin-resistant Enterobacterales as top priorities for new antibiotic development among MDR pathogens (World Health Organization, 2024). Resistance to these antibiotics is largely driven by the production of various β-lactamases, with over 7,000 different β-lactamases identified so far, and new variants being reported frequently (Naas et al., 2017).

β-lactamases are classified based on sequence similarities according to the Ambler classification (classes A to D) (Ambler et al., 1980) or functionally by the Bush–Jacoby–Medeiros (Bush et al., 1995; Bush & Jacoby, 2010) system (groups 1 to 3). Group 1 includes cephalosporinnases (Ambler class C), which were originally chromosomally encoded. Group 2 encompasses all other serine β-lactamases, spanning several subgroups (Ambler classes A and D). Group 3 consists of metallo-β-lactamases (MBLs, Ambler class B) (Noster et al., 2021).

Extended-spectrum β-lactamases (ESBLs), which have been increasingly detected since the 1980s in both hospitals and the community, are particularly concerning (Noster et al., 2021). These enzymes can hydrolyze penicillins, monobactams, and 3rd generation cephalosporins. ESBLs typically belong to Ambler classes A and D, with class A ESBLs generally being sensitive to inhibitors like clavulanic acid (Noster et al., 2021). Examples of β-lactamases relevant to *Enterobacterales* for each class are illustrated in Figure 15.

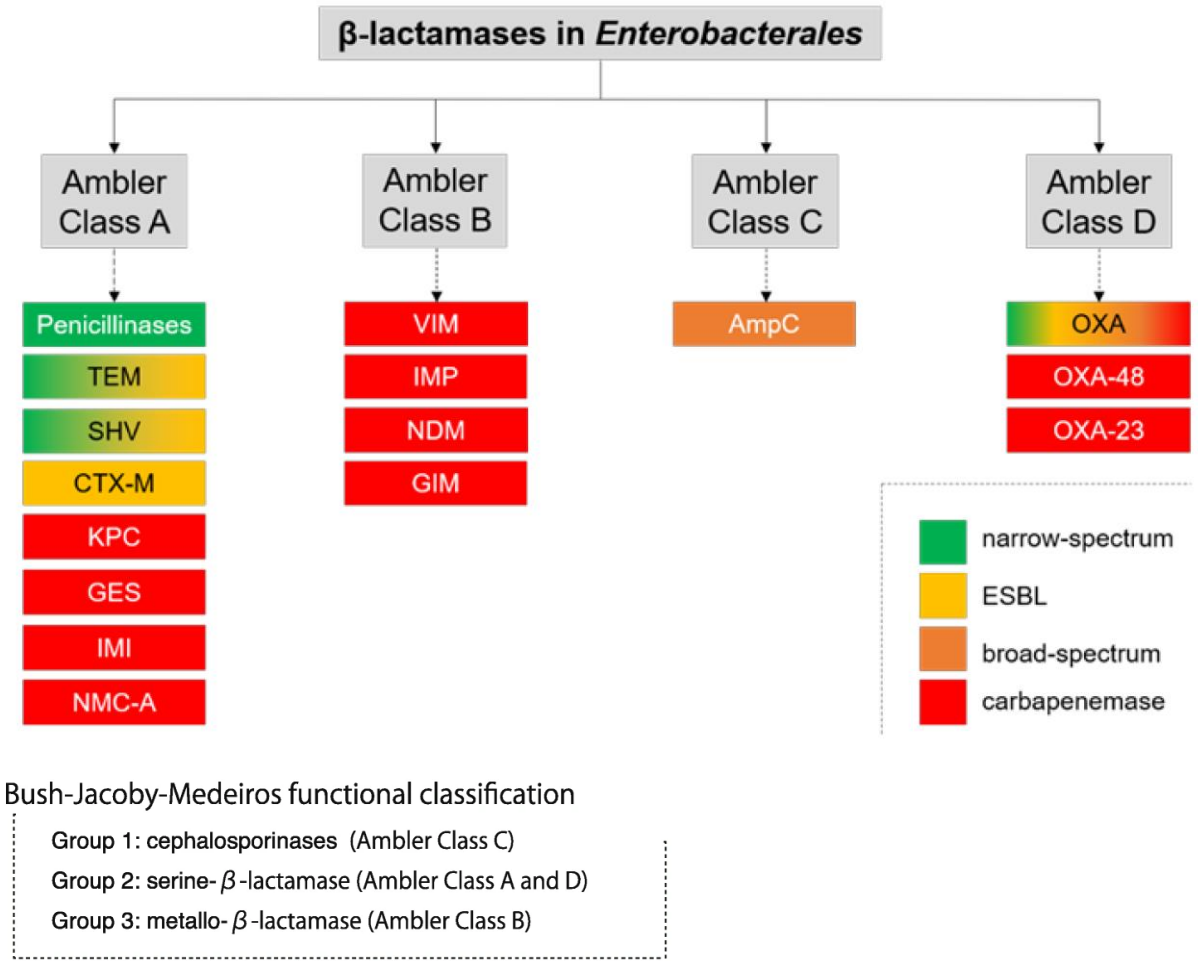


Figure 15 - Classification of β-lactamases. Molecular structure classification using the Ambler method (Ambler et al., 1980) and functional classification using the Bush-Jacobi-Medeiros method (Bush et al., 1995; Bush & Jacoby, 2010) have both been employed. In the Ambler classification system, β-lactamases are divided into four classes—A, B, C, and D—based on specific motifs in their primary amino acid sequences. Classes A, C, and D are serine-based enzymes, meaning they use a serine residue at their active site to hydrolyze β-lactam antibiotics. In contrast, class B β-lactamases utilize zinc ions as cofactors for their enzymatic activity. The Bush-Jacoby-Medeiros functional classification groups β-lactamases into three categories (groups 1 to 3) based on their ability to degrade different β-lactam antibiotics and their response to inhibitors (Noster et al., 2021; Sawa et al., 2020).

Extended spectrum β-lactamases

β-lactamases have become widespread due to the overuse of β-lactam antibiotics. In Gram-negative bacteria, broad-spectrum β-lactamases, like TEM-1 and SHV-1, emerged after the introduction of first- and second-generation cephalosporins. With the subsequent use of extended-spectrum β-lactam antibiotics, resistant enzymes evolved, particularly ESBLs. They are classified as class A serine β-lactamases in the Ambler system and according to the functional classification scheme of Bush et al., they are clustered in group 2be (Bush et al., 1995). They can hydrolyze a wide range of β-lactam antibiotics, including extended-spectrum cephalosporins and monobactams, but not carbapenems or cephamycins (cefoxitin) (Castanheira et al., 2021).

Multiple ESBL families exist, but the majority of the ESBLs are represented by TEM, SHV, and CTX-M genes (Castanheira et al., 2021). Five major CTX-M ESBL groups, based on ≥10% variance in amino acid sequence identity exist — CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (D'Andrea et al., 2013). ESBLs have been detected in humans (Wyres, Hawkey, et al., 2019), animals (Debergh et al., 2022; Rodrigues et al., 2022), the food chain (Daehre, Projahn, Friese, et al., 2018) and the environment in *K. pneumoniae* (Ben Said et al., 2015; Conte et al., 2017). In *K. pneumoniae*, the most prevalent ESBL in humans is $bla_{\text{CTX-M-15}}$ (Wareth & Neubauer, 2021).

Carbapenemases

Carbapenems have been effective in treating infections caused by ESBL-producing bacteria, but their extensive use has led to the spread of another significant group of β-lactamases: carbapenemases. These enzymes vary in the resistance they confer. Class A includes serine β-lactamases like *K. pneumoniae* carbapenemase (KPC), non-metallo carbapenemase (NMC), and imipenemase (IMI). Class B carbapenemases are metallo-enzymes capable of hydrolyzing all β-lactams except monobactams, with notable examples including NDM, VIM, and IMP. Class D carbapenemases include oxacillinases and OXA-48 derivatives (Noster et al., 2021). The presence of carbapenemases has been described in *K. pneumoniae* in humans (Campos-Madueno et al., 2022; Sun et al., 2024), animals (Sellera et al., 2021), the food chain (Chaalal et al., 2020) and the environment (Montenegro et al., 2023; Nasri et al., 2017; X. Zhang et al., 2012).

β-lactamase inhibitors

The development of β-lactam/β-lactamase inhibitor combinations has been a key strategy to combat β-lactamase-mediated resistance. Following the discovery of clavulanic acid, penicillin-inhibitor combinations (such as amoxicillin–clavulanate) became widely used, but they are limited in their spectrum and do not cover certain resistant β-lactamases like KPC. The rise of more resistant β-lactamases has driven the need for better treatment options, particularly against Gram-negative bacteria. Recent advancements include the introduction of diazabicyclooctanones (DBOs), such as avibactam, which is a non-β-lactam β-lactamase inhibitor. Avibactam, when combined with ceftazidime, is effective against class A β-lactamases, including KPC, and is used to treat complex infections such as intra-abdominal, urinary tract infections, and pneumonia. This success has led to the development of other DBOs, like relebactam, which is combined with imipenem. Despite the clinical success of DBOs, there is ongoing research to understand their interactions with β-lactamases, the stability of their acylenzyme complexes, and the mechanisms behind potential resistance. While resistance to DBOs is still emerging, laboratory studies have shown that mutations can reduce their effectiveness, especially in KPC and AmpC enzymes (Tooke et al., 2019).

2.9.1.6 Antimicrobial tolerance of biofilms

Biofilms are structured aggregates of bacterial populations with distinct properties compared to planktonic cells. Biofilm formation begins with bacterial adherence to biotic or abiotic surfaces, followed by maturation involving extracellular matrix production, and culminates in a dispersal phase influenced by environmental factors. The transition from planktonic to biofilm growth is regulated by the secondary messenger c-di-GMP, with high levels promoting biofilm formation and low levels triggering dispersal. The biofilm matrix, composed of polysaccharides, proteins, extracellular DNA, and other components, creates a microenvironment with nutrient and oxygen gradients, leading to physiological heterogeneity. Over time, biofilms generate persister cells and mutants, and dispersed bacteria can attach to new surfaces, potentially spreading infections to nearby or distant tissues, such as from dental biofilms to heart valves or implants to bone tissues. (Ciofu et al., 2022). They are a major contributor to infections and antibiotic failure, are implicated in a wide range of infections, including medical devices and implants, lung, bladder, dental, skin, ear, nose and throat, sinusitis, and orthopedic infections (de la Fuente-Nunez et al., 2023; Hancock et al., 2021).

Biofilms exhibit metabolic diversity, with active populations located on the oxygenated surface and dormant, non-growing populations in the central anoxic zones. Antimicrobial tolerance in biofilms arises from three key mechanisms: metabolic heterogeneity, impaired antibiotic penetration, and activation of stress-adaptive responses (Figure 16). Active subpopulations on the biofilm surface increase efflux pump and β-lactamase expression, while dormant subpopulations, with low growth rates, show reduced expression of antibiotic targets and decreased active antibiotic uptake. Matrix components, such as negatively charged polysaccharides, extracellular DNA (eDNA), and filamentous phages, bind to positively charged antibiotics like aminoglycosides and polymyxins, slowing their penetration. Additionally, stress responses, including stringent and SOS pathways, along with transient resistance via upregulated β-lactamase production or lipopolysaccharide modifications, further contribute to biofilm antibiotic tolerance by impairing antimicrobial efficacy (Ciofu et al., 2022).

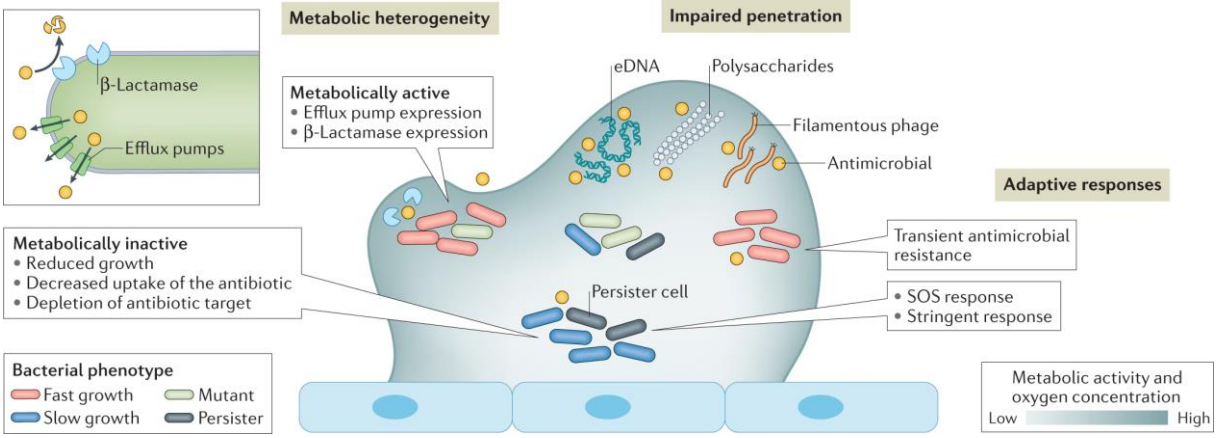


Figure 16 - The mechanisms of antimicrobial tolerance of a biofilm (Ciofu et al., 2022).

2.9.2 Transmission of antibiotic resistance

Figure 17 shows the mechanisms through which HGT can take place; through transformation (direct uptake of naked DNA from the environment), transduction (transfer of DNA between two bacteria through bacteriophages) and conjugation (transfer of DNA through direct contact between two cells) (Castañeda-Barba et al., 2024; Gyles & Boerlin, 2014).

Various mobile genetic elements (MGEs) function as vectors for genetic material transfer, either within a single genome (intracellular) or between different cells (intercellular) (Partridge et al., 2018). Among intracellular elements, transposable elements are well-characterized, utilizing transposases for movement and categorized into groups such as insertion sequences (IS) and transposons (Tn). Other intracellular elements, like integrons, are not mobilized by transposases but use site-specific recombination to move resistance genes between defined sites (Partridge et al., 2018). Intercellular gene transfer is facilitated by elements such as bacteriophages, conjugative and mobilizable plasmids, and genomic islands. Integrated conjugative elements (ICEs) are a type of MGE capable of both intra- and intercellular mobility (Partridge et al., 2018).

Horizontal gene transfer can result in co-resistance when multiple resistance genes are transferred together on the same MGE, in contrast to cross-resistance, where a single mechanism confers resistance to multiple compounds. Both co- and cross-resistance can lead to co-selection of resistance across different compounds, such as various classes of antibiotics or heavy metals, following exposure to a single agent (Pal et al., 2017).

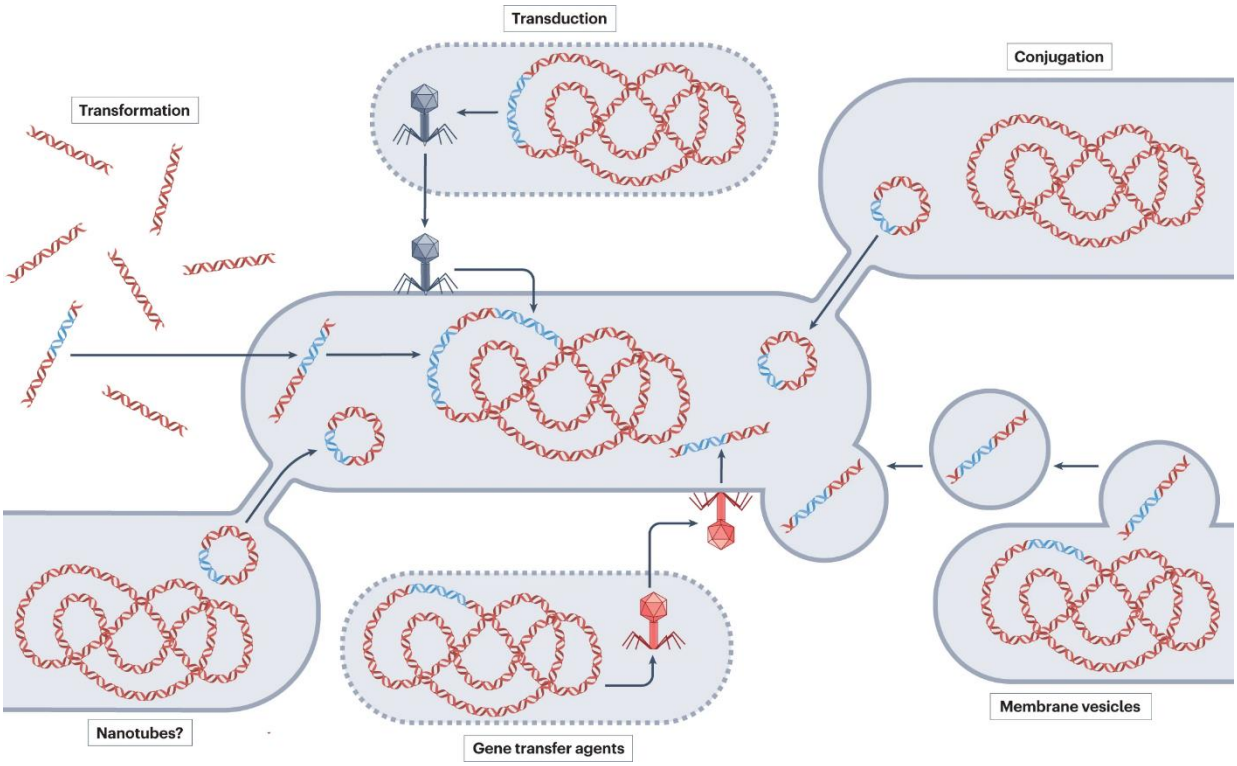


Figure 17 - Mechanisms of horizontal gene transfer. Horizontal gene transfer (HGT) mechanisms encompass transformation (top left), transduction (top middle), conjugation (top right), nanotubes (bottom left), gene transfer agents (bottom middle) and membrane vesicles (bottom right). Transformation involves the uptake of exogenous DNA from the environment; transduction is the process where virus-mediated transfer of genetic material occurs; conjugation occurs through direct cell-to-cell contact, where plasmids or other conjugative elements are transferred; nanotubes are membranous connections between bacteria that allow exchange of genetic material; gene transfer agents are virus-like particles capable of gene transfer; and membrane vesicles are spherical nanostructures secreted by Gram-negative bacteria that can carry genetic material. While transformation, transduction, and conjugation are the most recognized mechanisms in the spread of antimicrobial resistance (AMR), the roles of gene transfer agents, nanotubes, and membrane vesicles are less understood and remain largely unexplored in this context. It's worth noting that recent studies suggest that nanotubes might result from cell death rather than functioning in plasmid transfer (indicated by question mark). (Castañeda-Barba et al., 2024)

2.9.2.1 Plasmids

Plasmids are circular or linear double-stranded DNA molecules that replicate independently and can be transferred between bacterial species. Most plasmids are identified through the positive selection of traits such as antimicrobial resistance or virulence.

The machinery required for bacterial conjugation, which enables the transfer of DNA from a donor to a recipient bacterium, can be encoded either by plasmids or by integrative conjugative elements (ICEs). These elements not only ensure the mobilization of their own DNA but can also facilitate the transfer of ARGs on other MGEs, thus acting as vehicles for the dissemination of resistance genes across diverse bacterial species. Some conjugative plasmids have the additional ability to mobilize genes from plasmids and even chromosomes in the plasmid recipient back to the donor, a process known as

retromobilization. This ability highlights the ecological role of conjugative plasmids, as they could potentially allow a plasmid carrier to tap into the broader pool of ARGs within a microbial community (Castañeda-Barba et al., 2024).

Most plasmids contain specific regions known as replicons, which encode functions responsible for initiating and regulating replication. Since 2005, PCR-based replicon typing (PBRT) has been used to detect the major plasmid families in *Enterobacteriaceae*, initially covering 18 major incompatibility (Inc) groups (Carattoli et al., 2014). With the rapid increase in WGS and whole-plasmid sequence data generated by high-throughput sequencing platforms, there is a growing need to identify plasmids using raw sequence data or contigs. Caratolli et al. described the development of two user-friendly web tools designed for the quick identification of plasmids in *Enterobacteriaceae*, which are particularly relevant for epidemiological and clinical microbiology investigations into the plasmid-mediated spread of antimicrobial resistance. The PlasmidFinder tool, based on a curated plasmid replicon database, allows microbiologists without advanced bioinformatics skills to identify plasmids in WGS data from *Enterobacteriaceae*. It supports the analysis of raw high-throughput reads, assembled contigs, or Sanger sequences and not only detects replicons but also assigns plasmids to specific lineages, linking them to established knowledge on Inc groups and suggesting potential reference plasmids for each lineage (Carattoli et al., 2014).

Plasmids, which vary in size from a single kilobase to several thousand kilobases, are highly diverse genetic elements. They are broadly classified as conjugative, mobilizable, or non-mobilizable, depending on whether they encode their own conjugative machinery or rely on existing systems. The plasmid 'backbone' contains genes essential for conjugation, stable maintenance, regulatory control, and replication. In addition to these core regions, plasmids often carry 'accessory' regions that encode functions promoting their own survival, such as toxin-antitoxin systems, as well as host survival, including genes for metal and antibiotic resistance, virulence, and various metabolic functions (Castañeda-Barba et al., 2024). These accessory regions are highly variable between plasmids, largely due to frequent recombination events mediated by transposons, insertion sequences, and integron gene cassettes. This recombination leads to the creation of mosaic plasmids—plasmids composed of genetic elements from distinct sources. Mosaic plasmids are characterized by a significantly higher proportion of transposase genes and ARGs and are more commonly found in clinically relevant genera such as *Escherichia*, *Klebsiella*, and *Salmonella* (Castañeda-Barba et al., 2024).

K. pneumoniae has been associated with a wide range of plasmid replicon types and exhibits a higher median number of plasmids per genome compared to other species (Wyres & Holt, 2018). This suggests *K. pneumoniae* is highly permissive to plasmids, facilitating its role as a recipient and vector of plasmid-borne AMR genes from diverse horizontal gene transfer donors. Lower fitness costs

associated with plasmid carriage in *K. pneumoniae*, as seen in several studies, likely enhance its ability to maintain and transmit plasmids over time. However, plasmid permissiveness and AMR gene distribution are not uniform across all *K. pneumoniae* lineages, suggesting variations in their capacity to act as plasmid donors or recipients, which requires further investigation (Wyres & Holt, 2018).

2.9.3 AMR characterization methods

Several methods can be used to detect antimicrobial resistance, namely phenotypic and genotypic methods. Phenotypic tests enable the deduction of the type of resistance expressed by the bacteria, while genotypic tests, reveal the resistance gene(s) present. The combination of these two methods enables the understanding of not only what is written in the genes, but also what is expressed.

Phenotypic methods include, among others, culture based screening methods and broth microdilution methods. Genotypic methods include PCR based methods, multiplex LCR methods and whole genome sequencing.

2.9.3.1 Phenotypical methods

Screening media

Selective media are commonly used to screen samples for ESBL- and carbapenemase-producing Enterobacterales. These media often contain chromogens that facilitate presumptive identification of species based on species-specific enzyme activity. Various commercially available agars for ESBL detection include CHROMagar ESBL (CHROMagar, Paris, France), chromID ESBL agar (bioMérieux, L'Etoile, France), ESBL ChromoSelect Agar (Merck, Darmstadt, Germany), and Brilliance ESBL agar (Oxoid, Basingstoke, UK). In addition to ESBL agars, chromogenic agars for the detection of CPE are commercially available: Brilliance CRE (Oxoid, Basingstoke, UK), Chromatic CRE (Liofilchem, Roseto degli Abruzzi, Italy), chromID CARBA and chromID OXA-48 (bioMérieux, Marcy-I'Etoile, France) (Noster et al., 2021).

Broth microdilution

The reference method for antimicrobial susceptibility testing (AST) is broth microdilution, which assesses the MIC (minimum inhibitory concentration) of an organism against an antimicrobial agent. The MIC is defined as the lowest concentration of an antibacterial agent expressed in mg/L that, under strictly controlled in vitro conditions, completely inhibits the visible growth of the organism (The European Committee on Antimicrobial Susceptibility Testing, 2003). Clinical breakpoints determine whether the organism is categorized as susceptible at normal dosing (S), susceptible at increased exposure (I) or resistant (R) to the agent in question. Epidemiological cut-off values (ECOFFs) and tentative epidemiological cut-off values (TECOFFs) distinguish microorganisms without (wild type) and with phenotypically detectable acquired resistance mechanisms (non-wild type) to the agent in question (Supplementary table 5 and Supplementary table 6) (EUCAST, n.d.). ECOFFS were used in this research for determining the MIC of all *K. pneumoniae* isolates from the various sources. The first panel includes a screening for presence of ESBL production (cefotaxime and ceftazidime) and carbapenem resistant (meropenem) isolates. In case of resistance observed towards these antimicrobials, the second panel is tested, providing a detailed panel to test for ESBL/AmpC/carbapenem resistant phenotype. Phenotype determination of ESBL/AmpC/Carbapenem was based on the MIC values from EUVSEC2 and values are displayed in Figure 18 (EFSA & ECDC, 2023). The levels of antimicrobial resistance are described according to the following EFSA criteria : “rare: <0.1%”, “very low: 0.1% to 1.0%”, “low: 1% to 10.0%”, “moderate: 10.0% to 20.0%”, “high: 20.0% to 50.0%”, “very high: 50.0% to 70.0%”, “extremely high:> 70.0%” (EFSA & ECDC, 2023).

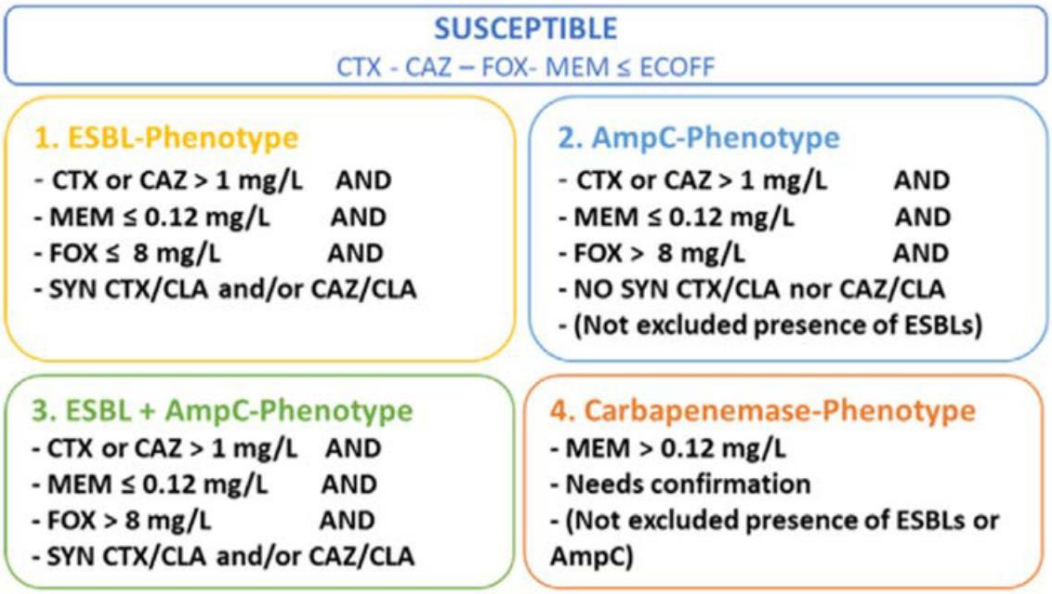


Figure 18 - Phenotypes inferred based on the resistance to the β-lactams included in EUVSEC 2. Synergy is defined as a ≥ 3 twofold concentration decrease in an MIC for either antimicrobial agent tested in combination with clavulanic acid vs. the MIC of the agent when tested alone (EFSA & ECDC, 2023).

2.9.3.2 Genotypical methods

Molecular methods have the highest sensitivity and specificity for the detection of resistance genes and can be applied to cultured isolates or directly to clinical specimens. They can provide accurate information on the presence of ARGs, the type of ESBL enzyme or carbapenemase and have a relatively short time-to-result.

Polymerase chain reaction

PCR is a technique developed in the 1980s by Kary Mullis (Saiki et al., 1988) that revolutionized molecular biology by enabling the rapid and exponential amplification of target DNA sequences. It uses a forward and reverse PCR primer, along with the enzyme DNA polymerase, in the presence of deoxyribonucleotides. Conventional PCR involves three steps: (i) denaturation of double-stranded DNA at 95°C, (ii) annealing of the PCR primers at 50 to 60°C, and (iii) extension of the DNA at 72°C. This technique is routinely used in microbiology labs to detect genes in bacteria, provided that a DNA sequence for the whole or partial gene is available to design PCR primers. The amplified gene product can be visualized by running agarose gels and staining the DNA with ethidium bromide or other fluorescent dyes (Anjum et al., 2017).

Since PCR's development, several advances have emerged, including real-time PCR (RT-PCR) and isothermal amplification methods, such as loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA). The main difference between conventional PCR and RT-PCR is that in RT-PCR, amplification is monitored in real time due to the presence of fluorescent dyes, which is why it is also called quantitative PCR (qPCR) (Anjum et al., 2017).

Multiplex PCR, which allows the simultaneous amplification of several target DNA fragments, can be performed using either conventional or RT-PCR. This has simplified the detection of multiple antimicrobial resistance (AMR) genes in bacteria, making it a widely used technique to replace single-gene amplification methods. In a multiplex PCR assay, different resistance genes can be detected simultaneously using various primers included in the assay mix (Anjum et al., 2017).

Digital PCR (dPCR), which splits a PCR sample into thousands or even millions of subsamples, each containing either a single or no copy of the target DNA, is another more recent PCR technique. dPCR relies on microfluidics and can be either droplet-based or chip-based. It enables absolute quantification of DNA/RNA copy numbers without the need for standard curves used in qPCR. Additionally, dPCR supports multiplex PCR and can amplify DNA samples with varying ratios of abundant and rare DNA molecules (H. Zhu et al., 2020).

The AMR-ARRAY

Detecting ARGs using single polymerase chain reaction (PCR) assays is possible, but it requires multiple separate tests to cover a broad range of genes. (Timmermans et al., 2022). Multiplex PCR and microarrays are the preferred methods for simultaneous detection of multiple target genes, providing a comprehensive overview of an isolate's resistance profile. These methods are particularly advantageous as they can detect resistance genes even at low expression levels or with low catalytic activity. However, their limitation lies in their ability to detect only the genes specifically targeted by the assay, necessitating frequent updates to include newly emerging resistance genes. Although several in-house multiplex PCR assays have been published, commercially available PCR assays are now widely preferred for their convenience, despite often being limited in target range and relatively expensive (Timmermans et al., 2022).

Timmermans et al. (2022) developed an in-house multiplexed bead array (the AMR-ARRAY) to detect ARGs and mutations commonly linked to resistance against β-lactams, (fluoro)quinolones, colistin, macrolides, and aminoglycosides in Enterobacteriaceae (Timmermans et al., 2022). This 53-plex assay integrates ligase chain reaction (LCR) with Luminex® technology and targets specific genetic markers. The AMR-ARRAY boasts several strengths: (i) cost-effectiveness, (ii) a broad analytical range covering 50 markers across 5 AB families, (iii) a user-friendly single-tube design with 4 successive steps, (iv) a turnaround time of under 8 hours, (v) the ability to detect allelic variations at critical SNPs, and (vi) an open-access, easily upgradable format with freely available probe sequences, procedures, and software source code (Timmermans et al., 2022).

Next generation sequencing

Nearly 25 years after the discovery of the DNA structure, the first DNA sequencing method (Sanger sequencing) was developed (Sanger et al., 1977; Watson & Crick, 1953). This technique involved adding chain-terminating dideoxynucleotides, which were initially radioactively labeled and later fluorescently labeled, to sequence a DNA strand complementary to the target template. The resulting fragments were then size-separated and analyzed using gel electrophoresis to determine the sequence. This method, known as Sanger sequencing, saw significant improvements with the advent of capillary electrophoresis, which enhanced its efficiency. Sanger sequencing became widely accepted as the "first-generation sequencing" method, enabling the sequencing of a range of genomes, from bacteria and phages to humans. However, its throughput was limited by the fact that only one sequencing reaction could be analyzed at a time (Hu et al., 2021).

Between 2004 and 2006, "next-generation sequencing (NGS)" technologies were introduced, revolutionizing DNA sequencing with their high throughput and single-molecule sequencing

capabilities. Second-generation platforms, like Illumina and Ion Torrent, typically involve DNA fragmentation, end-repair, adapter ligation, surface attachment, and in-situ amplification, enabling the massively parallel sequencing of short reads (250-800 bp). The short-read nature of these technologies presents challenges when reassembling long stretches of DNA, particularly in regions with structural variations or low complexity (Boolchandani et al., 2019).

Third-generation sequencing platforms, such as Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT), offer significantly longer read lengths, exceeding 10 kb. These long-read technologies address the limitations of short-read sequencing, especially in detecting genome-wide repeats and structural variants. Third-generation sequencing involves minimal library preparation and directly sequences unfragmented DNA in real-time, with the primary requirement being the generation of high molecular weight DNA. Although early third-generation technologies initially faced issues with read accuracy compared to second-generation methods, continuous improvements in software analysis have significantly enhanced their accuracy over time (Boolchandani et al., 2019).

A multitude of software packages are available for species identification, MLST typing, and AMR detection. Lam et. al (2021) developed a genomic surveillance framework and genotyping tool for *K. pneumoniae* and its related species complex, called Kleborate. The open source tool allows identification of species, the presence of acquired virulence determinants, acquired ARGs, and serotype prediction. Furthermore, based on the detected virulence and AMR determinants, the organism is given a virulence and resistance score, respectively (Lam et al., 2021a). The virulence score is based on the presence of the virulence determinants yersiniabactin, colibactin, aerobactin and salmochelin. The resistance score is determined by the presence of an ESBL, a carbapenemase or a carbapenemase plus colistin resistance.

For this doctoral research, a pipeline specific for *K. pneumoniae* was developed in Galaxy, which is freely available for non-commercial use at https://galaxy.sciensano.be (registration required). The *Klebsiella* pipeline enables comprehensive characterization from raw FASTQ data, including pre-processing, quality control, detection of AMR and virulence genes, characterization of STs and MGEs. A minimum spanning tree based on cgMLST can be generated and SNP-based phylogenomic investigations can be performed using the pipeline.

3 Aspergillus fumigatus

3.1 Generalities about fungal infections

Fungi represent one of the most diverse groups of organisms on the planet, playing an essential role in ecosystem processes and functioning (Hyde, 2022). While fungi have significant economic value and beneficial applications, such as antibiotic production, biotechnology, food processing, and fermentation, they are also known to cause infections in humans. Currently, over 600 fungal species are identified as pathogenic to humans, with an estimated annual incidence of 6.5 million invasive fungal infections and 3.8 million deaths, of which about 2.5 million are directly attributable to fungal infections (Denning, 2024). For a fungal species to become a human commensal or pathogen, it must thrive at 33-37°C (Fisher et al., 2012). However, due to climate change and fungi's ability to adapt and evolve towards thermotolerance, a gradual adaptation to increasing temperatures could result in a rise in the number of human pathogenic fungi (Nnadi & Carter, 2021).

3.2 Generalities about *Aspergillus fumigatus*

Aspergillus fumigatus is a widespread saprophytic mould that naturally lives on decaying plant material and in soil (J. P. Latgé, 1999). This highly sporulating mould is commonly found in compost from household and green waste, mouldy hay, and woodchips (Jeanvoine et al., 2017a; Shelton et al., 2022). Due to its small size (2.5 to 3 µm), they are easily released in the air where they stay airborne both indoors and outdoors (J. P. Latgé, 1999). It is estimated that humans inhale hundreds of *A. fumigatus* conidia per day. Although exposure is common, only a minority will develop disease (Kosmidis & Denning, 2015). The disease usually manifests in the lungs, although dissemination to all organs is possible. *A. fumigatus* is not considered a primary human pathogen, however with an increase of the immunocompromised community, it has become an opportunistic pathogen (J. P. Latgé, 1999).

3.3 Taxonomy of *Aspergillus fumigatus*

Aspergillus is a diverse genus which belongs to the Order Eurotiales (Table 1, Figure 19 and Figure 20) and contains a large number of species possessing a worldwide distribution (Tsang et al., 2018). They are ubiquitous and can be found in the air, soil, vegetation and indoor environments. The order Eurotiales includes several economically significant genera, such as *Aspergillus*, *Penicillium*, *Rasamsonia*, and *Talaromyces* (Samson et al., 2014; Tsang et al., 2018). Species within this order exhibit a wide range of characteristics and include some of the most notable organisms responsible for food spoilage (e.g., *A. proliferans* [Eurotium morph], *Paecilomyces variotii]), mycotoxin producers (e.g., *A. flavus*; aflatoxins), human pathogens (e.g., *A. fumigatus*, *A. flavus*), and indoor contaminants (e.g., *A. versicolor*, *A. penicillioides*) (Frisvad et al., 2019; Houbraken et al., 2020; Samson et al., 2019). Despite

their negative impact on human activities, these species are also harnessed in food fermentation processes (e.g., *A. oryzae*: soy sauce, miso), in biotechnology for the production of organic acids and enzymes (e.g., *A. niger*: citric acid), and in pharmaceutical manufacturing (e.g., *A. terreus* produces the statin lovastatin as a secondary metabolite, which is used to reduce high blood cholesterol) (Frisvad et al., 2019; Houbraken et al., 2012, 2014). The classification and identification of *Aspergillus* has been based on phenotypic characters but is now strongly influenced by molecular and chemotaxonomic characterization (Samson et al., 2014).

Table 1 - Taxonomy of *Aspergillus fumigatus*. From domain to species level.

Taxonomic rank	Taxonomic identification
Domain	Eukaryota
Kingdom	Fungi
Phylum	Ascomycota
Class	Eurotiomycetes
Order	Eurotiales
Family	Aspergillaceae
Genus	*Aspergillus*
Subgenus	*Fumigati*
Section	*Fumigati*
Species	*Aspergillus fumigatus*

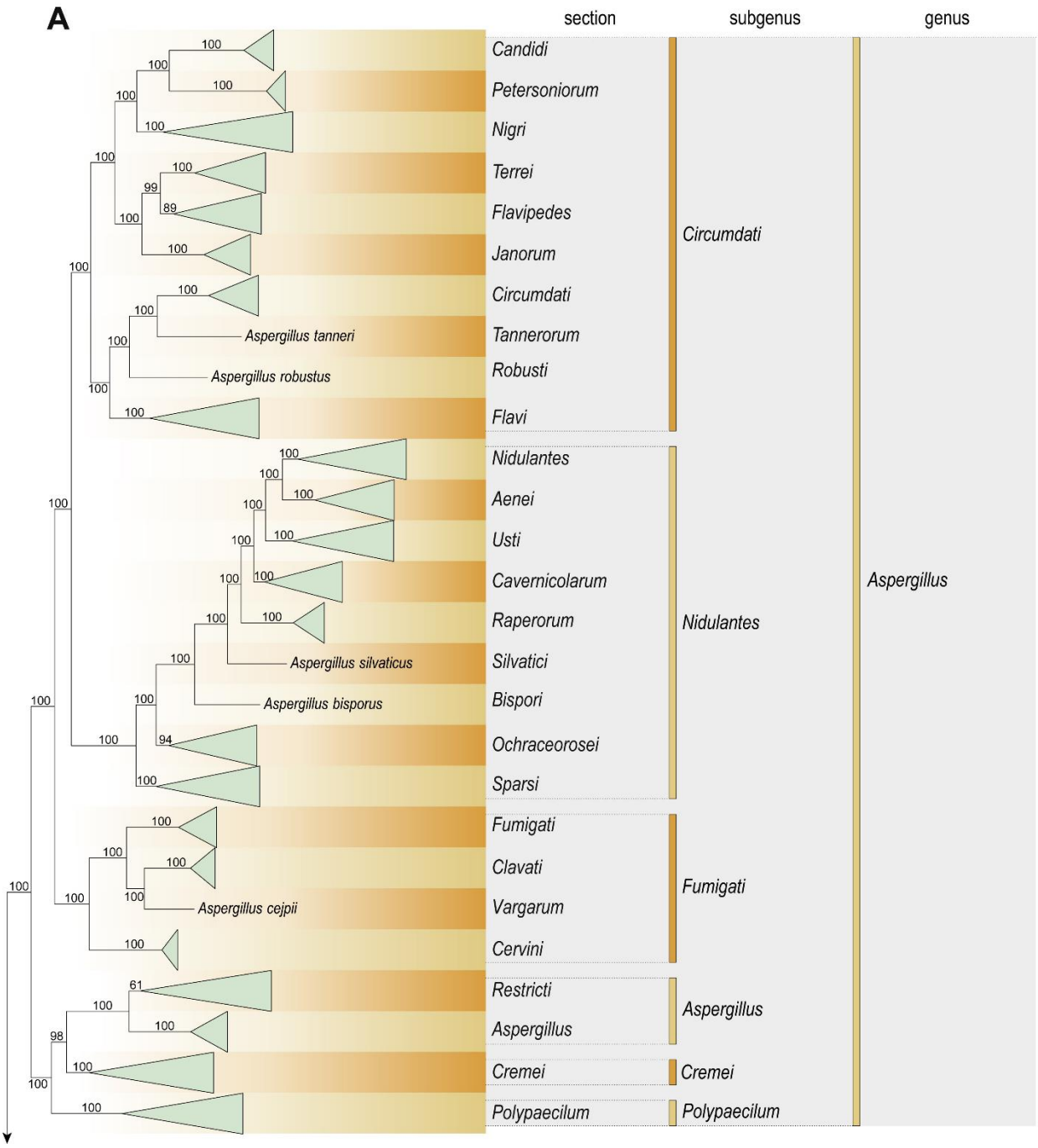


Figure 19 - Combined phylogeny of the genus *Aspergillus* using nine loci (RPB1, RPB2, Cct8, Tsr1, CaM, BenA, SSU, LSU, ITS). In the phylogram, only the subgenera and sections of *Aspergillus* are shown; the other genera are collapsed as one outgroup clade (Houbraken et al., 2020).

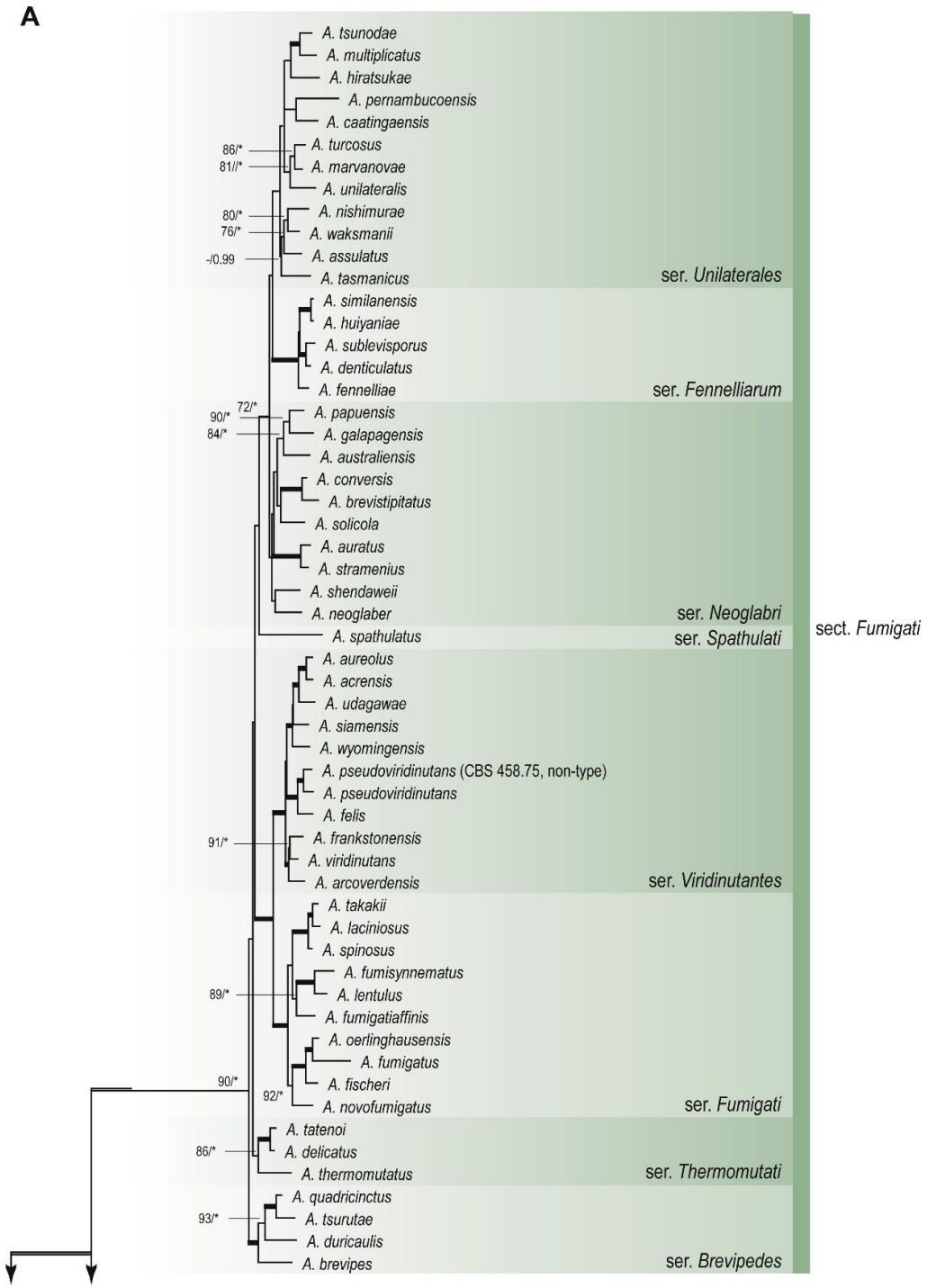


Figure 20 -Combined phylogeny for BenA, CaM and RPB2 data sets showing the phylogenetic relation of species, series and sections within *Aspergillus* subgen. *Fumigati*. The BI posterior probability (pp) values and bootstrap percentages of the maximum likelihood (ML) analysis are presented at the nodes; fully supported branches are thickened. Values less than 70 % bootstrap support (ML) or less than 0.95 posterior probability (Bayesian analysis) are indicated with a hyphen or not shown. The bar indicates the number of substitutions per site. The phylogram is rooted with *Hamigera avellanea*. (Houbraken et al., 2020).

The organism is characterized by green, echinulate conidia measuring 2.5 to 3 µm in diameter, produced in chains in a basipetal manner from greenish phialides that are 6 to 8 µm by 2 to 3 µm (Figure 21). Some isolates of *A. fumigatus* lack pigmentation and produce white conidia. The conidial chains are borne directly on broadly clavate vesicles, 20 to 30 µm in diameter, without the presence of metulae (Fig. 1). No sexual stage has been identified for this species. *A. fumigatus* exhibits rapid growth, with colonies reaching approximately 4 ± 1 cm within one week on Czapek-Dox agar at 25°C. It is a thermophilic species, capable of growing at temperatures as high as 55°C and surviving at temperatures up to 70°C (J. P. Latgé, 1999).

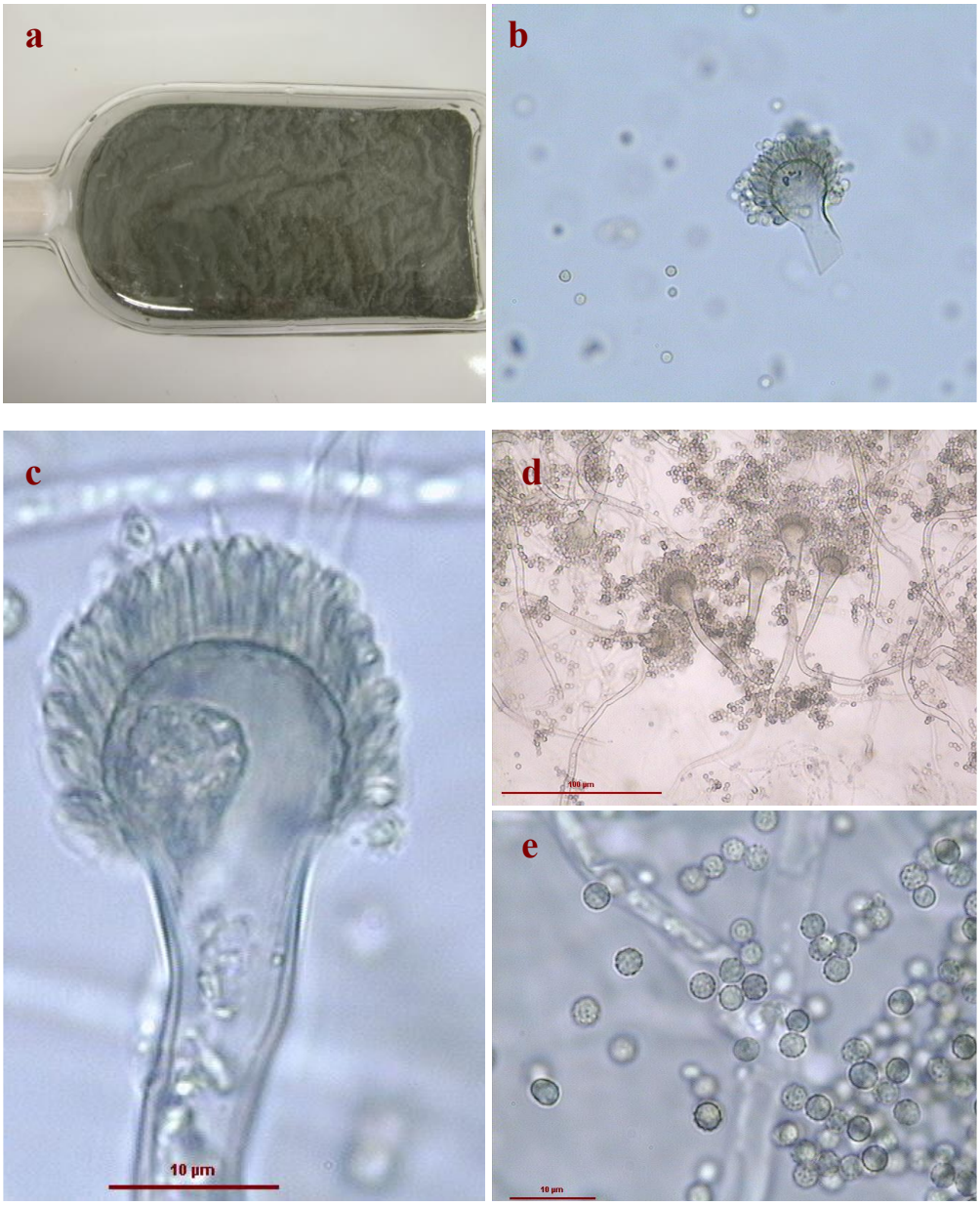


Figure 21 – Morphology of *Aspergillus fumigatus* IHEM 3007 from the BCCM/IHEM collection. a. Appearance of the colony at the time of harvest on Synthetic Liquid Medium (SLM) in stationary culture after 7 days at 24°C; b. Microscopic view of dry matter; c. Conidiophore, vesicle, and phialide (uniseriate *Aspergillus*); d. Conidial heads; e. Conidia.

3.4 Reproduction cycle and adaptation strategies of *Aspergillus fumigatus*

Three processes of reproduction are recognized in *A. fumigatus*: asexual, sexual, and parasexual (Figure 22A). A fungal colony initiates from the germination of a single spore, which develops into a network of hyphae. After a few days, vegetative hyphae exposed to air form aerial hyphae that produce asexual reproductive structures known as conidiophores, each generating up to 10^4 asexual spores. Consequently, within one week, a colony may produce up to 10^9 spores that become airborne. During each mitotic division, there is a small probability of mutation. However, due to the large number of mitotic divisions during asexual reproduction, this provides a substantial supply of spontaneous mutations, such as those conferring azole resistance in an azole-rich environment (Figure 22B) (Verweij, Zhang, et al., 2016).

Sexual reproduction in fungi requires two parental strains of opposite mating types, MAT1-1 and MAT1-2. Following fertilization, a fruiting body known as a cleistothecium develops, producing around 10^4 sexual spores from a single zygote. These spores exhibit genetic diversity due to recombination during meiosis, enhancing genetic variation in the parental strains through genotype reshuffling. Genes and alleles linked to low-level resistance can evolve into more resistant phenotypes within different genetic backgrounds (Figure 22C). Persistent or increased azole pressure promotes the emergence of high-level resistant phenotypes through natural selection. Although sexual reproduction has been observed only under specific laboratory conditions and can take several months to complete, it is recognized as a primary source of increased variation in fungi (Camps, van der Linden, et al., 2012; O'Gorman et al., 2009). However, this process has not yet been observed in nature or in the human host for *A. fumigatus* (Dyer & O'Gorman, 2012; Verweij, Zhang, et al., 2016).

Parasexual recombination also increases genetic variation by reshuffling genes, which can occur in genetically different but compatible hyphae. Hyphal fusion allows nuclear fusion, resulting in a temporary diploid phase where mitotic recombination might occur before returning to the normal haploid stage (Figure 22D). This process increases existing genetic variation, enabling the production of genotypes better adapted to new environments (Verweij, Zhang, et al., 2016). This process has been observed as in-host adaptation strategy in patients with cysitic fibrosis where azole resistance occurs through parasexual recombination involving long-term hyphal colonization and a temporary ploidy change from haploid to diploid. (T. Engel et al., 2020).

While asexual and sexual reproduction are common among many microorganisms, parasexual recombination is a fungal strategy to generate genetic diversity rather than a mode of reproduction. These aspects of the lifecycle contribute to increased genetic diversity through spontaneous mutations and reshuffling of genotypes, enhancing the likelihood of producing offspring better adapted to new environments, e.g. the lungs. Although azoles are not inherently mutagenic or recombinogenic, their exposure imposes strong selection pressure for azole resistance (Verweij, Zhang, et al., 2016).

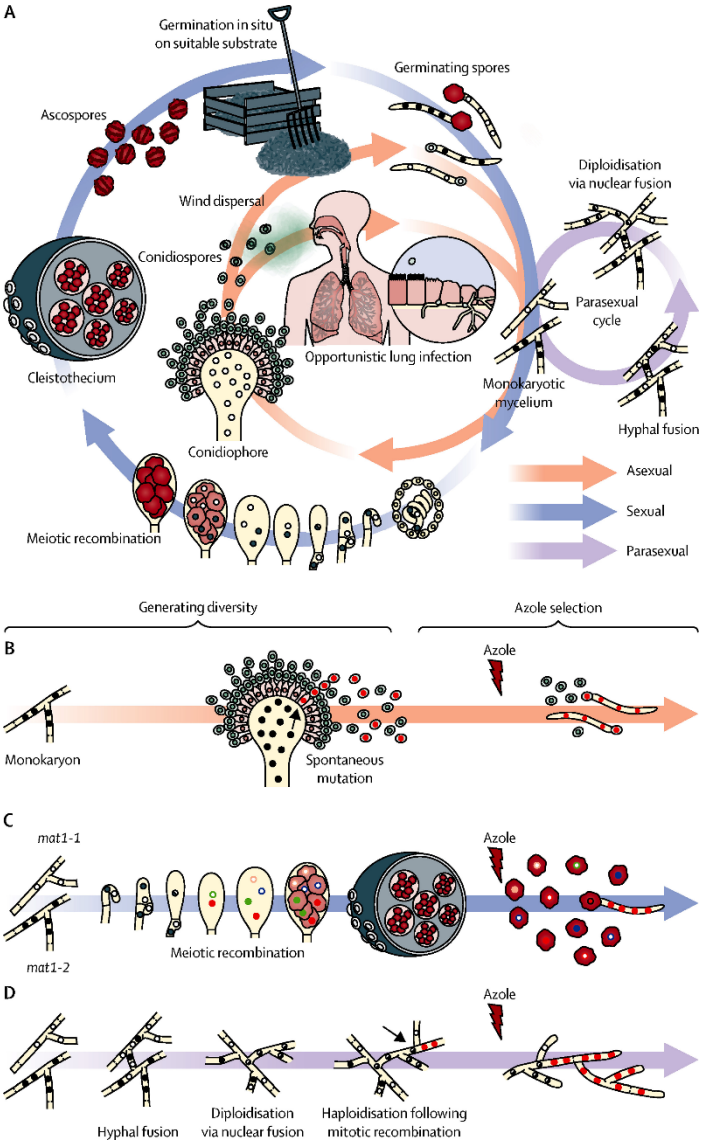


Figure 22 - Reproduction processes in *Aspergillus fumigatus* and adaptation strategies in response to azole exposure. (A) The diagram illustrates asexual reproduction (orange line), sexual reproduction (blue line), and parasexual recombination (purple line). (B) Asexual reproduction contributes to the accumulation of spontaneous mutations through mitotic divisions during the formation of numerous conidiospores. Exposure to azoles can select for rare resistance mutations. (C) Sexual reproduction is a key mechanism for increasing genetic diversity in fungi via meiotic recombination. (D) The parasexual cycle involves the fusion of genetically different but compatible hyphae, followed by nuclear fusion. This process enables mitotic recombination in a transient diploid stage, resulting in increased genetic variation. Although it remains unclear whether the presence of azoles in the environment impacts these reproductive modes—by potentially increasing mutation frequency or altering the rates of reproduction—it is evident that azoles exert significant selective pressure once resistant phenotypes arise. Black indicates wildtype nuclei, red indicates azole-resistant nuclei, and other colors represent genetic variability among nuclei. (Verweij, Zhang, et al., 2016).

3.5 Pathogenesis

The opportunistic pathogen *A. fumigatus* can cause a wide spectrum of diseases, ranging from chronic or allergic pulmonary infections to acute invasive aspergillosis (IA). The clinical syndrome represented is dependent on the host immune status, being either a dysfunction or hyperactivity of the immune system (Kosmidis & Denning, 2015). Roughly, the clinical representations can be classified into three groups: immunocompetent, atopic or allergic and severe immunodeficient (J.-P. Latgé & Chamilos, 2019).

Aspergillosis in the immunocompetent patient can lead to chronic, non-invasive forms of infection, ranging from a fungus ball, or aspergilloma, to chronic pulmonary aspergillosis (CPA). Aspergilloma usually involves colonization and proliferation of *A. fumigatus* in a preexisting pulmonary cavity, such as those developed during pulmonary tuberculosis (TB) or sarcoidosis (Harmouchi et al., 2020). CPA is a destructive pulmonary disease caused by a fungal infection, affecting mainly those individuals suffering from predominantly pulmonary diseases: classical TB, pneumothorax, chronic obstructive pulmonary disease (COPD), lung cancer survival, pneumonia and asthma (Zarif et al., 2021). CPA can be further divided into five subtypes: single aspergilloma, chronic cavitary pulmonary aspergillosis (CCPA), chronic fibrosing pulmonary aspergillosis (CFPA), *Aspergillus* nodule(s), and subacute invasive aspergillosis (SAIA). These subtypes represent overlapping presentations, and the disease can evolve from one subtype to another over time, rather than being entirely distinct categories (Zarif et al., 2021).

In the group of atopic individuals, a hypersensitivity reaction develops after sensitization to *A. fumigatus* allergens. The most severe form in this category, allergic bronchopulmonary aspergillosis (ABPA), is commonly described in patients with cystic fibrosis or poorly controlled asthma patients (J.-P. Latgé & Chamilos, 2019).

Acute IA is the most severe form of aspergillosis and has become a leading cause of mortality among immunocompromised patients (J.-P. Latgé & Chamilos, 2019). IA primarily affects the respiratory tract but occasionally the gastrointestinal tract and skin can be involved. Without a functioning immune system in invasive pulmonary aspergillosis (IPA), *Aspergillus* conidia can start germinating into hyphae, which may invade the underlying tissue and blood vessels (angio-invasion), resulting in thrombosis, necrosis, and hemorrhage (Heylen et al., 2024). In rare cases this can lead to hematogenous dissemination to other organs such as the kidneys or the central nervous system (van de Veerdonk et al., 2017). In the past, IPA was recognized as an opportunistic infection affecting the immunocompromised population presenting host factors as defined by the European Organization for Research and Treatment of Cancer and Mycosis Study Group Education and Research Consortium

(EORTC/MSGERC) consensus definitions (Donnelly et al., 2020). Nowadays, IPA is increasingly recognized in critically ill patients admitted to the intensive care unit without the classical predisposing immunodeficiencies. This new susceptible group includes patients with COPD exacerbations, severe liver disease, and severe viral pneumonia, including influenza A and B and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Bulpa et al., 2007; Heylen et al., 2024; Lahmer et al., 2022; Prattes et al., 2022; Schauwvlieghe et al., 2018; Wauters et al., 2012).

The true disease burden of IPA is difficult to estimate due to a lack of comprehensive surveillance data. Fungal diseases are not required to be reported, and many cases of invasive fungal diseases (IFDs) remain undiagnosed worldwide (Heylen et al., 2024). Based on the limited available data, the global incidence of IPA is estimated to be at least 300,000 cases per year, but this is likely an underestimation of the actual disease burden (Bongomin et al., 2017). Global estimates indicate that >1.2 million patients have CPA (Denning et al., 2016) and 4.8 and 10 million have ABPA and severe asthma with fungal sensitization, respectively (Denning et al., 2013).

Evidence indicates that *A. fumigatus* employs various mechanisms to survive in hostile environments, which contribute to its pathogenicity, evasion of the immune response, and ability to invade tissues. Among these mechanisms is an extensive secondary metabolism, allowing the production of a large array of specific secondary metabolites, including mycotoxins such as gliotoxin, fumagillin, and pseurotin A. The production of these metabolites likely aids the fungus in adapting to diverse conditions, enhancing its competitiveness against other microbes or the host immune system during infections. Several of these secondary metabolites are considered key virulence factors in the genomic and phenotypic profile of this pathogen (Gomez-Lopez et al., 2022).

3.6 Diagnosis of aspergillosis

According to the proposed criteria of the EORTC/MSGERC, IA can be categorized as “proven,” “probable,” or “possible” invasive fungal disease (IFD) based on a combination of host factors, clinical features, and mycological evidence. Host factors include conditions such as hematologic malignancy or receipt of a solid organ transplant. Clinical features might involve dense, well-circumscribed lesions, cavities, or an air crescent sign. Mycological evidence includes a positive culture from sputum or bronchoalveolar lavage (BAL), the presence of galactomannan antigen, or a positive *Aspergillus* PCR test (Donnelly et al., 2020). IA is classified as "proven" only when histopathologic evidence of fungal invasion is present. If direct evidence is not obtainable, patients are further classified based on the specified criteria. A diagnosis of "probable" IA is made by combining host factors, clinical features, and mycological evidence. "Possible" IA is diagnosed when the criteria for host factors and clinical features are met, but mycological evidence is lacking (Donnelly et al., 2020).

3.7 Antifungal treatment in *A. fumigatus*

Although fungi possess a distinct cell wall and differences in cell membrane composition, their metabolic processes are quite similar to those of mammalian cells, offering limited pathogen-specific targets. One crucial fungal-specific component is ergosterol, which is essential for maintaining the integrity and fluidity of the fungal cell membrane. As a result, the ergosterol biosynthesis pathway has been a primary target for antifungal therapies. Systemic antifungal agents are typically categorized based on their mechanism of action within this pathway in pathogenic fungi (R. E. Lewis, 2011). Antifungal drug classes that can be used for monotherapy in the treatment of aspergillosis include azoles, polyenes and echinocandins (Figure 23) (Ullmann et al., 2018).

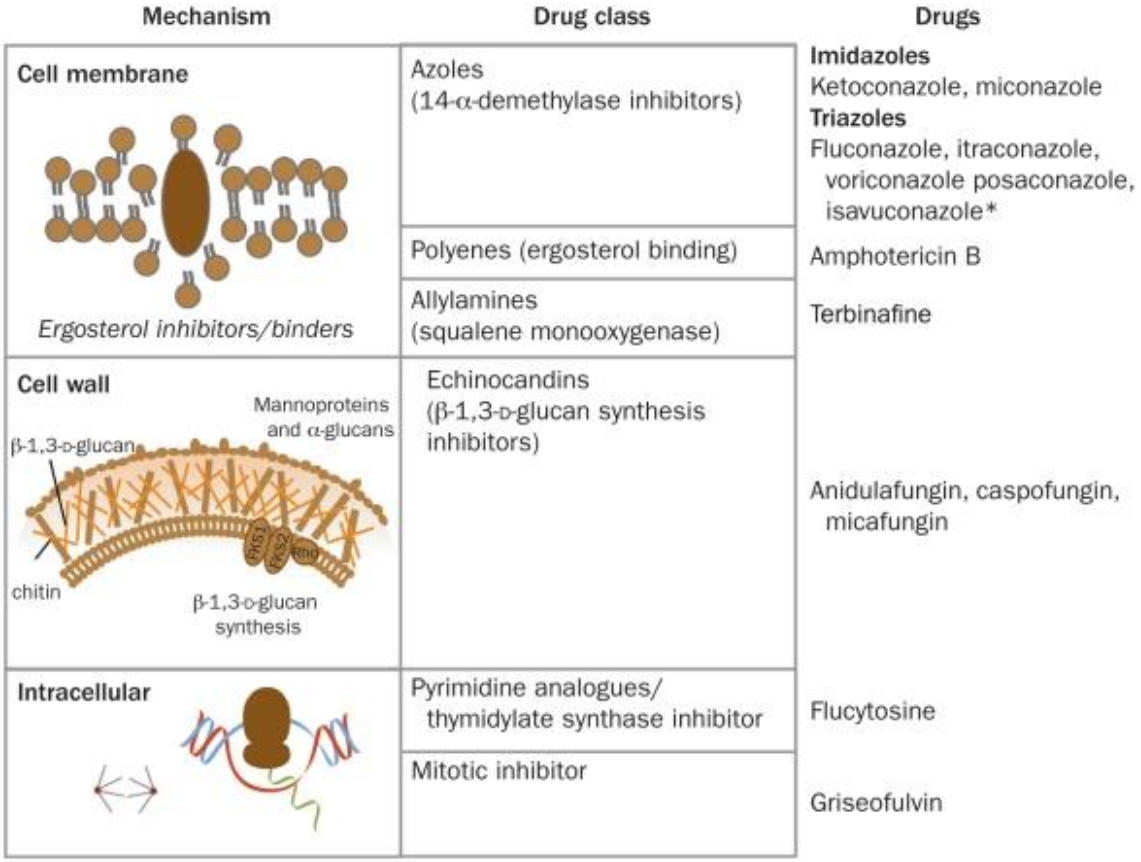


Figure 23 - Sites of action and mechanisms of systemic antifungal agents. Azoles inhibit the 14-α-demethylase enzyme, responsible for ergosterol production. The polyenes, such as Amphotericin B target the fungal cell membrane by binding to ergosterol, forming complexes that create pores, leading to leakage of intracellular contents and cell death.Allylamines, like terbinafine, inhibit squalene monooxygenase to prevent the formation of ergosterol and cause an accumulation of squalene, weakening the cell wall of fungal cells. Echinocandins act on the cell wall by inhibiting the β-1,3-D-glucan synthesis. By competitevily binding to the cell was, echinocandins deplete glucan in the cell wall, making it vulnerable to osmotic lysis, especially in rapidly growing cells. The FKS1 and FKS2 catalytic subunits of the glucan synthase complex are the putative target binding site of echinocandin antifungals. Rho is a cell wall–regulating protein Flucytosine is selectively taken up by cytosine permease and cytosine deaminase, and is converted to cytostatic 5-fluorouracil in fungal cells, where the active drug inhibits thymidylate synthase and causes RNA miscoding. Griseofulvin is a systemic antifungal agent that binds to tubulin, interfering with microtubule formation. Because the drug concentrates in keratinocytes, it is only used for noninvasive dermatophyte infections. (R. E. Lewis, 2011).

3.7.1 Azoles

Triazole antifungals are currently the cornerstone for both prophylaxis and treatment of *Aspergillus* diseases. Itraconazole and posaconazole are used for chronic conditions and prophylaxis, while voriconazole and isavuconazole serve as the first-line treatments for IA. First-line combination antifungal therapy is not routinely recommended (Tissot et al., 2017). Alternative therapies include liposomal amphotericin B (L-AmB), other lipid formulations of AmB, and echinocandins, which are primarily used in combination therapy unless other antifungal classes are contraindicated.

Voriconazole and isavuconazole offer better clinical responses and improved survival rates compared to lipid formulations of AmB, with fewer infusion-related toxicities and less nephrotoxicity. Isavuconazole appears to be as effective as voriconazole for the treatment of IA and has a better safety profile (Tissot et al., 2017). Clear guidelines regarding the diagnosis and management of *Aspergillus* diseases, including the need for therapeutic drug monitoring, are summarized in the European Society for Clinical Microbiology and Infectious Diseases, the European Confederation of Medical Mycology and the European Respiratory Society (ESCMID-ECMM-ERS) guideline (Ullmann et al., 2018).

Azole compounds are cyclic organic molecules and can be differentiated into two groups, based on their chemical structure: imidazoles and triazoles. Imidazole derivatives, with a two-nitrogen azole ring in their structure include miconazole and ketoconazole and were introduced in the early 1980's (Vandeputte et al., 2012). Triazoles contain three nitrogen atoms (Figure 24). In the treatment of *Aspergillus* infections, only triazoles are used (Ullmann et al., 2018). Triazole antifungals target the biosynthetic pathway of ergosterol, a component of the fungal cell membrane. They bind to the enzyme 14-alfa-demethylase (*cyp51*) to inhibit the conversion of lanosterol to ergosterol. Two isoforms of the ezyme are produced: *cyp51A* and *cyp51B*. This inhibition occurs through the binding of the free nitrogen atom of the azole ring to the iron atom of the heme group of the enzyme (Vandeputte et al., 2012). The resulting ergosterol depletion and the accumulation of lanosterol and other 14-methylated sterols interfere with the functions of ergosterol as a membrane component (Georgopapadakou & Walsh, 1996).

The complex treatment strategies are however even more challenged by the emergence of triazole resistance in *A. fumigatus*, which has been reported in human, veterinary and environmental isolates on a global level (Debergh et al., 2023, 2024; Fisher et al., 2018; Schoustra et al., 2019; Sewell, Zhu, et al., 2019). Due to the increasing resistance to antifungal drugs, there is a pressing need for new antifungal agents and drug targets. Recently, several new promising antifungal drugs have been developed, such as Fosmanogepix and Olorofim (Vahedi-Shahandashti & Lass-Flörl, 2020).

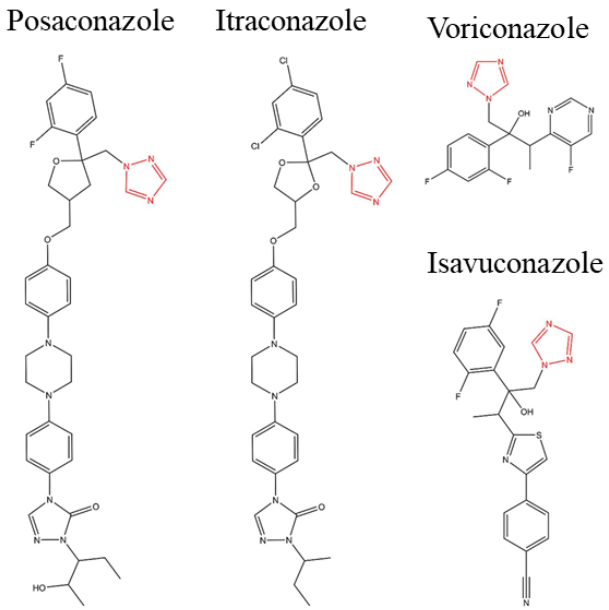


Figure 24 - Structures of the systemic triazole antifungals with the common triazole moiety marked in red. (Jäger et al., 2023)

3.7.2 Polyenes

Amphotericin B, introduced in 1958 as the first effective treatment for systemic fungal infections, is a broad-spectrum polyene antifungal derived from *Streptomyces nodosus*. It targets the fungal cell membrane by binding to ergosterol, forming complexes that create pores, leading to leakage of intracellular contents and cell death. While amphotericin B has a higher affinity for ergosterol-rich fungal membranes than cholesterol-rich mammalian membranes, its use at high doses can cause nephrotoxicity, particularly in the kidneys. To reduce its toxicity, lipid-based formulations - liposomal and lipid complex - were developed in the 1990s, which limit the drug's distribution to the kidneys and are now preferred over conventional formulations. Despite its potency, amphotericin B can trigger infusion-related reactions such as fever and chills due to the release of proinflammatory cytokines. Resistance to amphotericin B is rare but can occur through mechanisms such as the substitution of alternative cell wall sterols and increased resistance to oxidative damage (R. E. Lewis, 2011).

3.7.3 Echinocandines

Echinocandins are semisynthetic lipopeptides and were first introduced with caspofungin in 2001, followed by micafungin in 2005 and anidulafungin in 2006. These antifungals are unique in their ability to specifically target the fungal cell wall by competitively inhibiting the synthesis of β-1,3-D-glucan polymers, essential structural components of the cell wall in certain pathogenic fungi. By binding to the glucan synthase enzyme complex, particularly to the *FKS1* and *FKS2* subunits, echinocandins deplete glucan in the cell wall, making it vulnerable to osmotic lysis, especially in rapidly growing cells.

This results in fungicidal activity against *Candida* species and fungistatic activity against *Aspergillus* species. While echinocandin resistance is uncommon, it can occur through point mutations in the highly conserved "hot spot" regions of *FKS* genes, leading to reduced drug efficacy and higher minimum inhibitory concentrations (MICs) (R. E. Lewis, 2011). Some *Candida* species, such as *C. parapsilosis* and *C. guilliermondii*, are intrinsically less susceptible to echinocandins due to naturally occurring point mutations in the *FKS* gene regions, resulting in a decreased enzymatic activity of 1,3-β-D-glucan synthase (Kizny Gordon et al., 2017; R. E. Lewis, 2011).

3.7.4 The antifungal pipeline

Over the past two decades, the epidemiology of invasive fungal infections has shifted, with broad-spectrum antifungal prophylaxis reducing prevalence and improving survival in high-risk patients. However, selective pressure from prophylaxis and advances in molecular testing have contributed to the emergence of rare fungal pathogens, often resistant to current antifungal treatments. Diagnosing these breakthrough infections is challenging due to the limitations of current diagnostics. Notable threats include *Candida auris*, azole-resistant *Aspergillus fumigatus*, and cryptic species. Additionally, infections like COVID-19-associated aspergillosis and mucormycosis complicate treatment due to drug interactions. Despite a lack of new antifungal drug classes in the past two decades, several promising drugs in late-stage clinical development offer hope, including fosmanogepix, ibrexafungerp, olorofim, opelconazole, and rezafungin (Figure 25). These new drugs, currently under clinical evaluation, may broaden the available treatment options (Figure 26) (Hoenigl et al., 2021).

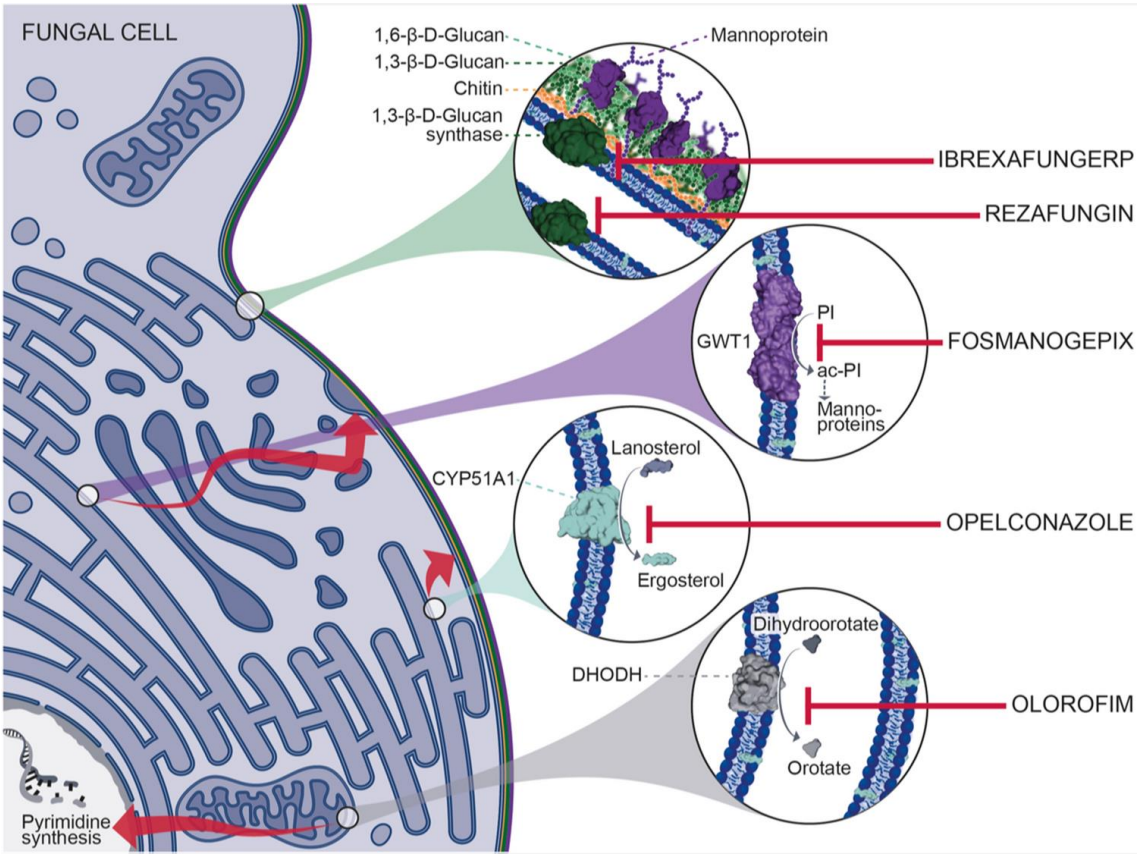


Figure 25 - Mechanism of action of novel antifungal drugs in the pipeline. Ibrexafungerp is a first-in-class oral glucan synthase inhibitor, resulting in the inhibition of the biosynthesis of 1,3-beta-D-glucan in the fungal cell wall. Rezafungin is considered the first member of second-generation echinocandins with enhanced PK/PD pharmacokinetics. The antifungal activity of rezafungin is carried out by the inhibition of the cell-wall enzyme complex β-1,3-d-glucan synthase. Fosmanogepix is rapidly converted after administration to the active moiety manogepix. Manogepix has a novel mode of action that targets glycosylphosphatidylinositol-anchored protein maturation through inhibition of the fungal enzyme Gwt1, an inositol acyltransferase that is essential for trafficking and anchoring mannoproteins to the fungal cell membrane and wall. Opelconazole is a first-in-class inhaled triazole drug. It was was designed and optimized for inhalation via commonly available nebulizers. Olorofim is a member of the novel class of antifungal drugs, the ortomides. Olorofim inhibits fungal growth through inhibition of the fungal dihydroorotate dehydrogenase enzyme involved in pyrimidine synthesis, and without any significant crossreactivity with the human dihydroorotate dehydrogenase, limiting the compounds on target drug toxicity. **DHODH = dihydroorotate dehydrogenase (Hoenigl et al., 2021).**

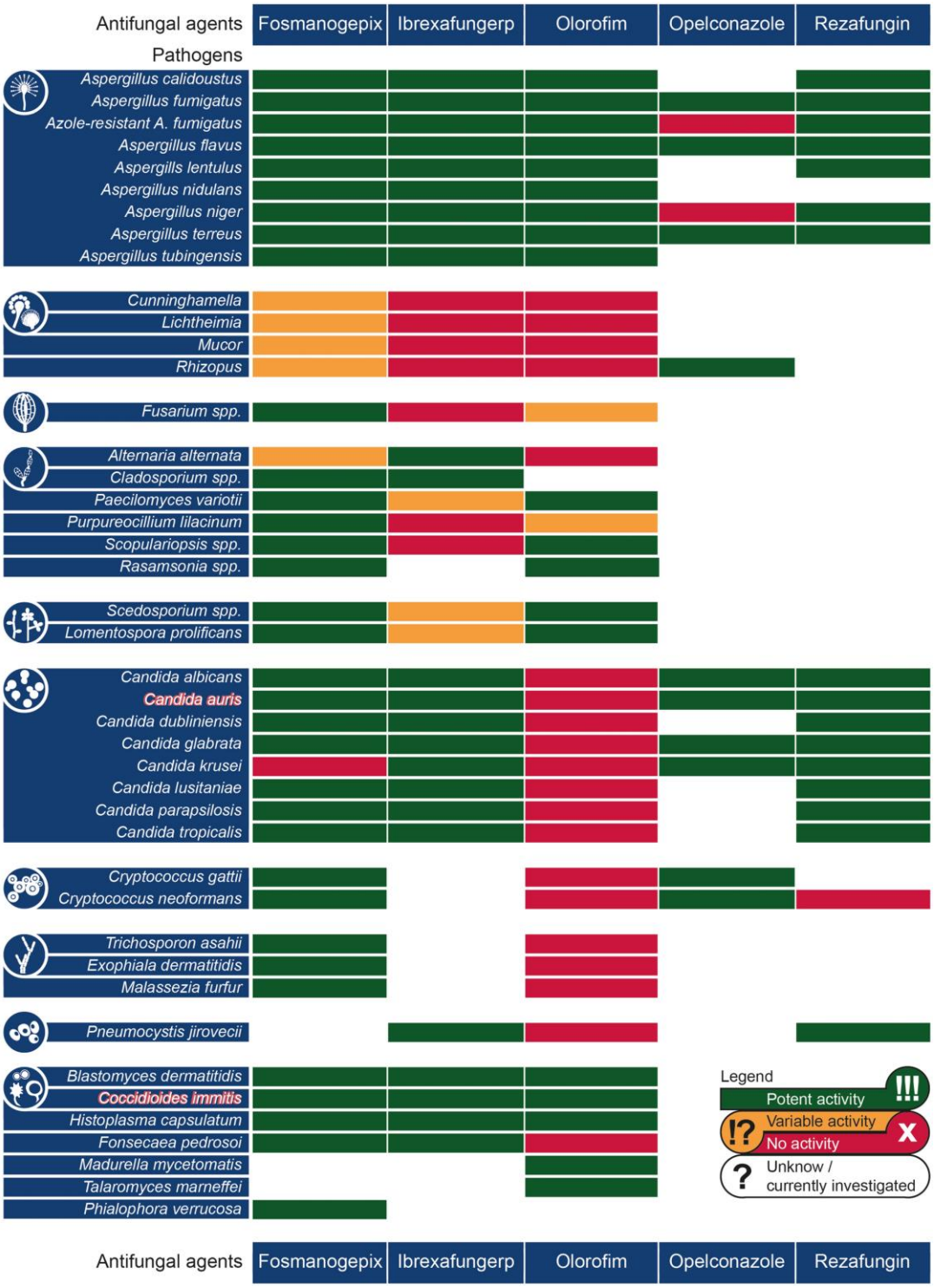


Figure 26 - Activity of the new antifungal drugs in the pipeline against most common fungal pathogens. (Hoenigl et al., 2021)

3.8 Triazole resistance

Triazole resistance refers to the ability of fungal strains to withstand doses of azole drugs that are typically effective against susceptible isolates (Pérez-Cantero et al., 2020). This is defined as *in vitro* resistance of this fungus to at least one triazole antifungal agent having a minimal inhibitory concentration (MIC) value higher than the clinical breakpoints (CBPs) (EUCAST, 2020) or higher than the epidemiological cutoff values (ECOFFs) (Clinical & Laboratory Standards Institute, 2017). ECOFFs categorize isolates into wild-type (WT) or non-wildtype (non-WT), with a non-WT phenotype being indicative of decreased susceptibility to a particular antifungal agent. This reduction in susceptibility may be associated with potential acquired resistance mechanisms. However, unlike CBPs, it cannot be used to predict a patient's response to therapy (Pérez-Cantero et al., 2020).

The EUCAST committee recently introduced the concept of an area of technical uncertainty (ATU) to provide cautionary guidance for clinical laboratories. This applies to triazole MICs that cannot be definitively categorized without additional information, necessitating further decisions before reporting results to the physicians (Table 2). *A. fumigatus* isolates can be categorized as resistant to a single azole (e.g. itraconazole resistance) to multiple azoles (multi-azole resistance) or to all medical azoles (pan-azole resistance) (Verweij, Howard, et al., 2009).

Triazole resistance can be intrinsic or acquired. Intrinsic resistance is when a species is naturally resistant to a certain drug without the occurrence of mutations or gain of genes (Van Der Linden et al., 2011). Some non-*A. fumigatus Aspergillus* species may exhibit intrinsic resistance to certain classes of antifungal agents. These species often have higher MICs for amphotericin B and azoles compared to A. *fumigatus.* For example, *A. lentulus, A. udagawae, A. fumigatiaffinis* and *A. viridinutans,* members of *Aspergillus* section *Fumigati,* display reduced susceptibility to AmB and azoles. The differences in susceptibility profiles may be clinically relevant, but data concerning the role and prevalence of sibling species of *A. fumigatus* as causative agents of IA and other diseases associated with *Aspergillus* spp. is scarce (Fernandez-Pittol et al., 2022; Van Der Linden et al., 2011).

Acquired triazole resistance can be divided into two main groups: *cyp51A* mediated and non-*cyp51A* mediated mechanisms. The *cyp51A* gene encodes for the lanosterol-C14-α-demethylase which converts lanosterol into ergosterol, a vital element of the fungal cell wall (Resendiz Sharpe et al., 2018). Mutations in the *cyp51A* protein are the most frequently reported mechanism of acquired triazole resistance in *A. fumigatus.* Mutations in this gene may be either tandem repeats (TRs) in the promotor region, single nucleotide polymorphisms (SNPs), or both. They reduce the affinity between the azole drug and its target by modifying the binding site and restricting the entry of azole drugs (Pérez-Cantero et al., 2020).

Some SNPs in the *cyp51A* gene have been linked with azole resistance. Mutations at glycine 54 (G54) and glycine 138 (G138) cause cross-resistance to itraconazole (ITC) and posaconazole (POS), while the glycine 448 (G448S) mutation results in voriconazole (VRC) resistance with some reduction

in ITC and POS susceptibility. An amino acid substitution at methionine 220 (M220) is also associated with various patterns of reduced triazole susceptibility (Bellete et al., 2010; Chowdhary et al., 2017; Howard et al., 2009; Mellado et al., 2004; Pelaez et al., 2012; Snelders et al., 2010). Point mutations such as M220I/V/T/K and G54E/R/V are predominantly reported in patients undergoing long-term azole therapy for chronic aspergillosis, typically for about four months, ranging from three weeks to 23 months (Bellete et al., 2010; Diaz-Guerra et al., 2003; Howard et al., 2009; Pelaez et al., 2012). Other sporadically reported mutations include P216L, F219C, F219I, A284T, Y431C, G432S, and G434C (Albarrag et al., 2011; Howard et al., 2009; van der Linden et al., 2011). Frequently reported polymorphisms like F46Y, M172V, N248T, D255E, and E427K, alone or in combination, have been detected in both azole-susceptible and azole-resistant strains. These strains often exhibit higher MICs than wild-type strains, though not always exceeding the clinical breakpoint (Garcia-Rubio et al., 2018; Howard et al., 2009; Rodriguez-Tudela et al., 2008; van der Linden et al., 2011). Environmental isolates harboring the G54 mutation have been recovered from sources in India, Tanzania, Romania, Germany and Belgium (Bader et al., 2015; Debergh et al., 2023; Sharma et al., 2015).

Alterations in the *cyp51A* gene, leading to pan-azole-resistant *A. fumigatus*, have been reported globally from clinical, veterinary, and environmental sources (Astvad et al., 2014; Debergh et al., 2023, 2024; Hurst et al., 2017; Siopi et al., 2020). These alterations involve tandem repeats in the promoter region and specific mutations. The TR_{34}/L98H mutation, the most frequently reported resistance mechanism, involves a 34-base pair (bp) tandem repeat in the promoter region and a leucine to histidine substitution at codon 98. This mutation causes overexpression of the *cyp51A* gene, increasing protein levels and altering triazole docking, leading to pan-azole resistance with particularly high itraconazole MIC values (Snelders et al., 2011). The second most common mutation, TR_{46}/Y121F/T289A, consists of a 46-bp tandem repeat in the promoter and amino acid changes at codons 121 and 289 . This mutation confers high-level voriconazole resistance and variable itraconazole and posaconazole MIC values (Chowdhary et al., 2017; Sewell, Zhu, et al., 2019; Snelders et al., 2015). The third resistance mechanism involves a 53 bp tandem repeat insertion (TR_{53}) without mutations in the *cyp51A* gene, conferring a pan-azole-resistant phenotype (Hodiamont et al., 2009). Recently, two new mutations have been discovered in the Netherlands, consisting of three ($TR46_3$) and four ($TR46_4$) copies of the 46 bp tandem repeat (J. Zhang, Snelders, et al., 2017). The TR_{120}/F46Y/M172V/E427K has also been reported but to a much lesser extent (Hare et al., 2019).

Table 2 - Interpretation of *Aspergillus fumigatus* minimal inhibitory concentrations for the two reference broth microdilution methods (Clinical & Laboratory Standards Institute, 2017; EUCAST, 2020)

Triazole	EUCAST clinical breakpoints[1]			CLSI[2]	Comments on the ATU
	S ≤	R >	ATU	ECOFF	
Itraconazole	1	1	2	1	Report as R with the following comment: "In some clinical situations (non-invasive infections forms) itraconazole can be used provided sufficient exposure is ensured".
Voriconazole	1	1	2	1	Report as R with the following comment: "In some clinical situations (non-invasive infections forms) voriconazole can be used provided sufficient exposure is ensured".
Isavuconazole	1	2	2	1	If voriconazole wild-type (MIC ≤1 mg/L) report as isavuconazole S and add the following comment: The MIC of 2 mg/L is one dilution above the S breakpoint but within the wild-type isavuconazole MIC range due to a stringent breakpoint susceptibility breakpoint. If voriconazole non wild-type report as isavuconazole R and refer to reference laboratory for *cyp51A* sequencing and confirmation of MICs."
Posaconazole	0.125	0.25	0.25	0.25	If S to itraconazole report as S and add the following comment: "The MIC is 0.25 mg/L and thus one dilution above the S breakpoint due to overlapping wt and non-wt populations". If not S to itraconazole report as R and refer to reference laboratory for *cyp51A* sequencing and confirmation of MICs.

[1]EUCAST Antifungal Clinical Breakpoints Table v. 10.0 valid from 2020-02-04; [2]CLSI Epidemiological Cutoff Values for antifungal susceptibility testing M38 3rd (2017), M59-Ed3 (2020); [3] XXX; EUCAST = European Committee on Antimicrobial Susceptibility Testing; CLSI = Clinical Laboratory Standards Institute; S = Susceptible; R = Resistant, ATU = area of technical uncertainty

Non-*cyp51A* gene-mediated mechanisms, though less frequently reported, include: efflux pumps, *cyp51B* overexpression, cholesterol import, and *HapE* mutation. The efflux pumps are adenosine triphosphate-binding cassette (ABC) transporters and play a significant role in overcoming intracellular toxin accumulation. Overexpression of the ABC transporters *AfuMdr4* and *Cdr1B* is associated with azole-resistance to voriconazole and itraconazole, respectively (Fraczek Mg et al., 2013; Rajendran et al., 2011). In addition to the *cyp51A* gene, *A. fumigatus* possesses a closely related homolog, the *cyp51B* gene, which shares 60% sequence identity. Both genes encode fungal 14-alpha-sterol-demethylase enzymes involved in ergosterol biosynthesis and cell surface stress responses. Their functions overlap and compensate for each other's absence (Handelman et al., n.d.; Mellado et al., 2001; Roundtree et al., 2020). Overexpression or mutations in the *cyp51B* gene are rare but have been observed in some triazole-resistant *A. fumigatus* isolates (Buied et al., 2013; Gonzalez-Jimenez et al., 2020). However, the specific role of *cyp51B* in conferring triazole resistance remains underexplored. Cholesterol import by *A. fumigatus* helps compensate for ergosterol depletion, conferring resistance to triazoles (Xiong et al., 2005). The sterol regulatory element-binding protein *SrbA* directly regulates *cyp51A* transcription, mediating resistance to fluconazole and, to a lesser extent, voriconazole. In *SrbA*-null mutants, the absence of *SrbA* leads to increased susceptibility to fluconazole (Willger et al., 2008). Lastly, mutation P88L in *HapE*, an important subunit of the CCAAT-binding transcription factor complex was found to confer azole resistance in *A. fumigatus* (Camps, Dutilh, et al., 2012). Overall, the diverse and complex mechanisms of azole resistance in *A. fumigatus* illustrate the adaptive capability of the fungus to various environmental pressures.

3.9 Triazole resistance selection pathway

Triazole resistance is a form of acquired resistance occurring after azole exposure either due to prolonged treatments (patient route) or due to environmental exposure to agricultural fungicides (environmental route) (Figure 27).

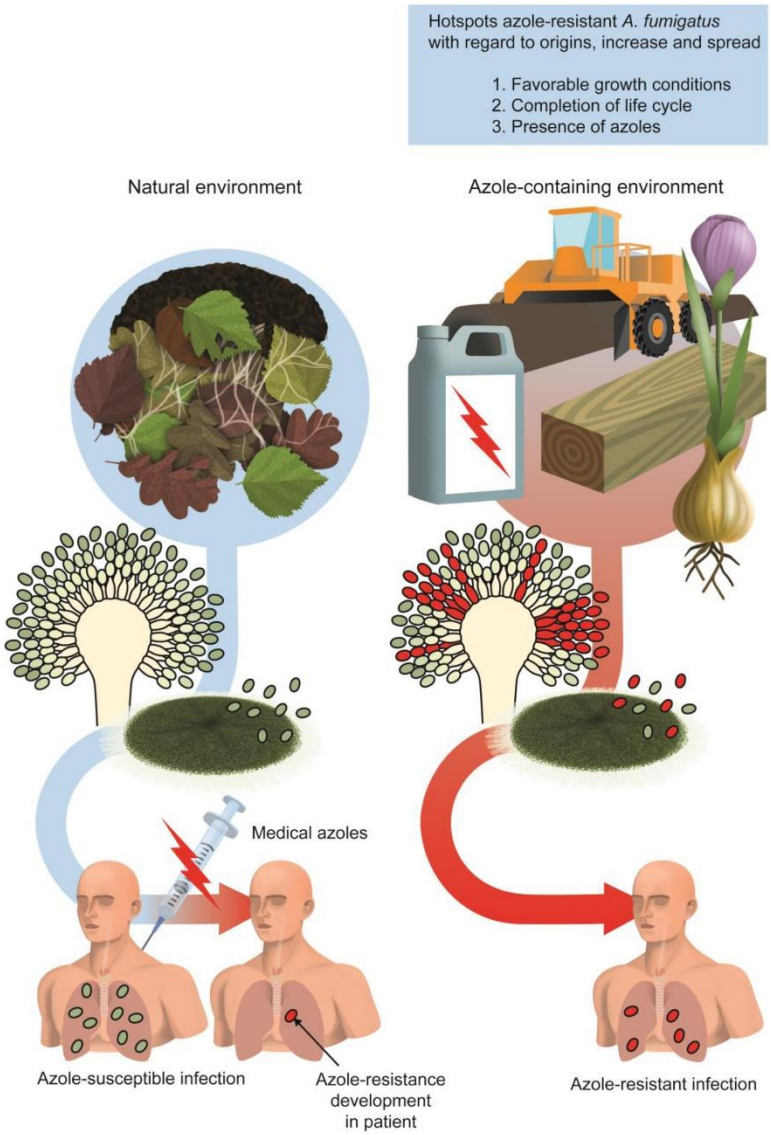


Figure 27 - Routes of azole resistance selection through medical treatments and in specific conditions in the azole-containing environment (Verweij et al., 2020).

3.9.1 Resistance selection through patient exposure

Resistance selection through the patient route occurs after long-term azole therapy. Pathogenesis involves infection of the patient with an azole-susceptible *A. fumigatus* isolate that becomes resistant due to azole pressure of the prolonged treatment regimen. It has been reported in patients with aspergilloma and cavitary lung disease who have been treated with azoles (J. Chen et al., 2005; Howard

et al., 2009). The risk of developing phenotypic resistance appears to be influenced by the mode of reproduction of molds. In patients with aspergilloma, resistance may develop as aspergilli reproduce asexually through sporulation within the lung cavity. This process allows for multiple generations to form, facilitating mutations due to the selective pressure of azoles (Anderson, 2005). In patients with acute invasive aspergillosis, the infection progresses through hyphal elongation rather than asexual reproduction by sporulation. Spontaneous mutations are likely to occur in the nuclei within the hyphae. The development of phenotypic resistance during treatment in these patients is highly unlikely unless the fungus undergoes asexual reproduction, such as when the infection progresses to a cavitary lesion (Verweij, Snelders, et al., 2009). As a consequence, the mechanisms involved include point mutations in the *cyp51A* gene, frequently leading to the development of multiple mutations within the same patient (Howard et al., 2009).

3.9.2 Resistance selection through environmental exposure

The isolation of triazole-resistant strains from triazole-naive patients implies that patients might also acquire triazole-resistant *Aspergillus fumigatus* from environmental sources. This hypothesis is supported by several key observations. Firstly, the prevalence of specific resistant mutations (primarily TR34/L98H and TR46/Y121F/T289A) in over 80% of clinical isolates from epidemiologically unrelated patients across different centers, where environmental and airborne isolates exhibited genetic clustering with these clinical isolates (Snelders et al., 2008, 2009; van der Linden et al., 2011). Secondly, the geographical dissemination of these mutations across multiple countries and continents further supports this notion (Chowdhary et al., 2014; Debergh et al., 2024; Macedo et al., 2021; Sewell, Zhu, et al., 2019; Siopi et al., 2020). Thirdly, the presence of multiple genomic alterations suggests a more complex reproductive mechanism, such as sexual reproduction, which, while not reported in human infections, is likely to occur in the environment (Snelders et al., 2009). Lastly, the selection for triazole-resistant *A. fumigatus* could result from exposure to fungicides routinely used in agriculture and horticulture for crop protection and material preservation (Burks et al., 2021; Cao et al., 2020; Jeanvoine et al., 2017a; Snelders et al., 2009).

The introduction of demethylation inhibitors (DMIs) of the triazole class, which are sterol-biosynthesis inhibiting (SBI) fungicides molecularly similar to medical triazoles, coincided with the first reports of resistance. Cross-resistance between these agricultural DMIs and medical triazoles has been documented (Snelders et al., 2012). Additionally, extensive cross-resistance to medical triazoles was observed following in vitro experimental evolution of triazole-susceptible *A. fumigatus* under pressure from environmental DMI fungicides, further implicating DMIs in the environmental development of triazole resistance (Faria-Ramos et al., 2014; J. Zhang, van den Heuvel, et al., 2017). In one study, the immediate home environment of a patient with probable IA due to TR_{46}/Y121F/T289A harbored isolates with the same resistance mutation and identical genotype, suggesting the environment as a source of

human infection (Lavergne et al., 2017). Furthermore, plant bulbs from the Netherlands containing the TR_{46}/Y121F/T289A mutation were found to contaminate their immediate environment near a hospital in Ireland, although no direct genetic link to patient infection was established (Dunne et al., 2017). The effect of changing azole-treated bulbs for organic tulip bulbs in the hospital environment on the rate of ARAf was investigated by a French research group. They report a decrease of ARAf prevalence from 71% to below 3% (Rocchi et al., 2021).

Environments such as flower bulb waste, green waste, and wood chipping waste management sites, are referred to as "hotspots" (Schoustra et al., 2019). These hotspots are defined by three characteristics: (1) the physical, biotic, and abiotic conditions facilitate the growth and dissemination of the fungus, (2) the fungus can grow for prolonged periods, completing all stages of its growth cycle and (3), azole fungicides (DMIs) are present in varying concentrations sufficient to exert selective pressure on fungal populations (Verweij et al., 2020). These hotspots facilitate the emergence, amplification, and spread of mutations conferring triazole resistance in *A. fumigatus*. However, it remains unclear how resistant mutants arise in these hotspots—whether through de novo acquisition or by selecting pre-existing resistant strains—and how subsequent patient infection from these hotspots occurs, as they are not always in close proximity to patients (Schoustra et al., 2019).

The presence of tandem repeats in *A. fumigatus* has been widely associated with environmental routes of selection, whereas amino acid substitutions have been linked to patient routes of selection. Nonetheless, these selection routes are not mutually exclusive. Single amino acid resistance mutations have been identified both in the environment and in patients with no prior triazole exposure (Buil et al., 2019). Additionally, the in vivo development of a triazole-resistant *A. fumigatus* isolate harboring a TR_{120} mutation (TR_{120}/F46Y/M172V/E427K) isogenic to a previously cultured susceptible isolate has been reported after prolonged triazole exposure in a CPA patient (Hare et al., 2019). Thus, the selection of TR-associated resistance mechanisms may not be exclusively linked to the environmental route (Buil et al., 2019). Currently, specific resistance mechanisms dominate particular habitats, but continuous surveillance is essential to detect future changes. Further investigation is required to determine the conditions favoring the development of specific resistance mechanisms. Furthermore, in order to reduce the global spread of environmental resistance mutations, mitigation strategies are needed that prevent or reduce the burden of resistance induced by fungicide use (Buil et al., 2019).

3.10 Diagnosis of azole resistance

Screening methods for the identification of triazole-resistant *A. fumigatus* isolates is performed using agar-based methods (Montesinos et al., 2017). The commercially available 4-well agar plate dilution method VIPCheck® (MediaProducts, Groningen, The Netherlands) provides an easy and less costly screening method for testing multiple colonies from one sample. This results in a sensitive and

specific selection of presumptive azole resistant *A. fumigatus* (ARAf) isolates that require broth microdilution method for confirmation (Buil et al., 2017).

The reference method for determining the MIC values of *A. fumigatus* colonies and their antifungal resistance profile for the four medical azoles (voriconazole, itraconazole, isavuconazole and posaconazole) remains broth microdilution. Both EUCAST (EUCAST, 2020) and CLSI (Clinical & Laboratory Standards Institute, 2017) can be used as a reference method, with the former providing clinical breakpoints, guiding clinical decision making, and the latter providing ECOFFs. Confirmation of the resistance mechanisms require sequencing of the *cyp51A* gene and its promotor region (Otto et al., 2023).

3.11 Epidemiology using a One Health approach

The global awareness of pathogenic fungi has heightened due to their impact on agriculture and their ability to cause infections in animals and humans (Fisher et al., 2012; Geddes-McAlister & Shapiro, 2019). Among humans, immunocompromised individuals are particularly susceptible to these invasive fungal pathogens, with infections often associated with high mortality rates, necessitating effective antifungal therapy (Wiederhold & Verweij, 2020). The occurrence of fungal diseases in plants has both direct and indirect effects on human health, as pathogenic fungi are responsible for epidemics that reduce the yield of staple crops like rice, wheat, and corn, which are crucial for feeding billions of people (Fisher et al., 2012). Critically, the application of fungicides in agriculture to prevent crop diseases exposes environmental fungi to antifungal agents, fostering the evolution and development of resistance, which compromises treatment efficacy in medical and veterinary settings. From a One Health perspective, the essential role of azole fungicides in ensuring food security must be balanced with the need to preserve the efficacy of structurally related azoles used in clinical practice (FPS Health, Food Chain Safety and Environment, 2021; Verweij et al., 2020). Therefore, it is crucial to identify the drivers behind the development of resistance to azole fungicides released into the environment. In this context, the European Centre for Disease Prevention and Control (ECDC) advocates for active environmental surveillance across all its member states (European Centre for Disease Prevention and Control., 2013).

In retrospect, the first azole-resistant *A. fumigatus* isolates in human medicine were identified in the late 1980s on the West Coast of the USA in California, cultured from two patients treated with itraconazole (Denning et al., 1997). A Dutch study later reported three itraconazole-resistant *A. fumigatus* isolates in 1997 from a lung transplant recipient following long-term itraconazole treatment (Verweij et al., 2002). Following these findings, a French study identified four itraconazole-resistant isolates in 1999 (Dannaoui et al., 1999). In 2007, a comprehensive study of nine cases of azole-resistant IA revealed that four out of nine patients had never previously received azole therapy (Verweij et al., 2007). Furthermore, the resistant *A. fumigatus* isolates exhibited the same resistance mechanisms found

in environmental and airborne isolates (Snelders et al., 2008). Remarkably, the genetic relatedness of European clinical and environmental ARAf isolates, measured using a panel of highly polymorphic genetic markers, showed a close relationship (Camps, Rijs, et al., 2012). This led to the hypothesis that these patients had acquired azole-resistant *A. fumigatus* strains from the environment. This suggested the development of a second route for resistance induction through a fungicide-driven pathway, due to environmental fungicide exposure and selection of the fungus to azoles (Verweij, Snelders, et al., 2009). The characteristics of these two routes have been clearly established in the last decade (Snelders et al., 2009; Verweij, Chowdhary, et al., 2016; Verweij et al., 2015).

Since then, reports of ARAf have increased around the world (Meis et al., 2016). While a spectrum of resistance mechanisms to azoles has been characterized in *A. fumigatus*, azole resistance is frequently the result of mutations in the *cyp51A* gene (Astvad et al., 2014; Fraczek Mg et al., 2013; Gonçalves et al., 2020; Hare et al., 2019; Meneau et al., 2016). The majority of the mutations conferring azole resistance include tandem repeats, with the TR_{34}/L98 (60.7%) and TR_{46}/Y121F/T289A (15.0%) allele predominantly reported (Burks et al., 2021). Both mutations have been reported on every continent, with the exception of Antarctica (Burks et al., 2021). Most of the studies reporting on the presence of the TR_{34}/L98H allele originate from Europe (71.1%), followed by the Middle East (8.2%) and India (7.8%) (Burks et al., 2021). The TR_{46}/Y121F/T289A is far less common than the TR_{34}/L98H allele and were especially prevalent in Europe (46.1%), South America (23.6%), and East Asia (14.0%). This allele was especially common in South America, where the number of resistant isolates with this allele was far greater than the TR_{34}/L98H allele: 4.3% of the resistant isolates reported with the TR_{34}/L98H mutations vs 60% with the TR_{46}/Y121F/T289A mutation (Alvarez-Moreno et al., 2017, 2019; Burks et al., 2021; Le Pape et al., 2016).

Numerous studies on the surveillance of ARAf in the clinics are available. According to a multicenter international surveillance network, the incidence of triazole resistance in *A. fumigatus* ranged from 0.6% to 4.2%, with the TR34/L98H mutation being the most common (van der Linden et al., 2015). Reported resistance rates by country include: The Netherlands (0.8%–9.4%, with voriconazole resistance rates as high as 26%–29% in ICU patients from one hospital), Belgium (5.5%), the UK (6.6%–27.8%), Germany (3.2%, with 30% in hematopoietic stem cell transplant patients), Spain (0.3%–4.2%), Denmark (4%–6%), Greece (2.7%), Poland (4.13%), and Turkey (10.2%) (Resendiz Sharpe et al., 2018). In Belgium, surveillance data from the tertiary care center University Hospitals Leuven indicated an average azole resistance prevalence of 7.1% [95% CI 6.6-7.7%] from 2016 to 2020, with 73% of isolates resistant to all three tested antifungals (Resendiz-Sharpe et al., 2021). Additionally, the first cases outside Europe were reported from China in 2011, with a resistance prevalence of 5.8% (Lockhart et al., 2011). Other countries reporting resistance include India (1.7%), Iran (3.2%), Japan (6.1%), Thailand (3.2%), Australia (2.6%), Tanzania (13.9%, environmental), Colombia (9.3%, environmental), and the United States (0.6%–11.8%) (Resendiz Sharpe et al., 2018). It is important to interpret resistance prevalence across countries cautiously due to differences in isolate sources, sample

sizes, geographic regions, laboratory methods, and patient conditions (Resendiz Sharpe et al., 2018). Routine *A. fumigatus* susceptibility testing is not common in many centers, leading to underdiagnosed resistance. Retrospective testing revealed that the first TR34/L98H isolate in Italy was cultured as early as 1998 (Lazzarini et al., 2015), and the United States identified a TR46/Y121F/T289A isolate in 2008, one year before its discovery in the Netherlands (Wiederhold et al., 2016). It remains unclear when and how triazole resistance emerged in A. *fumigatus*, and whether it developed once and spread or arose independently in different regions. Although triazole resistance has now been detected on six continents, the prevalence remains unknown in many countries. Most epidemiological data on triazole resistance comes from chronic aspergillosis (aspergilloma, chronic pulmonary aspergillosis, and chronic colonization) and acute IA. In chronic aspergillosis, resistance may result from point mutations in the *cyp51A* gene or infection by an environmentally resistant isolate. In IA, resistance typically arises from environmentally triazole-resistant isolates (Snelders et al., 2008; Verweij et al., 2007). Notably, no clear patient risk factors predict resistant IA, as surveillance studies found that up to two-thirds of patients with triazole-resistant IA had no prior triazole treatment (van der Linden et al., 2011).

Recent genetic-relatedness analysis of worldwide triazole-susceptible and triazole-resistant *Aspergillus fumigatus* isolates, harboring either the TR34/L98H or TR46/Y121F/T289A mutations from both clinical and environmental origins, revealed that triazole-resistant isolates with these mutations are closely related and globally disseminated through clonal proliferation. These findings indicate that large geographic distances do not hinder the potential worldwide distribution of triazole-resistant *A. fumigatus*, particularly those isolates with these specific mutations, thereby posing an international public health concern (Rhodes et al., 2022).

The widespread use of azole fungicides in the environment is considered one of the cornerstones in the development of azole resistance in *A. fumigatus*. In agriculture, azole fungicides, such as tebuconazole (107534-96-3), epoxiconazole (133855-98-8), propiconazole (60207-90-1) and bromuconazole (116255-48-2) are or have been intensively used for crop protection (Godeau et al., 2023; Schoustra et al., 2019; Snelders et al., 2009). In 2019, these were, respectively, the second, fourth, seventh and tenth most sold triazoles in agriculture in Belgium (*Sold Volumes of Plant Protection Products for Agriculture in Belgium*, 2019). Both epoxiconazole and propiconazole are now banned from use at the EU-level under Regulation (EC) No 1107/2009, because of suspected fertility, developmental and endocrine perturbation properties (European commission, 2009). The largest volumes of azoles are sold in Asia and Europe, accounting for more than two-thirds of all azoles used worldwide (Jørgensen & Heick, 2021).

The use of these azoles in ornamental plants for manipulating the shape, size, and aesthetic quality, in countries such as the Netherlands and Denmark, is significant and is linked to their market value (Jørgensen & Heick, 2021). Consequently, waste materials from agriculture and horticulture, along with input material from wood, forestry, paper, household and garden waste, are likely to contain fungicide residues. These waste streams are generally composted by industrial composting facilities,

where compost is processed in different stages and the rows are frequently turned to improve oxygenation and to control heat and moisture levels. Research from the Netherlands (Schoustra et al., 2019; J. Zhang, Lopez Jimenez, et al., 2021) and the United States (Hurst et al., 2017) showed that composting material is a hotspot for the development and release of a large number of ARAf spores. To date, limited data is available on the presence of azole resistant *A. fumigatus* in the environment in Belgium. A pilot study performed in Belgium found an overall resistance rate of 2.6% of ARAf in horticulture and composting facilities (Debergh et al., 2024).

Furthermore, aspergillosis, primarily caused by the fungus *A. fumigatus*, poses a significant health risk to both birds and mammals, leading to illness and death. Aspergillosis in birds is quite common and it can affect a wide variety of species such as domestic, free-ranging or captive wild animals (Arné et al., 2021). Aspergillosis is the most common fungal infectious disease affecting penguins in zoos, with up to 99% of cases attributed to *Aspergillus* section *Fumigati*. It represents a major limiting factor for the rehabilitation of penguins in captivity (Beernaert et al., 2010; Seyedmousavi et al., 2015; Silva Filho et al., 2015a; Stidworthy, n.d.; Xavier et al., 2007). On the contrary, aspergillosis in free-ranging birds is only rarely described (Ewbank et al., 2021; Graczyk & Cockrem, 1995). In comparison, aspergillosis is less prevalent in mammals, regardless of the increasing number of immunocompromised animals (Tell, 2005). Exceptions include canine sinonasal aspergillosis, equine guttural pouch mycosis and bovine mycotic abortion caused by *Aspergillus* spp (Dobesova et al., 2012; Elad & Segal, 2018; Tell, 2005). Predisposing factors for the development of aspergillosis in birds and mammals consists of immunosuppression, previous debilitating illnesses, stress factors and environmental factors (Higgins & Pusterla, 2006; U. P. Melo et al., 2024; Tell, 2005). These latter factors can consist of contaminated feed, soil and bedding or poor husbandry such as poor ventilation, high humidity, and warm temperatures (Tell, 2005)**.** The mode of entry is presumed to be oropharyngeal during inhalation, as such the most frequently affected areas are the head region for mammals, and lungs and air sacs in birds (Tell, 2005). Diagnosis often occurs late and diagnostic tools are scarce, inaccurate or expensive, which limits the chance of timely treatment (Desoubeaux et al., 2022). Additionally, because of the concurrent severe underlying diseases in mammals, treatment or prophylaxis is challenging (U. P. Melo et al., 2024). The emergence of azole resistance in *A. fumigatus* in the clinics and the environment has become a major concern. The surveillance of azole resistance in *A. fumigatus* in clinical strains is a common practice, however, this is not the case for avian aspergillosis with only few epidemiological studies published (Barber, Scheufen, et al., 2020; Beernaert et al., 2010; Cateau et al., 2022; A. M. Melo et al., 2021). This emergence of resistance also creates additional difficulties for the treatment of aspergillosis in veterinary medicine (Beernaert et al., 2009; Debergh et al., 2023; Martinez et al., 2022; Ziołkowska et al., 2014).

All this makes the pathogen *A. fumigatus* a perfect candidate to illustrate the interconnectedness of human, animal, and environmental health that are taken into account in the framework of the One Health approach (Panel (OHHLEP) et al., 2022).

CHAPTER 2 - OBJECTIVES

As outlined in the introduction, *K. pneumoniae* (in particular carbapenem - and 3GC-resistant *K. pneumoniae*) and *A. fumigatus* are both classified as critical pathogens in the WHO's Bacterial and Fungal Priority Pathogen Lists (BPPL and FPPL, respectively). However, significant gaps remain in our understanding of the epidemiology of these pathogens, particularly within the framework of the One Health approach.

The general objective of the thesis was to provide a better understanding of the resistance mechanisms and their transmission involved in *K. pneumoniae* and *A. fumigatus* for an adopted guidance towards developing a One Health framework. This doctoral thesis aims to answer the specific objectives listed in the schematic overview (Figure 28) and a detailed overview below.

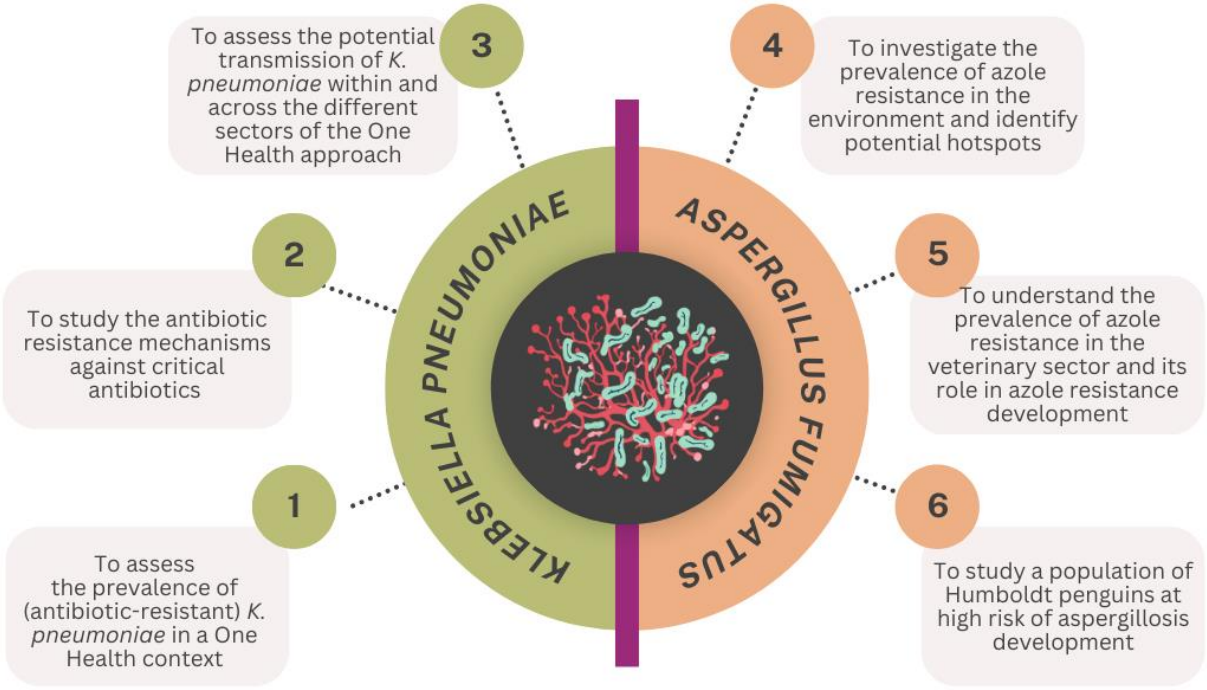


Figure 28 - Overview of the specific objectives of this doctoral research (created with canva.com)

The first specific objective of this thesis was **to assess the prevalence of (antibiotic-resistant) *K. pneumoniae* applying a One Health approach.** We performed a large sampling effort including the isolation and selection of (antibiotic-resistant) *K. pneumoniae* from the food chain, diseased animals, wastewater and the human clinical environment. We assessed the antimicrobial susceptibility profile by performing broth microdilution on a selection of isolates to obtain an overall prevalence rate of *K. pneumoniae* in the food chain and in wastewater samples in **study 1.**

The second objective was **to study the genetic antibiotic resistance mechanisms in *K. pneumoniae*** related to their phenotypical resistance profile. Here, were performed whole genome sequencing on a selection of isolates encompassing all sectors of the One Health approach (**Study 1**). A hybrid sequencing approach was used to elucidate a rare resistance mechanism conferring ertapenem resistance in a *K. pneumoniae* strain from a companion animal (**Study 2**).

The third specific objective was **to elucidate possible transmission events within and across sectors of the One Health approach**. A phylogenetic analysis was performed on a selection of isolates based on cgMLST and SNP-based phylogeny to assess genetic relatedness in **study 1.**

The fourth specific objective was **to investigate the prevalence of azole resistance in the environment and identify potential hotspots.** To obtain this objective, a large sampling effort was performed including air samples and soil/compost in agriculture, horticulture and composting facilities to analyse the presence of azole-resistant *A. fumigatus* in the Belgian environment (**Study 3**).

The fifth specific objective was **to understand the prevalence of azole resistance in *A. fumigatus* in the veterinary sector** and to elucidate the role of the veterinary sector in azole resistance development considering the One Health approach. Here, a study was executed to analyse the prevalence of azole resistance in *A. fumigatus* isolated from diseased birds and mammals and to investigate the main resistance mechanisms linked to the environmental or to the patient route (**Study 4**).

The sixth specific objective was **to study a population of Humboldt penguins from a Belgian zoo and the role of the environment**. Here, we aimed to find a link between the environmental route of resistance development and the resistance mechanisms observed in the Humboldt penguins **(study 5**).

CHAPTER 3 - EXPERIMENTAL SECTION

Experimental section

Study 1

One health genomic surveillance of *Klebsiella pneumoniae* in Belgium demonstrates large genomic diversity, presence of ESBLs and carbapenemases and circulation of clones across sectors

Preamble

The ESKAPE pathogens - *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp. - are major drivers of antimicrobial resistance (AMR) in hospitals, posing significant global health risks. *K. pneumoniae* is particularly concerning due to the occurrence of antimicrobial resistance to key antibiotics, including carbapenems and third-generation cephalosporins, making treatment options limited. Previous research shows that *K. pneumoniae* persists in various reservoirs, such as hospitals, animals, food, and wastewater, which increases the risk of transmission across sectors.

Samples were obtained from the food chain (n = 1508), wastewater treatment plants (160 samples covering 10 locations), diseased animals (n = 344), and clinical human isolates (n = 100). Antimicrobial susceptibility testing and whole-genome sequencing were conducted on selected isolates, mainly focusing on resistance to critical antibiotics such as cephalosporins and carbapenems. Phylogenomic analyses, including core-genome MLST and SNP-based phylogeny, were performed to assess genetic relatedness, with findings aiding in the understanding of antibiotic resistance transmission across sectors.

Antimicrobial resistance testing revealed significant resistance to critical antibiotics, with human clinical and wastewater isolates showing the highest resistance rates, particularly to third generation cephalosporins and carbapenems. Whole-genome sequencing of 286 isolates revealed 141 different sequence types (STs), with some STs shared across different sectors. Key findings included the detection of carbapenemase genes (bla_{KPC}, *bla*NDM, $_{bla\text{OXA-48-like}}$, and bla_{VIM}) and a high prevalence of plasmids carrying AMR genes, particularly in wastewater. Phylogenomic analysis identified clusters within and across sectors. Notably, clusters of high-risk global clones (e.g., ST15, ST147, ST307) were identified, suggesting human exposure from various sources, including the food chain, wastewater, and companion animals. This study highlights the interconnectedness of AMR transmission in the One Health context.

In this work, data analyisis was performed by Dr. Bert Bogaerts and Dr. Kevin Vanneste of the service Transversal activities in applied genomics, Sciensano, and statistical data analysis was performed by Dr. Inge Van Damme of the servive Foodborne pathogens of Sciensano.

Experimental section

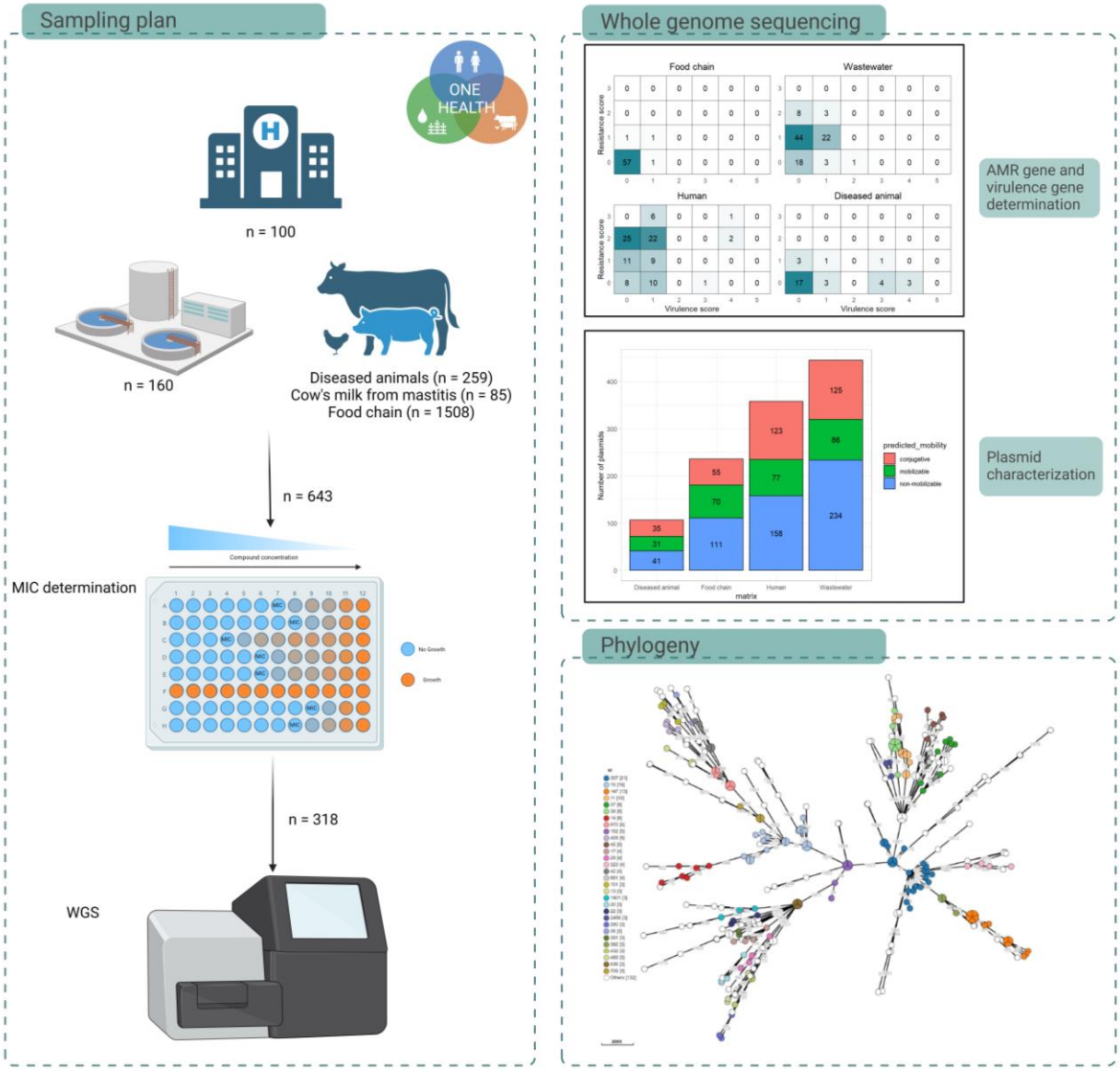
Study 1 :

One health genomic surveillance of *Klebsiella pneumoniae* in Belgium demonstrates large genomic diversity, presence of ESBLs and carbapenemases and circulation of clones across sectors

In preparation for submission in Science of the total environment

Hanne Debergh; Inge Van Damme; Bert Bogaerts; Cécile Boland; Kevin Vanneste; Koenraad van Hoorde; Pierre Bogaerts, Daniel Huang, Marc Saulmont, Nadine Botteldoorn, Claude Saegerman, Cristina Garcia-Graells

Graphical abstract



The color intensity increases with increasing number of isolates in the heatmap displaying the virulence and resistance score. MIC = minimal inhibitory concentration. WGS = whole genome sequencing. Created with BioRender.com

Abstract

Klebsiella pneumoniae is a major amplifier of antimicrobial resistance (AMR) genes, with multidrug-resistant strains such as ESBL-producing and carbapenem-resistant clones posing significant global health threats. Recognized by the WHO, *K. pneumoniae* is found across diverse reservoirs, including humans, animals, the food sector, and wastewater, with evidence of overlapping lineages. This study investigated the prevalence of *K. pneumoniae* in the One Health setting in Belgium, analyzing its resistance to critical antibiotics and exploring potential transmission routes.

A total of 2,112 samples were collected from the food chain (n = 1,508), diseased animals (n = 344), human clinical samples (n = 100), and wastewater (n = 160). *K. pneumoniae* was detected in 18.6% of fresh meat samples, 1.7% in primary production, and 69.4% of wastewater samples. Presumptive ESBL/AmpC-producers were detected in 54.4% of the wastewater samples. A total of 286 presumptive *K. pneumoniae* isolates were analyzed for their antibiotic resistance gene (ARG) content, virulence genes and plasmid replicons, with wastewater isolates harboring the highest plasmid counts, nearly half of which were potentially conjugative or mobilizable. Classic multi locus sequence typing (MLST) and core genome MLST were used for typing and initial clustering, and SNP-based phylogenies were used to investigate possible relationships between *K. pneumoniae* isolates within and between sectors. Related isolates were identified across sectors, including one cluster with identical isolates from cut poultry meat and wastewater. Other clusters across sectors included one isolate from a diseased companion animal and two human clinical isolates. These findings highlight the interconnectedness of human, animal, and environmental reservoirs, underscoring the importance of a One Health approach to monitor, prevent, and mitigate the spread of AMR in *K. pneumoniae*. Continued surveillance and targeted interventions are critical for managing this global threat.

Introduction

The antimicrobial resistance (AMR) crisis for the most difficult-to-treat infections in hospitals worldwide is driven by the ESKAPE pathogens: the Gram-positives *Enterococcus faecium* and *Staphylococcus aureus*, and the Gram-negatives *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. (Pendleton et al., 2013; Wyres & Holt, 2018). Typically, ESKAPE pathogens are environmental or commensal bacteria that cause opportunistic infections in hospitalized or immunocompromised patients, but they are generally not pathogenic otherwise. The Gram-negative ESKAPE pathogens pose the greatest threat due to the emergence of strains resistant to most or all available antibiotics (de Man et al., 2018). This accumulation of AMR is primarily facilitated by horizontal gene transfer (HGT) mediated by plasmids and mobile genetic elements (Wyres & Holt, 2018).

K. pneumoniae infections are associated with prolonged outbreaks, high disease burden and high mortality rates (Cassini et al., 2019; Murray et al., 2022). The prevalence of *K. pneumoniae* infections is increasing, and is primarily driven by extended spectrum β-lactamase (ESBL)-producing and carbapenem-resistant *K. pneumoniae* (CRKp) (Navon-Venezia et al., 2017; Wyres, Lam, et al., 2020). For example, the estimated EU incidence of bloodstream infections with carbapenem-resistant *K.pneumoniae* for 2022 increased by just under 50% compared to 2019 (European Centre for Disease Prevention and Control, 2022). These multidrug-resistant (MDR) *K. pneumoniae* clones pose a significant threat to the medical community due to limited antibiotic treatment options (de Man et al., 2018). Moreover, several AMR genes have been first detected in *K. pneumoniae* before they spread to other clinically relevant Gram-negative bacteria (Holt et al., 2015; Navon-Venezia et al., 2017). Accordingly, *K. pneumoniae* has been classified as a key amplifier and spreader of AMR genes (Wyres & Holt, 2018). Consequently, the ESBL-producing and carbapenem-resistant *K. pneumoniae* (CRKp), have been taken up into the priority pathogens list by the World Health Organization (WHO) as a critical public health threat (World Health Organization, 2022).

With 12.3%, *K. pneumoniae* was one of the most commonly reported bacterial species in EU/EEA countries in 2022 among invasive isolates originating from blood or cerebrospinal fluid (European Centre for Disease Prevention and Control, 2022). While the incidence of third generation cephalosporins (3GC) and carbapenem resistant *K. pneumoniae* in Belgium (18.3% and 1.4%, respectively) in 2022 is still far below the EU/EEA population weighted mean (32.7 % and 10.9%, respectively), prevention remains pivotal, which requires an understanding of the potential sources from which infecting organisms are acquired (European Centre for Disease Prevention and Control, 2022). The incidence is calculated by dividing the number of cases reported as R by the product of the national population, as reported to Eurostat, and multiplying this by the estimated population coverage, as reported to EARS-Net (European Centre for Disease Prevention and Control, 2022). The One Health concept, which recognizes the interconnectedness of human, animal, and environmental health,

provides a comprehensive framework for understanding and addressing the complex issue of antibiotic resistance and its transmission routes (The Food and Agriculture Organization of the United Nations (FAO) et al., 2021).

Population genomics analyses of *K. pneumoniae* have shown that hundreds of different lineages exist (Wyres, Lam, et al., 2020). These lineages correspond closely to the clonal groups (CGs), that are defined by core-genome multilocus sequence typing (cgMLST). A subset of these CGs disproportionately contribute to the global disease burden, which are also referred to as'global problem clones'. This subset includes CG258, CG15, CG147, CG101 and CG307. They are each widely geographically distributed and commonly implicated in MDR healthcare-associated infections (HAIs) and/or outbreaks. Collectively, we refer to these are also referred to as the 'global MDR clones (Navon-Venezia et al., 2017; Wyres, Lam, et al., 2020; Wyres & Holt, 2016).In contrast to these hospital-acquired global problem MDR clones, some lineages of hypervirulent *K. pneumoniae* strains (hvKp) exist. These community acquired (CA) invasive strains are generally susceptible to antibiotics and usually occur in healthy hosts. The hvKp strains are associated with the acquisition of mobile elements and/or large virulence plasmids encoding virulence determinants (M. J. Martin et al., 2023; Wyres, Lam, et al., 2020). The most common hypervirulent clonal group is CG23, followed by CG65 (including ST65 and ST375) and CG86 (Wyres, Lam, et al., 2020).

Previous studies have suggested that *K. pneumoniae* can persist in a broad range of reservoirs including the hospital environment (Clarivet et al., 2016; Mathers et al., 2019; Paskova et al., 2018), retail meat (Belmar Campos et al., 2014; Cardozo et al., 2021; Davis et al., 2015; Huizinga et al., 2019; Rodrigues et al., 2022), food-producing animals (Bonardi & Pitino, 2019; Daehre, Projahn, Semmler, et al., 2018; Köck et al., 2018), companion animals (Collineau et al., 2023; Debergh et al., 2022) and wastewater (Galler et al., 2014; Heljanko et al., 2024; Tiwari et al., 2022). Wastewater treatment plants (WWTPs), especially those receiving hospital effluents, can represent a hotspot for high-risk bacterial pathogens, which could be discharged into surface water due to inadequate wastewater treatment (Savin, Bierbaum, Mutters, et al., 2022). *K. pneumoniae* was added in the scope of the European Antimicrobial Resistance Surveillance network in Veterinary medicine (EARS-Vet) to monitor AMR in sick animals (Mader et al., 2022). In general, there are only limited data on lineage distributions in reservoirs others than human, but several reports exist on the overlap between lineages isolated from human clinical isolates and other sources. For example, the global problem clones ST11 have been isolated from a dog (Debergh et al., 2022) and from poultry (R. Zhang et al., 2019b), ST101 from pigs (De Koster et al., 2022), ST307 from a tortoise (Foster et al., 2020) and the hvKp lineage ST23 from non-human primates (Anzai et al., 2017).

In this study, we aimed to investigate *K. pneumoniae* in a One Health approach within Belgium, including clinical human isolates, isolates from diseased animals, the food chain and environmental strains. In order to gain insight in the antibiotic resistance profile, the virulence characteristics, and the genetic relatedness between the *K. pneumoniae* isolates, we performed antibiotic susceptibility testing and whole genome sequencing on a subset of samples. Our findings underscore the necessity of a One Health approach to address antibiotic resistance and highlight the urgent need for developing effective strategies at a global level.

Material and methods

1 Sample collection

1.1 The food chain

A total of 1508 samples from the annual Belgian AMR surveillance on bacteria isolated from the food chain were collected by the Federal Agency for the Safety of the Food Chain (FASFC) from September 2020 to April 2021, as part of a voluntary monitoring according to EFSA (EFSA, 2013). These included 905 samples (60%) from fresh meat (distribution sector) and 603 samples (40%) from primary production (feces and raw milk). Meat samples included fresh pork, beef, or turkey meat (n=422), whole poultry carcasses (n=96), and cut poultry with or without skin (n=387). In the primary production, fecal samples were taken at slaughterhouses from veal calves (n=111), fattening pigs (n=115), and broiler chickens (n=147), and at the farm level, 214 fecal samples of veal calves (younger than 7 months) or from poultry were collected. Additionally, 16 raw cow's milk samples were taken directly from bulk tanks at farms.

Samples were processed using the EURL-AR protocol to isolate ESBL-, AmpC-, and carbapenemase-producing *E. coli* (Hendriksen et al., 2023). This involved incubating 1g ± 0.1 g of caecal samples, 25g ± 0.5 g of meat samples or 25ml ± 0.5 ml of milk samples in 9ml, 225ml and 225ml buffered peptone water (BPW), respectively, at 37°C for 18-22 hours, followed by streaking 10 µl onto MacConkey (McC) agar plates and incubating again at 37°C for 18-22 hours. The agar plates were then sent to the Antimicrobial Resistance National Reference Laboratory (NRL-AMR, Sciensano), where three to five presumptive *K. pneumoniae* colonies were subcultured on nutrient agar (NA) for species identification using MALDI-TOF MS and antimicrobial susceptibility testing (AST). Colonies identified as *Klebsiella* spp. were stored in glycerol at -80°C for further analysis. All *K. pneumoniae* isolates from the food chain were subjected to AST. A total of 15 isolates were excluded due to microbiological contamination or the inability to reculture the isolate.

1.2 Wastewater

From April 2021 to March 2022, *K. pneumoniae* was isolated from ten wastewater treatment plants (WWTPs) serving a total of 2,536,616 inhabitants across ten locations in Belgium (Brussels North, Brussels South, Ghent, Bruges, Ostend, Leuven, Harelbeke, Grimbergen, Liederkerke, Aalst) (Supplementary table 1). The isolation of composite 24h influent wastewater samples was performed weekly during four non-consecutive months, covering each season. In total, 160 composite wastewater samples were analysed. The WWTPs processed both community and hospital wastewater. Samples were diluted 1:1000, and two 50 mL aliquots were filtered through a 0.45 µm nitrocellulose filter. The filters were placed on McC agar plates (non-selective McC agar for *Enterobacterales* isolation) and McC + cefotaxime (McC+CTX; 1 mg/L) agar plates (selective agar for the isolation of ESBL or AmpC producing *Enterobacterales*). After 24-hour incubation at 37°C, three typical colonies were subcultured

on NA and treated as described above. A total of 960 colonies were subjected to species identification. Selection criteria for AST consisted of 1 *K. pneumoniae* isolate per sampling day and per WWTP.

1.3 Diseased animals

A total of 242 isolates were obtained through passive surveillance of diseased animals between October 2019 and January 2021 from first line animal health laboratories according to the EARS-Vet scope by Animal Healthcare Flanders, Belgium (Mader et al., 2022). These included pigs (n=137), cattle (n=70), poultry (n=13), and others (n=22). Additionally, 17 *K. pneumoniae* isolates from diseased food-producing animals and a companion animal from January 2020 until September 2021 from Association Régionale de Santé et d'Identification Animales (ARSIA) , Belgium, were included in the study. Information on symptoms or antimicrobial treatment was not available. All isolates were screened for presumptive ESBL producers using McC+CTX (1mg/L) plates, and those identified were included for AST. Selection for AST of other isolates was based on animal species (cattle, pig and poultry) and gastrointestinal tract origin, totaling 126 *K. pneumoniae* isolates.

Furthermore, 58 *K. pneumoniae* isolates from individual milk samples from cows with mastitis were included, comprising clinical (n = 55) and subclinical (n = 3) cases. Milk samples (15-25 ml) were collected from one quarter, with 10 µl seeded onto Aesculin Blood Agar and McC agar and incubated at 37°C for 24 hours. Biochemical identification tests were performed, and isolates were confirmed as *K. pneumoniae* by MALDI-TOF MS (Bruker Daltonics).

1.4 Human isolates

A total of 100 clinical *K. pneumoniae* isolates resistant to 3GCs and/or carbapenemases were collected from hospitals during the biennial mandatory clinical surveillance according to EARS-Net (European Centre for Disease Prevention and Control, 2022) of ESBL and carbapenem-resistant gram-negative bacteria in humans. One single isolate per patient corresponding to the timeframe and location of the wastewater sampling campaigns was included. Clonal strains from hospital outbreaks were excluded.

1.5 Species identification

All isolates were identified using MALDI-TOF MS following manufacturer's instructions (Bruker Daltonics, Bremen, Germany). MBT Compass Library Revision K (April 2019), Revision L (November 2020) and Revision M (July 2021) were used. Isolates identified as *K. pneumoniae* by MALDI-TOF MS will be referred to as presumptive *K. pneumoniae* until species confirmation by WGS.

2 Phenotypic Resistance Testing

Antimicrobial susceptibility testing (AST) using the broth microdilution method according to ISO 20776-1:2019 was performed on a selection of *K. pneumoniae* isolates (n = 643). The first panel of antimicrobials (EUVSEC 3 Sensititre™) was tested on all strains, whereas the second panel (EUVSEC 2 Sensititre™) was performed on those isolates (n = 214) that displayed phenotypical resistance to cefotaxime, ceftazidime and/or meropenem. *E. coli* ATCC 25922 was used for quality control in both panels. Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines, using epidemiological cutoff (ECOFF) values as is described in the Commission Implementing Decision 2020/1729 for *E. coli*, since *K. pneumoniae* is not part of the Decision 2020/1729 (Commission Implementing Decision (EU) 2020/1729 of 17 November 2020 on the Monitoring and Reporting of Antimicrobial Resistance in Zoonotic and Commensal Bacteria and Repealing Implementing Decision 2013/652/EU, 2020). In the absence of epidemiological cutoff values for *K. pneumoniae*, those for *E. coli*, the indicator *Enterobacterales* bacterium, were applied (Supplementary table 5 and Supplementary table 6). Isolates with a minimally inhibitory concentration (MIC) strictly higher than the EUCAST ECOFF are referred to as 'resistant' rather than 'microbiologically antimicrobial-resistant' organisms for brevity.

3 Whole-genome sequencing and comparative genomic analyses

3.1 Selection and flowchart for whole genome sequencing

Isolate selection for whole genome sequencing (WGS) was based on their source of origin and phenotypic antimicrobial resistance profile. Clinical isolates from diseased animals' gastrointestinal (GI) tracts were chosen, and only one *K. pneumoniae* isolate per WWTP per sampling date was included. Only food samples from Belgium were considered. Selection criteria included resistance to 3rd and 4th generation cephalosporins, carbapenems, colistin, ciprofloxacin, azithromycin, temocillin, and/or amikacin.

A total of 201 unique *K. pneumoniae* isolates with at least one of these resistance profiles were selected from wastewater (n = 110), the food chain (n = 62), and diseased animals (n = 31). Additionally, 10% of fully susceptible isolates (except to ampicillin) were included: 10 from wastewater, 5 from meat samples, 1 from primary production, 3 from veterinary sources, and 1 from milk from mastitis cases (Figure 29). A subset of 100 ESBL-AmpC-producing and/or carbapenem-resistant clinical human strains from the NRC for gram-negative bacteria (CHU UCL Namur Site Godinne) was included, with dates and locations matching the WWTPs. Three out of 100 clinical isolates were excluded for further analysis due to microbiological contamination. In total, 318 isolates were sequenced (Figure 29).

3.2 DNA extraction, DNA library preparation, and whole-genome sequencing

Genomic DNA was extracted from the 318 *K. pneumoniae* isolates using the Maxwell cultured cells DNA extraction kit (Promega, Wisconsin, USA). DNA purity was assessed with a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA), and concentration was measured with a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Library preparation was performed with the TruSeq DNA Nano sample preparation kit (Illumina, San Diego, CA, USA), and sequencing was conducted on an Illumina NovaSeq 6000 platform (S4 PE150 XP, Illumina, San Diego, CA, USA).

3.3 WGS data analysis

3.3.1 Quality control & pre-processing

Pre-processing of the sequencing data began with downsampling to approximately 100x coverage, based on the estimated genome size of 5,330,000 bp for K. *pneumoniae*, using the 'sample' command of seqtk v1.4 (available at https://github.com/lh3/seqtk). Read trimming was performed with Trimmomatic v0.39 (Bolger et al., 2014a) using the following settings: LEADING=10, TRAILING=10, SLIDINGWINDOW=4:20, MINLEN=40, and ILLUMINACLIP=NexteraPE-PE.fa:2:30:10. The processed reads were de novo assembled using SPAdes v3.15.5 (Prjibelski et al., 2020) with the '--cov_cutoff' parameter set to 10 and the '--isolate' option enabled. Assembly metrics were calculated using QUAST 5.2.0, with the processed FASTQ files and assembled contigs as input, and the *Klebsiella pneumoniae* subsp. *pneumoniae* HS11286 genome as reference (RefSeq accession NC_016845.1). Processed reads were mapped to the assembly using Bowtie2 v2.5.1 (Langmead & Salzberg, 2012) with the '--end-to-end' and '--sensitive' options. The median depth was determined using the 'depth' command from samtools v1.17 (H. Li et al., 2009) with the '-a' option enabled.

3.3.2 Isolate characterization

All assemblies were analyzed using Kleborate v2.3.2 (Lam et al., 2021b) with the '--all' option. Detection of antimicrobial resistance (AMR) mutations and genes was performed using AMRFinder+ v3.11.26 (Feldgarden et al., 2021) with the 2021-12-21.1 database. Virulence genes were identified using the 'VirulenceFactor' core database (L. Chen et al., 2016) and a blastn-based approach (Bogaerts et al., 2021; Camacho et al., 2009), considering genes present if covered for at least 60% with 90% sequence identity. The 'mob_recon' command of MOB-suite v3.1.4 (Robertson & Nash, 2018a) was used to screen for mobile genetic elements (MGEs), and contigs with detected resistance and virulence genes were cross-referenced with MOB-suite output to predict their potential association with MGEs. Sequence types (STs) were determined using a blastn-based approach with the MLST scheme from the Institut Pasteur Paris BIGSdb instance (Diancourt et al., 2005b) (accessed on the 17th of December 2023).

3.3.3 Phylogenomic analysis

Core-genome MLST (cgMLST) was performed using the scheme from the Institut Pasteur Paris BIGSdb instance (Bialek-Davenet et al., 2014b) (accessed on August 18, 2022) using the methodology detailed in the previous section. A minimum spanning tree was constructed from the filtered allele matrix after removing datasets with less than 90% of loci detected and loci present in less than 75% of samples. GrapeTree v2.2 was used to construct the phylogeny with the 'MSTreeV2' method. For each ST where at least two isolates had fewer than five allele differences, an additional SNP-based phylogenomic analysis was conducted (Table 3). This involved mapping processed reads to a close reference genome of the same ST or clonal complex, with the selected reference genomes listed in Supplementary table 2. Sequences other than the chromosome were removed from the reference genome FASTA files prior to the SNP analysis. The PACU v0.0.3 'map_to_ref' script was used with default options to map reads, and SNP-based phylogenies were constructed using PACU v0.0.3 with the '--use-mega' option enabled (Bogaerts et al., 2024).

Table 3 - Guidelines for the interpretation of SNP distances

Threshold	Interpretation
0-1 SNPs	Most likely linked
2-5 SNPs	Very likely linked
6-10 SNPs	Likely linked
11-20 SNPs	Possibly linked
>20 SNPs	Not linked

3.3.4 Availability

All tools to perform the bioinformatics analysis are freely available for non-commercial use at https://galaxy.sciensano.be (registration required). The *Klebsiella* pipeline enables comprehensive characterization from raw FASTQ data, including pre-processing, quality control, and all assays described in the isolate characterization section. If cgMLST is enabled, the pipeline output can be fed directly into the 'MLST phylogeny' tool to generate a minimum spanning tree. The 'PACU mapping helper' and 'PACU' tools are also available to perform the SNP-based phylogenomic investigation.

3.3.5 Statistical analysis

To determine the prevalence of *K. pneumoniae* in the food sector, the binomial proportion and 95% confidence interval were calculated per animal species using the binom package, using the Wilson interval. The proportion of resistant isolates were calculated per matrix and were visualised using ggplot2 (Wickham, 2016). To visualise the combination of resistance against different antibiotics, the package UpSetR was used (Conway et al., 2017; Gehlenborg N, 2019). All analyses were performed using R version 4.2.3. (R Core Team, 2023).

Results

1 Occurrence of K. *pneumoniae* in the food chain and in wastewater

1.1 Occurrence of K. *pneumoniae* in the food chain

The number of samples originating from the food chain stratified per origin is shown in Figure 29. *K. pneumoniae* was detected in 178 (11.80 %) samples, with a prevalence rate of 18.6% from fresh meat and only 1.7% in the primary production. More specifically, we observed a prevalence rate of *K. pneumoniae* of 16.6% [13.3% – 20.4%] in fresh pork, beef or turkey meat, 22.9% [15.6%-32.3%] in complete carcasses of poultry, 19.6% [16.0%-23.9%] in cut poultry with or without the skin. In the primary production we observed *K. pneumoniae* in 12.5% [3.5% – 36.0%] of raw milk samples (bulk tank), 2.0% [0.7% – 5.8%] in fecal samples of broiler chickens, 1.7% [0.5% – 6.1%] in fecal samples of fattening pigs, 0% [0.0% – 3.3%] in fecal samples of veal calves and in 1.4% [0.4% –4.0%] of fecal samples at farm level. A total of 18 isolates were excluded from phenotypical analysis due to contamination (n = 3) or due to unavailability of the isolate due to the inability to regrow the isolate (n = 15) (Figure 29). In total, 163 *K. pneumoniae* isolates were subjected to AST.

Furthermore, *K. oxytoca* and *K. variicola* were also detected in the food chain. A total of 60 samples were positive for the presence of *K. oxytoca* [fresh pork, beef or turkey meat (n = 41), complete carcass of poultry (n= 2), cut poultry with or without the skin (n = 15), fecal samples at farm level (n = 2)] and *K. variicola* was detected in four samples [fresh pork, beef or turkey meat (n = 1), cut poultry with or without the skin (n = 1), fecal samples from veal calves (n = 1), fecal samples at farm level (n = 1)]. These isolates were not included in this study for further analysis.

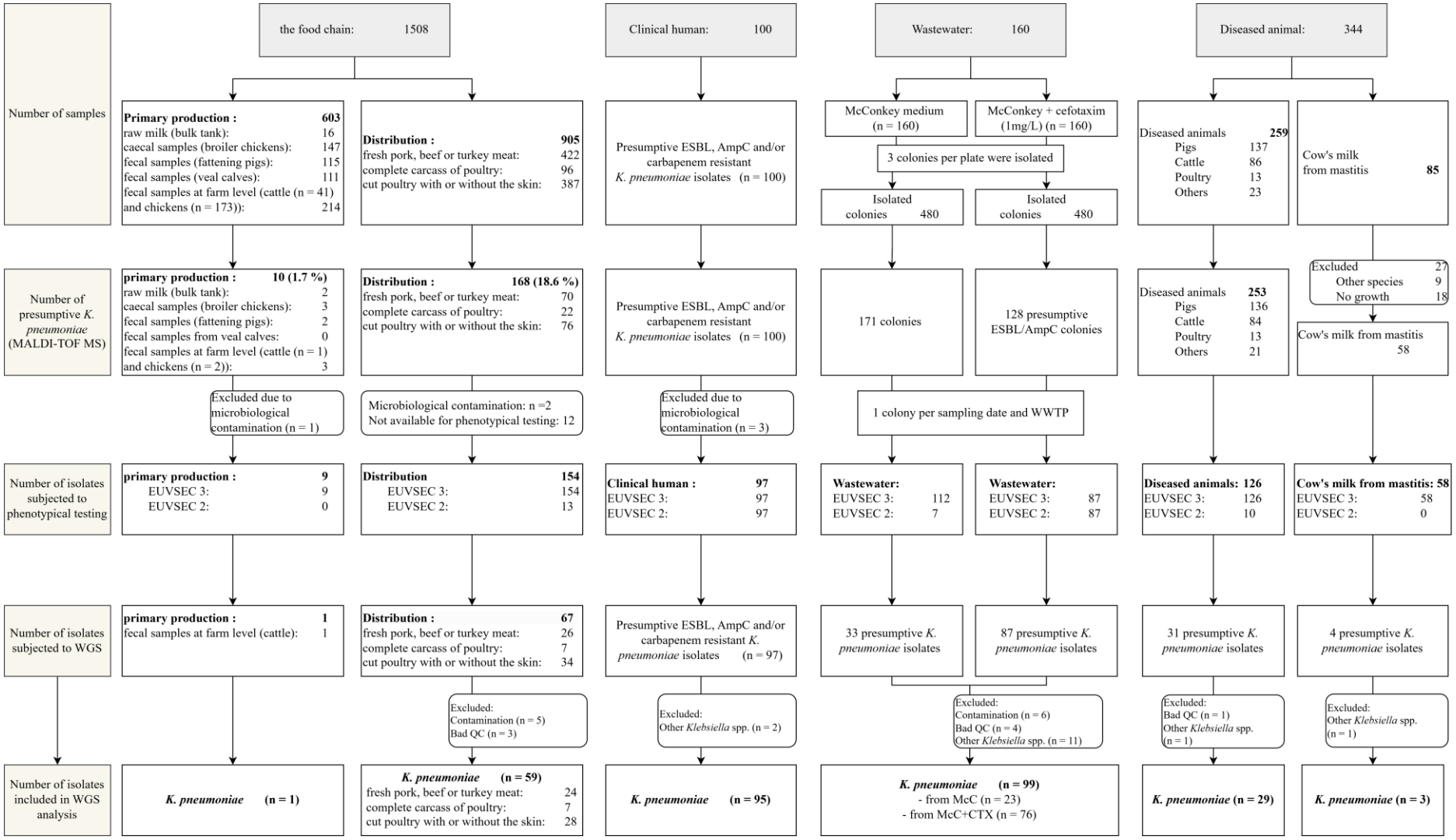


Figure 29 - Flowchart showing the number of samples from the food chain, clinical human isolates, wastewater and diseased animals, the number of samples positive for *K. pneumoniae* in this study and the final number of isolates subjected to whole genome sequencing.

1.2 Prevalence of *K. pneumoniae* in wastewater

A total of 160 wastewater samples were analyzed using two selective media. In total, species identification was performed on 960 colonies, resulting in 299 presumptive *K. pneumoniae* isolates (based on MALDI-TOF MS identification) from both isolation media (Figure 29). In total, we observed a prevalence of *K. pneumoniae* on McC agar of 69.4% (111/160) and 54.4 % (87/160) of presumptive ESBL- and/or AmpC- producing *K. pneumoniae* isolates from the McC+CTX agar.

2 Antimicrobial susceptibility testing

2.1 EUVSEC 3

A total of 643 presumptive *K. pneumoniae* isolates from the food chain (n = 163), diseased animals (n = 184), presumptive ESBL/CP from human origin (n = 97) and from wastewater (n = 199), were analyzed with broth microdilution using the Sensititre™ plates EUVSEC 3. Isolates resistant to 3rd and/or 4th generation cephalosporins and/or carbapenems (n = 214) were subjected to the EUVSEC2 panel for further confirmation and categorization of ESBL production and carbapenem resistance. The results are displayed in respectively Table 4 and Table 5. In total, 310 isolates did not display any other resistances than against ampicillin. Ampicillin sensitivity was observed in 14 isolates from wastewater (n = 8), the food chain (n = 4) and diseased animals (n = 2). A total of 18 isolates exhibited phenotypic resistance to amikacin, with 16 originating from human clinical samples, resulting in an amikacin resistance resistance proportion of 16.5% in the selection of presumptive ESBL producing and/or carbapenem resistant human clinical isolates. In comparison, resistance rates of 0.6% and 0.5% were observed in the food chain and wastewater, respectively. Resistance rates to azithromycin in human clinical isolates were 40.2%, 18.6% in isolates from wastewater, 9.8% in diseased animals, and 9.2% in the food chain. Ciprofloxacin resistance was particularly high in the clinical sector, with 88.7% of human clinical isolates showing resistance. Ciprofloxacin resistance rates of 37.2%, 24.5%, and 7.6% were observed in wastewater, the food chain, and diseased animals, respectively. Phenotypic resistance to colistin was identified in 11.3% of human clinical isolates, 8.6% of food chain isolates, 5.0% of wastewater isolates, and 4.9% of isolates from diseased animals. Meropenem resistance was detected in a total of 96 isolates. A total of 82 isolates originating from the presumptive ESBL producing and/or carbapenem resistant human clinical isolates (88.5%), followed by wastewater (n = 13; 6.5%) and the food chain (n = 1; 0.6%). Tigecycline resistance was only observed in human clinical strains. Notably, no meropenem-resistant isolates were found in diseased animals. In total, 214 isolates resistant to 3GCs were subjected to EUVSEC 2 testing for the identification of third- and fourth-generation cephalosporin (3GC and 4GC) and carbapenem-resistant strains.

Table 4 - Number of *K. pneumoniae* isolates and percentage of phenotypical resistance against each antimicrobial included in EUVSEC 3 of isolates from the four different sectors according to the ECOFF values (EUCAST, n.d.)

Antimicrobial	Wastewater (n = 199)		Human clinical (n = 97)		Food chain (n = 163)		Diseased animals (n = 184)		Total (n = 643)	
	n	%	n	%	n	%	n	%	n	%
Ami	1	0.5	16	16.5	1	0.6	0	0.0	18	4.4
Amp	191	96.0	97	100.0	159	97.5	182	98.9	629	98.1
Azi	37	18.6	39	40.2	15	9.2	18	9.8	109	19.4
Chl	25	12.6	41	42.3	18	11.0	18	9.8	102	18.9
Cip	74	37.2	86	88.7	40	24.5	14	7.6	214	39.5
Col	10	5.0	11	11.3	14	8.6	9	4.9	44	7.5
Fot	90	45.2	90	92.8	3	1.8	7	3.8	190	35.9
Gen	45	22.6	39	40.2	6	3.7	8	4.3	98	17.7
Mer	13	6.5	82	84.5	1	0.6	0	0.0	96	22.9
Nal	60	30.2	79	81.4	29	17.8	7	3.8	175	33.3
Smx	79	39.7	75	77.3	57	35.0	30	16.3	241	42.1
Taz	83	41.7	79	81.4	7	4.3	8	4.3	177	32.9
Tet	53	26.6	56	57.7	59	36.2	33	17.9	201	34.6
Tig	0	0.0	6	6.2	0	0.0	0	0.0	6	1.5
Tmp	72	36.2	72	74.2	45	27.6	30	16.3	219	38.6

n = number of tested *K. pneumoniae* isolates; Ami = amikacin; Amp = ampicillin; Azi = azithromycin; Chl = chloramphenicol; Cip = ciprofloxacin; Col = colistin; Fot = cefotaxime; Gen = Gentamicin; Mer = Meropenem; Nal = Nalidixic Acid; Smx = Sulfamethoxazole; Taz = Ceftazidime; Tet = Tetracycline; Tig = Tigecycline; Tmp = Trimethoprim. Human clinical isolates were part of a selection based on presumptive EBSL-producing or carbapenem resistant *K. pneumoniae* isolates possibly resulting in higher resistance rates towards other antimicrobials.

2.2 EUVSEC 2

Isolates displaying phenotypical resistance against cefotaxime, ceftazidime or meropenem were subjected to the Sensititre™ plates EUVSEC 2 (n = 214) (Table 5). A resistance rate to cefoxitin of 67.0% was observed in presumptive EBSL-producing and/or carbapenem resistant *K. pneumoniae* human clinical isolates (n = 65), of 30.0% in diseased animals (n = 10), and of 29.8% in wastewater isolates (n = 28). Nearly all clinical presumptive EBSL-producing and/or carbapenem resistant *K. pneumoniae* human isolates isolates exhibited phenotypic resistance to ertapenem (n = 91, 93.8%). Ertapenem resistance was observed in more than half (n = 52, 55.3%) of the wastewater isolates. Imipenem resistance was observed in 60.8% of the clinical isolates (n = 59) and in 12.8% of wastewater isolates (n = 12). No imipenem resistance was detected in isolates from the food chain or diseased animals. Temocillin resistance was found in 85.6% of the presumptive EBSL-producing and/or carbapenem resistant *K. pneumoniae* human clinical isolates and in 22.3% of wastewater isolates (n = 21). Phenotypical temocillin resistance was detected in 20.0% of the isolates from diseased animals (n = 2) and in 14.3% of *K. pneumoniae* isolates (n = 1) from the food chain.

Table 5 - Number of *K. pneumoniae* isolates and percentage of phenotypical resistance against each antimicrobial included in EUVSEC 2 of isolates from the four different sectors according to the ECOFF values (EUCAST, n.d.)

Antimicrobial	Wastewater (n = 94)		Human clinical* (n = 97)		Food chain (n = 7)		Diseased animals (n = 10)		Total (n = 214)	
	n	%	n	%	n	%	n	%	n	%
Cef	89	94.7	91	93.8	3	42.9	7	70.0	190	70.4
Fot	90	95.7	90	92.8	3	42.9	7	70.0	190	70.4
Fot+Cl	19	20.2	82	84.5	2	28.6	2	20.0	105	35.0
Fox	28	29.8	65	67.0	1	14.3	3	30.0	97	33.6
Etp	52	55.3	91	93.8	2	28.6	4	40.0	149	51.1
Imi	12	12.8	59	60.8	0	0.0	0	0.0	71	18.4
Mero	13	13.8	82	84.5	1	14.3	0	0.0	96	26.5
Taz	83	88.3	79	81.4	7	100.0	8	80.0	177	75.9
Taz+Cl	20	21.3	67	69.1	2	28.6	2	20.0	91	31.4
Tem	21	22.3	83	85.6	1	14.3	2	20.0	107	33.9

n = number of *K. pneumoniae* isolates; Cef =cefepime; Fot = cefotaxime; Fot+Cl = cefotaxime + clavulanic acid; Fox= cefoxitine; Etp = ertapenem; Imi= Imipenem; Mero = Meropenem; Taz = Ceftazidime; Taz+Cl = Ceftazidime + clavulanic acid; Tem = Temocillin. * Human clinical isolates were part of a selection based on presumptive EBSL-producing and/or carbapenem resistant *K. pneumoniae* isolates possibly resulting in higher resistance rates towards other antimicrobials.

2.3 Antimicrobial resistant profile of the ESBL/AmpC-producing and/or carbapenem resistant isolates

In Figure 30, the resistance profile of meropenem resistant isolates (n = 96) towards other critical antibiotics, such as colistin, gentamicin, azithromycin and ciprofloxacin included in EUVSEC 3 are displayed. Seven (7.3%) of these meropenem-resistant *K. pneumoniae* isolates also displayed colistin resistance, while 91.7% (n = 88) isolates displayed ciprofloxacin resistance (Figure 30). All meropenem resistant isolates were resistant to ertapenem (n = 96, 100%) and cefepime (n = 96, 100%), while 67.7% (n = 65) were resistant to imipenem, 88.5% (n = 85) were resistant to ceftazidime and 90.6% (n = 87) were resistant to temocillin (Figure 30). Azithromycin and gentamicin resistance was observed in 46.9% (n = 45) of the meropenem resistant *K. pneumoniae* isolates.

Figure 31 displays combinations of ESBL/AmpC-producing *K. pneumoniae* isolates (cefotaxime and/or ceftazidime resistance) (n = 196) and other critical antibiotics included in EUVSEC 3 and EUVSEC 2 (Figure 31). In total, 81.1% (n = 159) ESBL/AmpC-producing *K. pneumoniae* isolates were resistant to ciprofloxacin and 10.7% (n =21) were resistant to colistin (Figure 31). Of the 196 ceftazidime and/or cefotaxime resistant isolates, 95.4% (n = 187) isolates were resistant to cefepime, 75.0% (n = 147) resistant to ertapenem, 35.7% (n= 70) resistant to imipenem, 54.1% (n = 106) resistant to temocillin, 37.2% (n = 73) resistant to azithromycin and 43.4% (n = 85) resistant to gentamicin (Figure 31).

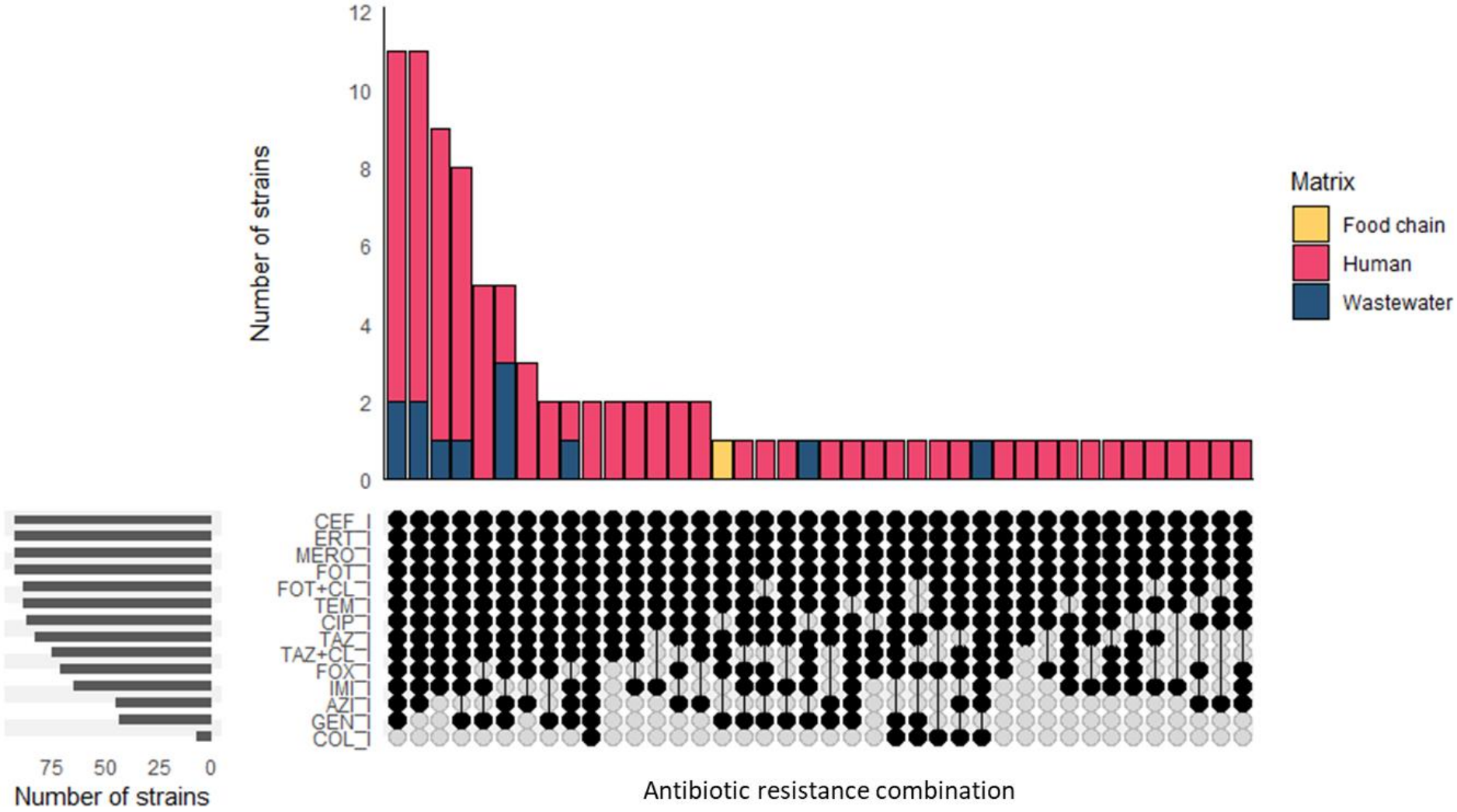


Figure 30 – UpSet plot of phenotypes of meropenem-resistant isolates with other critical antibiotics from the food chain, human clinical isolates and wastewater. A black circle means that the isolates showed phenotypical resistance against that antibiotic. A grey circle means the isolates were susceptible against the antibiotic. The black line links black circles, separated by grey circles, with eachother. Number of strains are stratified per isolation matrix (yellow = food chain, red = human, blue = wastewater). CEF = cefepime, ERT = ertapenem, MERO = meropenem, FOT = cefotaxime, FOT+Cl = cefotaxime + clavulanic acid, TEM = Temocillin, CIP = ciprofloxacin, TAZ = ceftazidime, TAZ+CL = ceftazidime + clavulanic acid, FOX = cefoxitin, IMI = imipenem, AZI = azithromycin, GEN = gentamicin, COL = colistin.

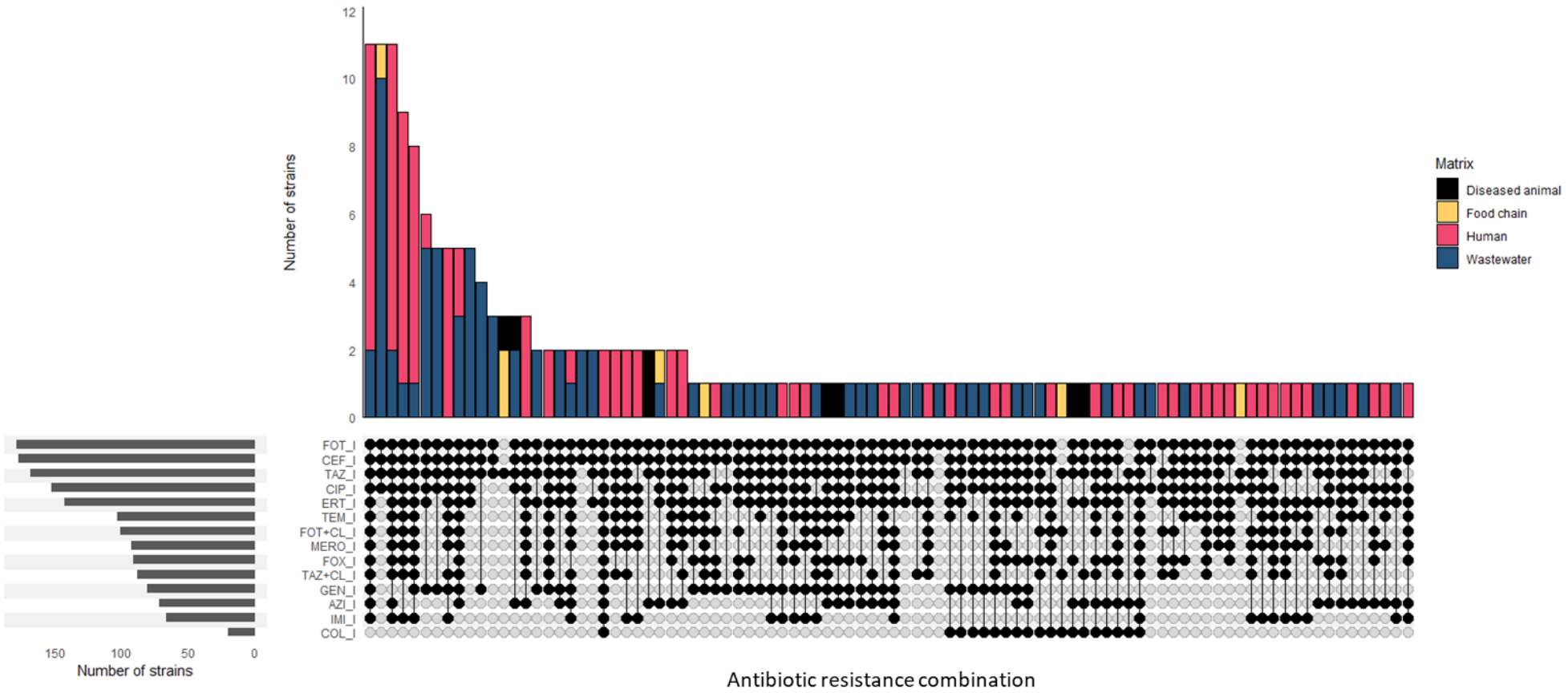


Figure 31 - UpSet plot of phenotypes of isolates resistant to cefotaxime or and/or ceftazidime with other critical antibiotics from diseased animals, the food chain, human clinical isolates and wastewater. A black circle means that the isolates showed phenotypical resistance against that antibiotic. A grey circle means the isolates were susceptible against the antibiotic. The black line links black circles, separated by grey circles, with eachother. Number of strains are stratified per isolation matrix (black = diseased animal, yellow = food chain, red = human, blue = wastewater). CEF = cefepime, ERT = ertapenem, MERO = meropenem, FOT = cefotaxime, FOT+Cl = cefotaxime + clavulanic acid, TEM = Temocillin, CIP = ciprofloxacin, TAZ = ceftazidime, TAZ+CL = ceftazidime + clavulanic acid, FOX = cefoxitin, IMI = imipenem, AZI = azithromycin, GEN = gentamicin, COL = colistin.

3 Genomic characterization using WGS

3.1 Diversity in AMR genes and virulence

A heatmap displaying resistance scores, virulence scores, and the number of isolates with these characteristics stratified by isolation source is shown in Figure 32. In terms of virulence scores, no isolates had a score of 5. However, six (6/286; 2.10%) isolates exhibited a virulence score of 4, comprising human isolates (n = 3) and diseased animal isolates (n = 3) (Table 6). Six (6/286; 2.1%) isolates had a virulence score of 3, including diseased animal isolates (n = 5) and human isolates (n = 1). All isolates from diseased animals with resistance scores of 3 and 4 originated from pigs. No environmental strains had a virulence score of 3 or higher, and no isolates from the food chain had a virulence score of 2 or above. None of the strains in our collection carried the salmochelin virulence gene, and only one isolate carried a colibactin gene (*clb3*). Additionally, no isolates belonging to hypervirulent lineages (ST23, ST65) were identified in our collection of *K. pneumoniae* isolates. Three isolates (3/286 = 1.05%), with a resistance score of 4 (n = 2) and 3 (n = 1) carried the accessory regulator gene *rmpA2*, which codes for hypermucoidy. Tests to confirm hypermucoidy or hypervirulence in a mouse model were not conducted. The aerobactin gene *iuc3* was only detected in strains from diseased pigs. To the best of our knowledge, this gene has only been detected before in *K. pneumoniae strains* in pigs (Kaspersen et al., 2023) and in pork meat (Crippa et al., 2023). The aerobactin producing gene *iuc1* has been detected before in clinical strains (Lam, Wyres, Judd, et al., 2018; Q. Zhu et al., 2023).

Table 6 - Isolates with a virulence score of 4 with their accompanying virulence genes. No colibactin and salmochelin genes were detected

Isolate	Source	ST	Yersiniabactin	Aerobactin	*rmpADC*	*rmpA2*
S22FP03118	Pig	113	*ybt 8; ICEKp3*	*iuc3*	-	-
S22FP03119	Pig	45	*ybt 10; ICEKp4*	*iuc3*	-	-
S22FP03161	Pig	530	*ybt 9; ICEKp3*	*iuc3*	-	-
S23FP03518	Human	2096	*ybt 14; ICEKp5*	*iuc1*	-	*rmpA2*_8-60%
S23FP03545	Human	101	*ybt 9; ICEKp3*	*iuc1*	-	-
S23FP03806	Human	709	*ybt 9; ICEKp3*	*iuc1*	*rmp 1; KpVP-1*	*rmpA2*_6*-47%

The columns '*rmpADC*' and '*rmpA2*' show if the respective genes are missing or truncated. Truncations are expressed as % amino acid length from the start codon. Nothing is reported in the column if the gene is present or not truncated.

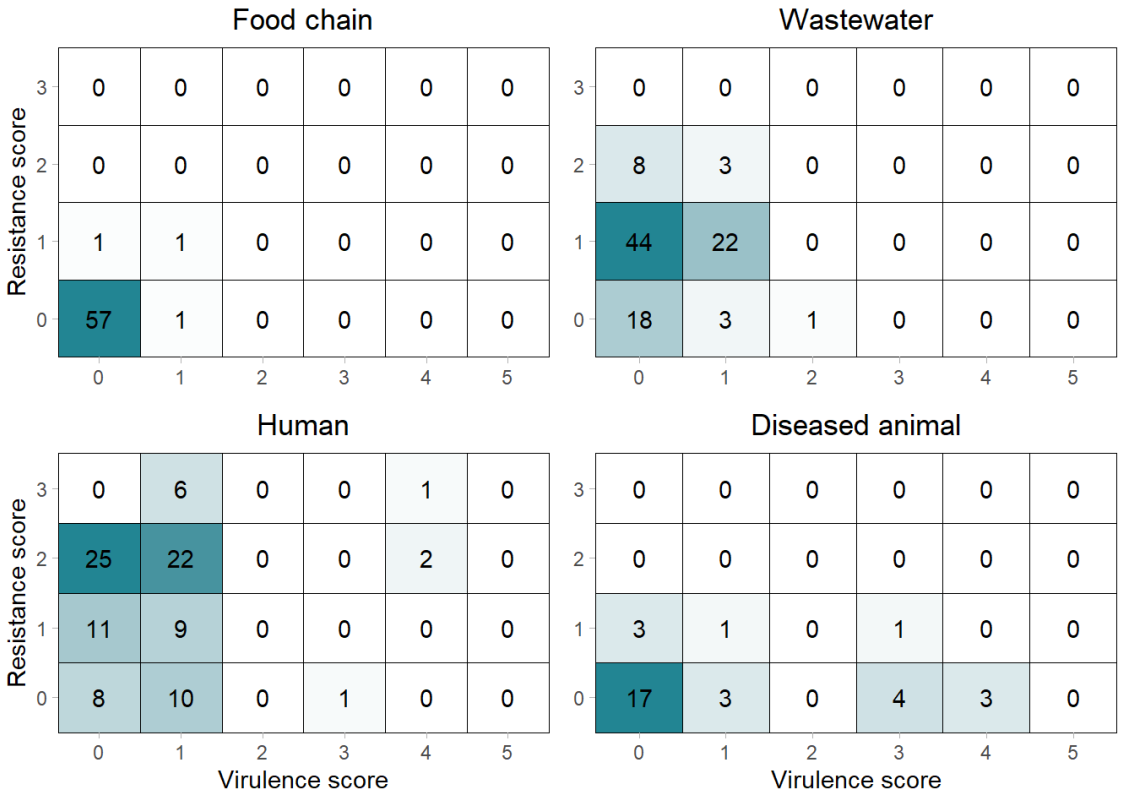


Figure 32 – Heatmap displaying resistance score and virulence score and the number of isolates with those characteristics stratified by isolation matrix. A total of 286 isolates are included from the food chain (n = 60), diseased animals (n = 32), the environment (n = 99) and human isolates (n = 95). Virulence score: 0 = no yersiniabactin, colibactin or aerobactin; 1 = yersiniabactin only; 2 = yersiniabactin and colibactin (or colibactin only); 3 = aerobactin only; 4 = aerobactin with yersiniabactin (no colibactin); 5 = yersiniabactin, colibactin and aerobactin. Resistance score: 1 = ESBL; 2 = Carbapenemase; 3 = Carbapenemase plus colistin resistance; 0 otherwise. Resistance scores and virulence scores were obtained by Kleborate (Lam et al., 2021b).

The most observed AMR genes/mutations according to the sector are provided in Supplementary table 3. In our collection, a resistance score of 3, indicating the presence of carbapenemase gene(s) plus colistin resistance, was observed exclusively in human isolates (n = 7). A detailed overview of their STs, ESBL resistance genes, carbapenemase genes, outer membrane protein mutations, and *mgrB* gene mutations conferring colistin resistance is provided in Table 7. None of these isolates carried a known *mcr* gene. Phenotypic colistin resistance was confirmed only in 2 isolates (S23FP03817 and S23FP03837). These seven isolates with a resistance score of 3 belong to lineages of global problem clones (ST101, ST15, ST11). No isolates with a resistance score of 2 were detected from the food chain or in diseased animals. Most of the human isolates had a resistance score of 2 (n = 49), while most environmental isolates had a resistance score of 1 (n = 66). In isolates from the food chain and diseased animals, a resistance score of 0 was most frequently observed. Caution is necessary when interpreting the results of the resistance scores and virulence scores as only a selection of the isolates of this collection has been subjected to WGS, alongside the selective inclusion of ESBL/AmpC-producing and/or carbapenem resistant isolates from human clinical strains and wastewater isolates.

Table 7 – Human isolates displaying a resistance score of 3 with their acquired ESBL and carbapenemase genes, the mutations in the outer membrane proteins and the mutations in the *mgrB* gene conferring colistin resistance.

isolate	ST	ESBL genes	carbapenemase genes	*OmpK* mutations	Col mutations*
S23FP03545	101	-	bla_{KPC-3}	*OmpK35*-17%; *OmpK36TD*	*mgrB* -0%
S23FP03563	15	$bla_{CTX-M-15}$	bla_{OXA-48}	*OmpK35*-70%	*mgrB* -6%
S23FP03804	11	$bla_{CTX-M-15}$	bla_{NDM-1};$bla_{OXA-181}$	*OmpK36GD*	*mgrB* -60%
S23FP03813	11	$bla_{CTX-M-15}$	bla_{NDM-1};$bla_{OXA-181}$	*OmpK36GD*	*mgrB* -60%
S23FP03817	15	-	bla_{OXA-48}	*OmpK35*-70%	*mgrB* -6%
S23FP03837	15	$bla_{CTX-M-15}$	bla_{OXA-48}	*OmpK35*-70%	*mgrB* -6%
S23FP03848	11	$bla_{CTX-M-15}$	bla_{NDM-1};$bla_{OXA-181}$	*OmpK36GD*	*mgrB* -60%

The column 'col mutations' shows if the *mgrB* gene is missing or truncated. Truncations are expressed as % amino acid length from the start codon. GD = Glycine-Aspartate, TD = Threonine-Aspartate

3.2 Number of acquired AMR gene load per strain

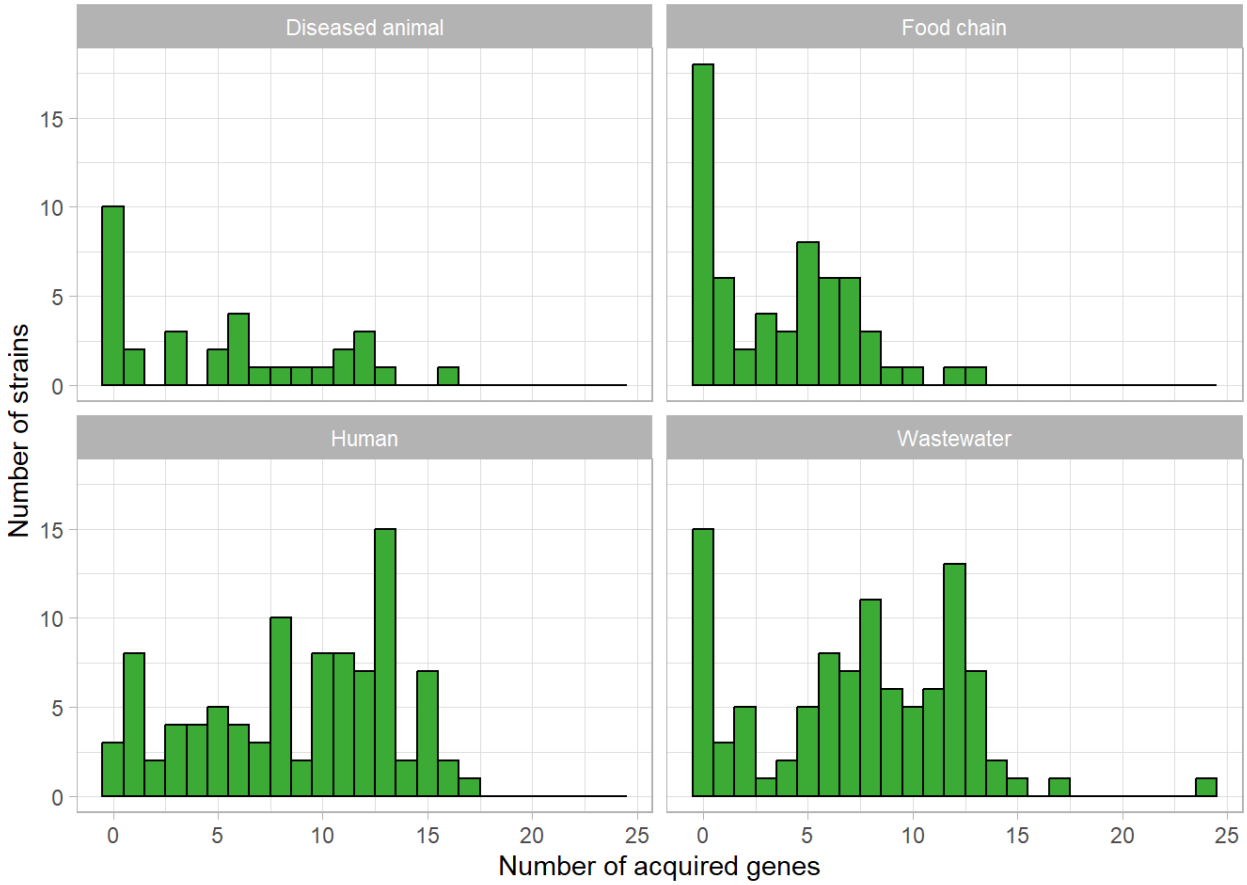


Figure 33 - Number of acquired antimicrobial resistance genes (as provided by Kleborate (Lam et al., 2021b)) load per strain, stratified by source of isolation

Since the majority of resistance to clinically relevant antibiotics in *K. pneumoniae* is associated with horizontally acquired AMR genes, the number of acquired genes per sector was mapped (Figure 33). Bimodal distributions are evident, with most strains either carrying zero acquired AMR genes (indicating susceptibility to all tested antibiotics except ampicillin) or possessing between five to ten acquired ARGs when isolated from the food chain or diseased animals. In contrast, human and environmental strains typically carry between 10-15 acquired ARGs (Supplementary table 4).

3.3 Distribution of carbapenemase genes among the isolates

Carbapenemase genes encoding KPC, NDM, OXA-48-like and VIM enzymes were detected in 67 isolates – 11 isolates from wastewater and 56 from human origin (Figure 34).

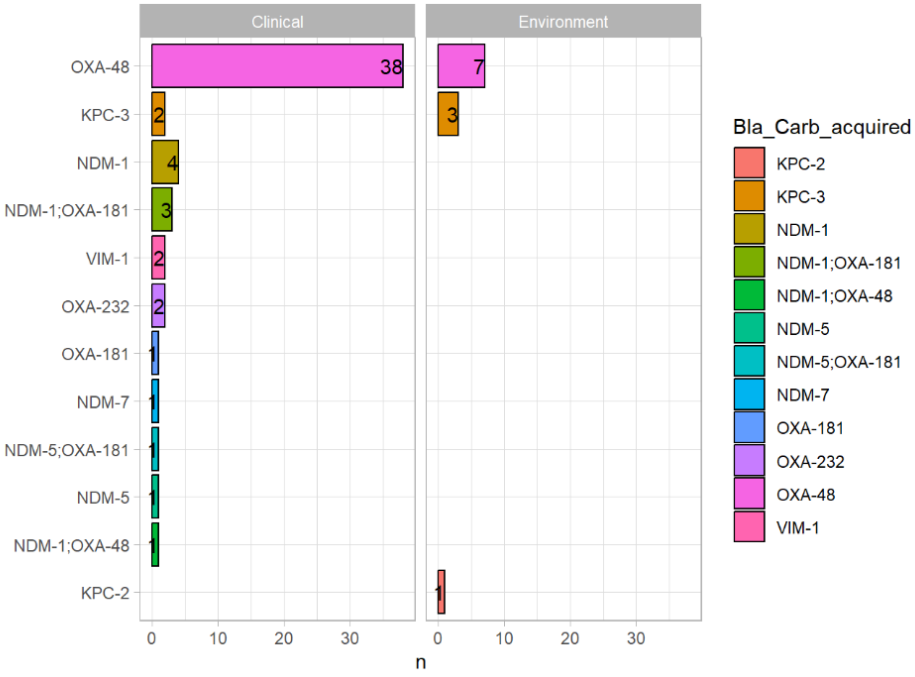


Figure 34 – Distribution of carbapenemase encoding genes (KPC, NDM, OXA-48-like and VIM) detected in carbapenem resistant isolates (according to EUCAST ECOFF values of meropenem >0.125 mg/L and imipenem >1 mg/L).

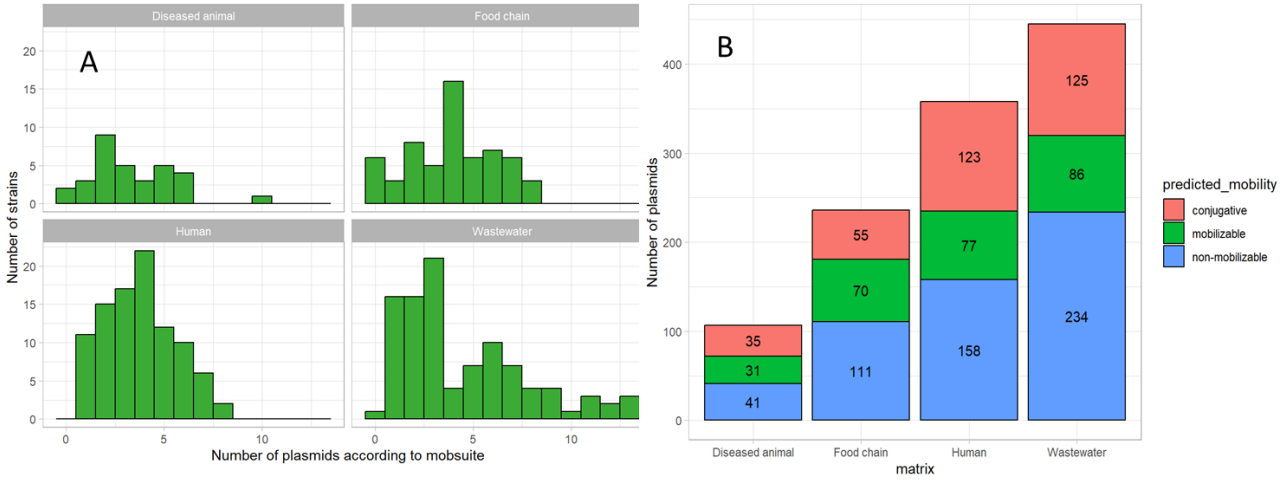
Among these, OXA-48-like β-lactamases (OXA-48, -181, -232), which are capable of hydrolyzing carbapenem antibiotics, were found in 79.10% of the isolates (n = 53), with $bla_{\text{OXA-48}}$ being the most prevalent (n = 46, 68.7%). In total, 59.70% of the $bla_{\text{OXA-48-like}}$ positive isolates (n = 40) also carried the $bla_{\text{CTX-M-15}}$ ESBL gene. Five human clinical isolates co-produced NDM-1 or NDM-5 enzymes alongside OXA-48-like enzymes. Isolates producing NDM-1 belonged to ST11 or ST14 and the isolate producing $bla_{\text{NDM-5}}$ belonged to ST16. A total of 9.0% of carbapenem resistant isolates (n = 6) carried a bla_{KPC} gene, of which only one was $bla_{\text{KPC-2}}$ and originated from wastewater. Two clinical isolates produced $bla_{\text{VIM-1}}$.

Given that carbapenem non-susceptibility can also arise from mutations in the outer-membrane proteins (*OmpK35* and/or *OmpK36*) in combination with the expression of ESBL and/or acquired AmpC β-lactamases, all strains were screened for known mutations in these genes. Variations in *OmpK35* and/or

OmpK36 were found in 16 isolates, 12 of which carried the ESBL gene $bla_{\text{CTX-M-15}}$. Among these, eight isolates were non-susceptible to all tested carbapenem antibiotics, while the remaining four were resistant to meropenem and ertapenem but susceptible to imipenem.

3.4 Plasmid replicons

The number of detected plasmid replicons and their predicted mobility stratified by the matrix is shown in Figure 35. The highest number of detected plasmid replicons per isolate was detected in wastewater, with 3 isolates carrying up to 13 plasmid replicons (Figure 35A). The most plasmid replicon content were observed in isolates from wastewater (n = 445; #isolates = 99), followed by human clinical isolates (n = 358; #isolates = 95), the food chain (n = 236; #isolates = 60) and diseased animals (n = 107; #isolates = 29). A total of 12 isolates did not carry any plasmid replicons. In total, 544 non-mobilizable plasmid replicons, 338 conjugative plasmid replicons and 264 mobilizable plasmid replicons were observed (Figure 35B). The plasmid replicon IncFIB(K) was most frequently detected (n = 182), with the highest occurrence in isolates from wastewater, followed by IncFII(K), IncR and IncL (Figure 35C). Plasmid replicon IncL was predominantly detected in human isolates (n = 38), with only low detection in wastewater (n = 7) and none in food chain or diseased animal isolates. We observed a low detection of plasmid replicon IncH1B(pNDM-MAR) in isolates originated from diseased animals (n = 2) and food chain (n = 7), but higher detection in humans (n = 17) and wastewater (n = 16).



C

Locus	Diseased animal	Food chain	Human	Wastewater	Total
IncFIB(K)_1	23	21	61	77	182
IncFII(K)_1	13	13	53	65	144
IncR_1	11	16	27	26	80
IncL_1	0	0	38	7	45
IncHI1B(pNDM-MAR)_1	2	7	17	16	42
IncFIA(HI1)_1	2	7	14	19	42
Col440I_1	1	2	11	22	36
IncFIB(K)(pCAV1099-114)_1	7	5	11	12	35
repB(R1701)_1	0	4	18	12	34
ColRNAI_1	7	0	17	6	30
Others	31	57	70	117	275
Total	**97**	**132**	**337**	**379**	**945**

Figure 35 – Plasmid replicon characterization. (A) Number of plasmid replicons according to MOB-suite (Robertson & Nash, 2018b) and their respective number of strains stratified per sector; (B) Number of plasmid replicons with their predicted mobility using MOB-suite (Robertson & Nash, 2018b) stratified per matrix and (C) the top ten detected plasmid replicons and their respective frequency of detection per matrix.

3.5 Population structure and phylogeny

A selection of 286 *K. pneumoniae* isolates were included in the genomic analysis coming from the food chain (n = 60), diseased animals (n = 29), the environment (n = 99) and human isolates (n = 95). Sequence typing revealed the presence of 141 different STs, with 92 of those only observed once in our collection of strains. Additionally, 17 of the 141 STs represented novel STs (isolates will be submitted in BIGsdB). The most frequently observed STs were ST307 (n = 21), ST15 (n= 18), ST147 (n = 13) and ST11 (n = 12) (Supplementary figure 1). Several clusters could be observed with five isolates (ST147, ST39), with three isolates (ST670, ST11, ST307, ST 363, ST152) and with two isolates (ST42, ST670, ST11, ST392, ST 307) (Supplementary figure 1). Figure 36 displays the distribution of STs that were observed twice or more in our collection by their source of origin. Several STs were only observed in environmental isolates (e.g., ST 584, ST 461 and ST253), in human isolates (ST20 and ST101) or in isolates coming from the food chain (e.g., ST485, ST46 and ST107). Other STs were shared between human and environmental isolates (ST 307, ST39, ST22, ST152, ST323, ST1401 and ST13), between diseased animals and the environment (ST661, ST29, ST17 and ST391), between isolates from the food chain and the environment (ST42, ST469, ST280 and ST3369) and between isolates from humans and diseased animals (ST35, ST353). Furthermore, some STs were observed in isolates from multiple origins (ST15, ST147, ST11, ST37, ST870, ST14, ST45 and ST405).

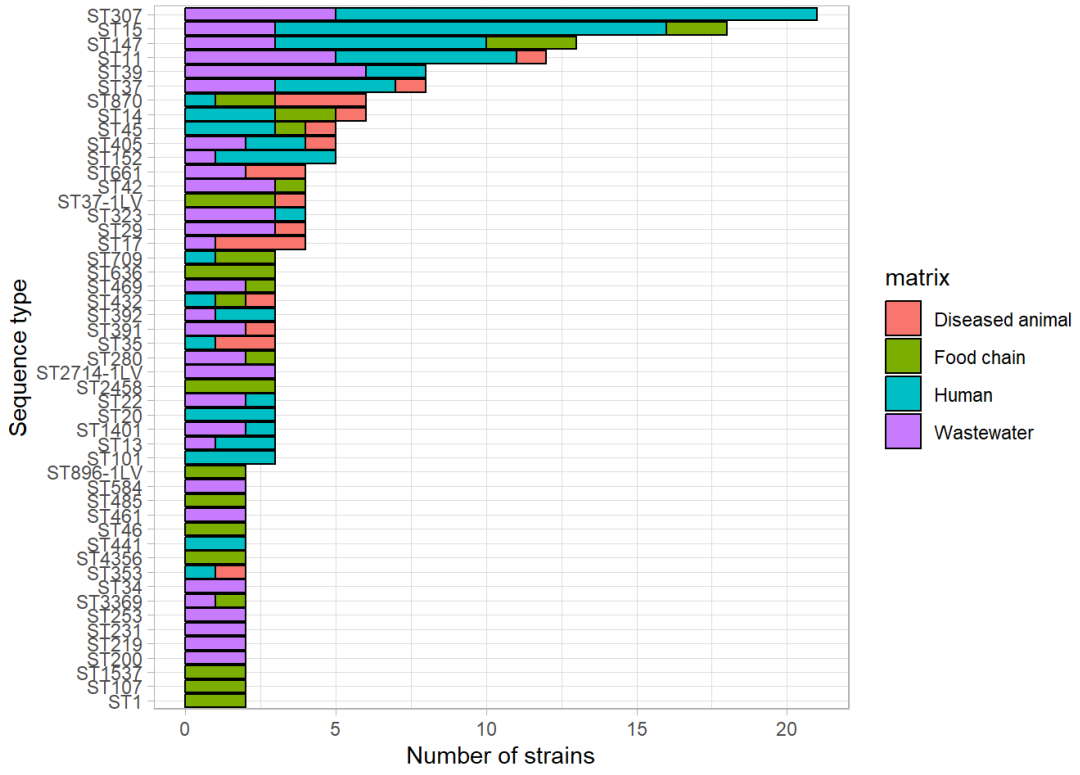


Figure 36 - Number of strains per observed sequence type. Results are stratified by origin (pink = diseased animal; green = food chain, blue = human, purple = wastewater); Only STs detected in two or more isolates are included in this figure.

3.6 Cluster analysis of *K. pneumoniae* within and across sectors

Cluster analysis within and across sectors was performed for those STs where at least two isolates had fewer than five allele cgMLST differences using SNP-based phylogenomic analysis. A total of 16 clusters could be observed, with 12 within sectors and 4 across sectors (Figure 37). The clusters within the same sector included: clusters of clinical isolates (ST11, ST15, ST147, ST152 and ST307), clusters within wastewater isolates (ST11, ST39 and ST307), clusters of isolates from diseased animals (ST870) and isolates from the food chain (ST870). Clusters across sectors were also observed: isolates from the food chain and from wastewater (ST147 and ST42) and clusters between human clinical isolates and environmental isolates (ST11) and between human clinical isolates and a veterinary strain (ST11).

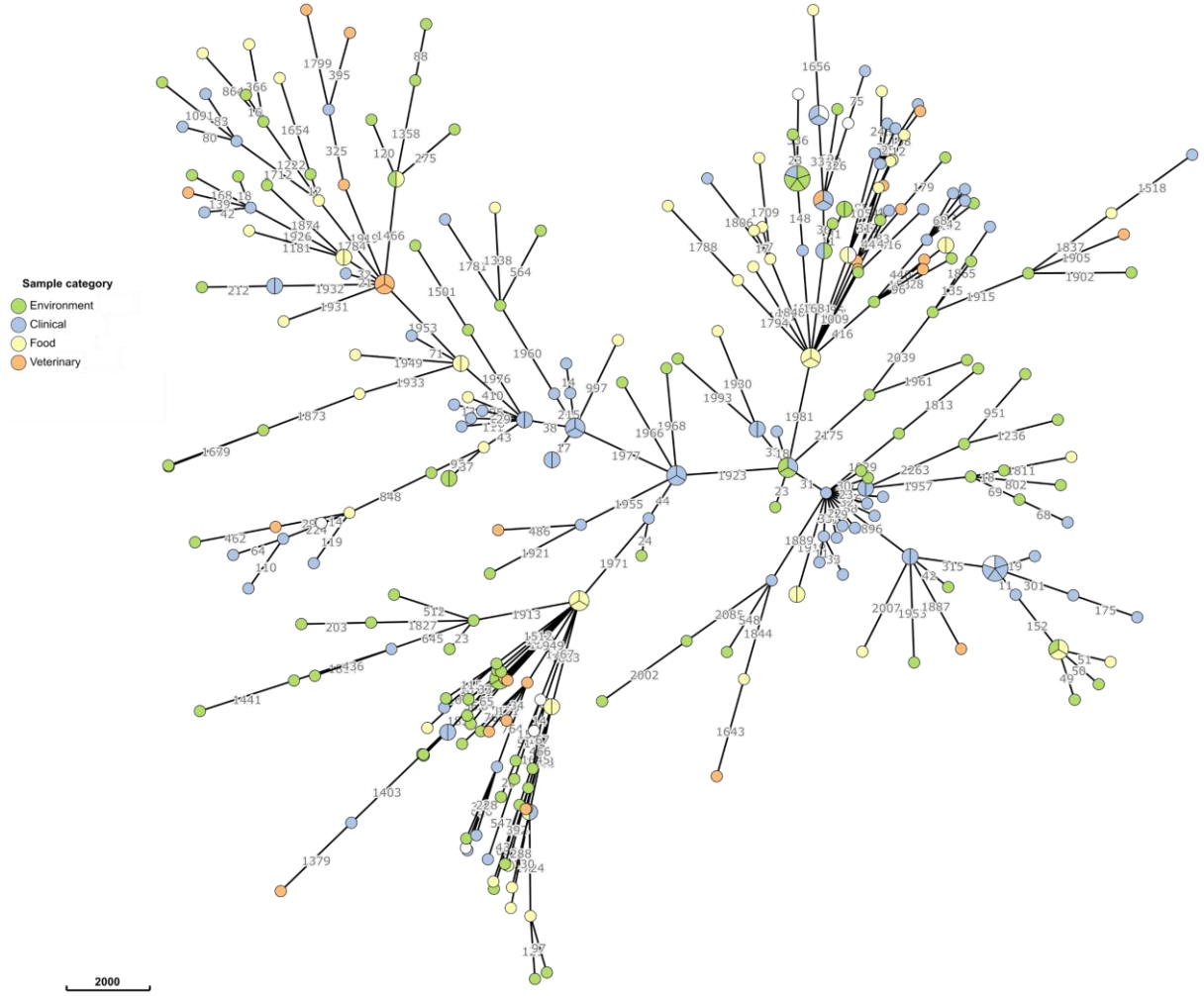


Figure 37 - Minimum spanning tree using core genome multilocus sequence type (cgMLST) phylogeny of 286 *K. pneumoniae* isolates stratified by sample category. Minimum spanning tree using core genome multilocus sequence type (cgMLST) phylogeny of 286 *K. pneumoniae* isolates stratified by sample category. Branches are collapsed at 10 alleles. Branch lengths and the scale bar represent the number of cgMLST allele differences on a logarithmic scale. The cgMLST scheme includes a total of 2,358 loci.

3.6.1 Cluster analysis within sectors

Several clusters consisting of human clinical isolates were observed. First, two different clusters consisting of *K. pneumoniae* ST11 isolates were observed. One of these consisted of 2 isolates differing by 2 SNPs, isolated on the same day from two patients submitted to the same hospital. The other cluster consisted of three *K. pneumoniae* ST11 isolates differing by 8 and 9 SNPs, all taken within three months from each other from the same hospital. Second, a cluster of two *K. pneumoniae* ST147 isolates was observed which differed by 7 SNPs. The strains were isolated 7 months apart from different hospitals. Third, a cluster with three clinical *K. pneumoniae* ST152 isolates were observed. The isolates differed by 7, 9 and 10 SNPs from each other. They were all isolated two weeks from each other and originate from three different hospitals. A fourth cluster with clinical *K. pneumoniae* ST15 isolates, all carrying the $bla_{\text{OXA-48}}$ gene, showing a 14-SNPs difference, consisted of two isolates from different patients in the same hospital in Tienen, collected approximately three weeks apart. The fifth cluster included three isolates (ST15) with SNP differences of three and six. These isolates were from different patients hospitalized in the same hospital in Leuven, all taken within three months of each other. Unfortunately, no additional information about the specific wards was available. Lastly, the sixth cluster involved two clinical *K. pneumoniae* isolates (ST307) carrying the $bla_{\text{OXA-48}}$ gene, differing by 3 SNPs, originating from 2 patients within the same hospital in Brussels, isolated about 1 month from each other.

A total of three clusters involving environmental isolates were identified. We observed a cluster of two environmental isolates (ST307), separated by only 1 SNP, which were detected in wastewater from Ghent and Bruges, isolated 1 week apart. A second cluster consisting of 2 *K. pneumoniae* ST11 isolates, isolated from the same WWTP (Harelbeke) 16 months aparts, differed by only 5 SNPs. Lastly, a cluster consisting of three *K. pneumoniae* ST39 isolates was found. This cluster represents a likely cluster of two environmental isolates collected on the same day (in Ghent & Brussels South) differing by only 1 SNP and a high similarity (difference of 15 and 16 SNPs) to another strain collect in Brussels South 6 month later.

Additionally, one cluster of two *K. pneumoniae* isolates (ST870) from the food chain with 13 SNPs difference was observed. Both isolates were isolated from cut poultry at distribution level. The strains were isolated about five months apart, however, no additional information regarding the meat processing location could be obtained. A second cluster consisting of three *K. pneumoniae* isolates (ST870) from diseased pigs was observed. A difference of 1 SNP was observed between two isolates, indicating a potential link between these isolates. Furthermore, only six and seven SNP differences were seen between these isolates and the other isolate, indicating that they are likely linked. The strains with one SNP difference were isolated on the same day and the other isolate was isolated about two months earlier. No information regarding the identity of the farms was available.

3.6.2 Transmission across sectors

Multiple clusters were observed across different sectors: between the food chain and the environment (ST42 and ST147), between clinical settings and the environment (ST11), and between clinical settings and the veterinary sector (ST11).

One notable cluster involved two *K. pneumoniae* isolates (ST147) from the food chain and one *K. pneumoniae* isolate (ST147) from a wastewater sample in Leuven. Remarkably, no SNP differences were detected between these three isolates. The food samples were isolated one day from each other, and the wastewater sample was taken two weeks later. The food samples originated from cut poultry meat, but no information about the meat processing location was available to confirm a direct link.

A second cluster involved two *K. pneumoniae* isolates (ST42) with 8 SNPs difference. The environmental sample was collected from the WWTP in Leuven while the food chain sample came from fresh beef at the distribution level. Information regarding the location of the meat processing factory was not available. The strains were sampled more than a year from each other.

A third cluster consisted of two clinical *K. pneumoniae* isolates (ST11) and one isolate from a diseased animal (ST11). The clinical strains differed by two SNPs and were from patients hospitalized on the same day in January 2022 at a hospital in Huy, Belgium. The veterinary isolate originated from a dog, as previously described (Debergh et al., 2022). There was an 11 to 13 SNP difference between the clinical isolates and the veterinary isolate, but no information was available about the isolation location of the dog. The veterinary strain was isolated in June 2020.

Lastly, an environmental-human cluster involving *K. pneumoniae* ST11 was observed. Both environmental isolates (S22FP05847 and S22FP05864) were from the WWTP in Leuven, collected in April 2021 and August 2021, respectively, and differed by 10 SNPs. The human clinical isolate came from a patient in a hospital in Geel in November 2021, located approximately 45 km away. The clinical isolate differed by 11 SNPs from S22FP05847 and by 15 SNPs from S22FP05864.

Discussion

Surveillance studies on *K. pneumoniae* that integrate the One Health approach—combining data from the food chain, diseased animals, environmental, and clinical sources—are relatively rare (Wyres, Lam, et al., 2020). *K. pneumoniae* is known for its extensive genetic diversity and its efficient ability to harbor and transmit ARGs (Wyres, Lam, et al., 2020). In this study, we examined the prevalence of *K. pneumoniae* in the food chain and wastewater and studied the occurrence of antibiotic resistance, particularly ESBL-producing and carbapenem-resistant *K. pneumoniae*, across the food chain, diseased animals, wastewater, and human clinical isolates.

The interpretation of our obtained results should be done with caution as there was difference in sampling strategy between the different sectors. In the food chain and in diseased animals, the McC medium was used, whereas in wastewater sampling the selective McC + CTX medium was used to select for ESBL-producing isolates. The included *K. pneumoniae* strains from the human clinical sector were presumptive ESBL-producing and/or carbapenem resistant isolates. This has led to a bias in the observed resistance and virulence profiles, leading to an overestimation of the proportion of resistant *K. pneumoniae* isolates in the human clinical sector and in wastewater samples. Furthermore, the McC medium was used for the isolation of *K. pneumoniae*, which has proven to be less efficient than the SCAI medium in the isolation of *K. pneumoniae* from food products. This could have potentially led to an underestimation of *K. pneumoniae* in the primary distribution, due to the commensal flora present in the intestines of animals, leading to difficulties in the identification and isolation of *K. pneumoniae*. Lastly, short read data was used for the analysis of plasmid replicon data, which is known to be error-prone, so results should be interpreted with caution.

We detected *K. pneumoniae* in 18.6% of fresh meat samples, which is lower than reported in other studies: 60% KpSc in chicken meat in a European study (Rodrigues et al., 2022), 47% in retail turkey, chicken, and pork products from the United states (Davis et al., 2015), and 46.7% in calf and chicken meat samples from Turkey (Gundogan et al., 2011). Several reasons may explain this difference. First, the isolation method used (McConkey agar) may have made it more difficult to differentiate and isolate *K. pneumoniae*, as McConkey agar is known to challenge the differentiation of *Klebsiella* species from other bacteria (Rodrigues et al., 2022). Some studies have identified Simmons citrate agar with 1% inositol (SCAI) as a more effective medium for isolating *Klebsiella* species (Rodrigues et al., 2022; Van Kregten et al., 1984). Second, the use of MALDI-TOF for species identification has been shown to be challenging for *K. pneumoniae* sensu stricto, leading to possible misidentification and, consequently, overestimation of *K. pneumoniae* sensu stricto (Rodrigues et al., 2018; Thorpe et al., 2022). This was confirmed in 15 isolates from our collection, which were initially identified as *K. pneumoniae* by MALDI-TOF MS but later reclassified as other species within the *Klebsiella pneumoniae* species complex (KpSC) through whole-genome sequencing (WGS). Lastly, large variations in prevalence of *K. pneumoniae* per country are expected, as shown in the study by Rodrigues et al. where prevalence of

of KpSC in chicken meat samples ranged between 43.3% and 46.6% for three of the countries (Austria, Italy, and Ireland), whereas in two other countries (France and Denmark), the prevalence was 72.5% and 90.0%, respectively (Rodrigues et al., 2022). Noteworthy, comparisons with other studies may be inaccurate, as many studies used selective isolation methods to detect colistin-resistant or ESBL- or carbapenemase-producing *K. pneumoniae*, which limits our understanding of the broader ecology and natural diversity of *K. pneumoniae* populations in food samples (Chaalal et al., 2020; Davis & Price, 2016; Huizinga et al., 2019; Ludden et al., 2020).

Despite the relatively high prevalence of *K. pneumoniae* in the fresh meat samples, ESBL-producing *K. pneumoniae* carrying the $bla_{CTX\text{-}M\text{-}15}$ gene was detected in only 0.6% (1 out of 168) of the samples, a rate comparable to the 1.3% observed in a European study (Rodrigues et al., 2022). This isolate showed resistance to ertapenem, but no resistance to meropenem or imipenem was detected, which aligns with other studies and is likely due to the ban on carbapenem use in livestock production (ANSES, 2022; Martak et al., 2024). No ARGs or outer membrane porin mutations were present that could explain the phenotypical ertapenem resistance. Carbapenemase-producing Enterobacteriaceae (CPE) contamination in food products is sporadic in Europe. For example, VIM-1-producing *E. coli* has been found in retail seafood and pigs in Germany and in retail meat in Belgium (Garcia-Graells et al., 2020; Köck et al., 2018; Roschanski et al., 2017). In contrast, CPE contamination of food products is more common in Africa and Asia, where carbapenemase-producing *E. coli*, *K. pneumoniae*, and *Enterobacter cloacae* have been identified in poultry, chicken meat, cows, milk, and pigs (Köck et al., 2018). In India and China, NDM producers are the most frequently identified CPEs in animals and food products (Köck et al., 2018).

In our study, the highest phenotypical antimicrobial resistance rates detected (excluding ampicillin due to instrinsic resistance) in *K. pneumoniae* from the food chain were to tetracyclines (36.2%), sulfamethoxazole (35.0%), and trimethoprim (27.6%). These findings reflect recent reports of antimicrobial sales for use in food-producing animals in various European countries from 2010 to 2022 (European Medicines Agency, 2023). More specifically, in Belgium, the highest sales of antimicrobial sales for use in food-producing animals were noted for penicillines, tetracyclines and sulfamethoxazole, respectively (European Medicines Agency, 2023). A low prevalence (1.7%) of *K. pneumoniae* was observed in primary production, specifically in the feces of food-producing animals.

K. pneumoniae was observed in 69.4% (111/160) of the wastewater samples after isolation on McC agar and 54.4% of the wastewater samples were positive for the presence of presumptive ESBL- or AmpC-producing isolates on McC+CTX agar. Cefotaxime resistance was found in 45.2% of the *K. pneumoniae* isolates (from both isolation media), closely aligning with the 42.5% of ESBL-producing *K. pneumoniae* reported by Ludden et al. after isolation on Brilliance™ ESBL agar (Oxoid, Basingstoke, UK) (Ludden et al., 2020). Additionally, high resistance rates of 41.7% to ceftazidime, 39.7% to sulfamethoxazole, 37.2% to ciprofloxacin, and 36.2% to trimethoprim were observed. Notably, 52 (55.3%) of the *K. pneumoniae* isolates from wastewater displayed ertapenem resistance, with 25.0%

(13/52) and 21.2% (11/52) of these isolates also resistant to meropenem and imipenem, respectively. A study from Germany (Savin, Bierbaum, Schmithausen, et al., 2022) reported higher meropenem resistance rates, though selective isolation of ertapenem-resistant isolates, compared to our ESBL-producing selective isolation, may have biased the occurrence of meropenem and imipenem resistance (Savin, Bierbaum, Mutters, et al., 2022).

The prevalence of *K. pneumoniae* resistant to 3GCs and carbapenems in municipal WWTPs poses a significant health risk. Phylogenomic analysis using cgMLST in this study revealed two clusters between human clinical isolates and those from WWTPs, suggesting the potential for transmission from hospital effluents into municipal WWTPs. For example, Crettels et al. studied the presence of ESBL-*E. coli* in two hospitals and their receiving WWTPs (Crettels et al., 2024). They observed in one hospital that ESBL-*E. coli* represents up to 25.7% of the total *E.coli* flora. In the untreated wastewater of the receiving WWTP, ESBL-*E. coli* made up 20.5% of the total *E.coli* flora. This reduced to 4.6% in the treated wastewater (Crettels et al., 2024). Other studies have similarly reported high concentrations of carbapenem-resistant *K. pneumoniae* (CRKp) in clinical wastewater, which is subsequently discharged into municipal sewer systems (Kehl et al., 2022; Kizny Gordon et al., 2017; Müller et al., 2018; Surleac et al., 2020). Although WWTPs reduce the concentrations of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) by approximately 2 orders of magnitude, they still release these contaminants into the environment (J. Wang et al., 2020). The presence of these bacteria in surface waters indicates that conventional biological treatment is insufficient to fully eliminate microbial loads, highlighting the negative impact of inadequately treated wastewater on surface waters (Cimen et al., 2023; Savin, Bierbaum, Schmithausen, et al., 2022).

A study from Austria reported two MDR *K. pneumoniae* ST985 isolates with identical cgMLST profiles, sampled from the same river at locations 200 km apart, demonstrating the potential survival distance of *K. pneumoniae* in river water (Lepuschitz et al., 2019). This phenomenon was possibly also observed in our strain collection, where a cluster of environmental *K. pneumoniae* ST307 isolates, separated by only 1 SNP, was detected in wastewater from Ghent and Bruges (approximately 50 km apart), sampled one week apart. However, this could also indicate the presence of a common reservoir or closely related strains that are present in both WWTP without transmission between the two WWTPs. A more in depth analysis is required to elucidate this. *Klebsiella* species in wastewater can retain clinically relevant traits, including those acquired through horizontal gene transfer (HGT), even after treatment, suggesting that the further dissemination of 3GC-resistant and CRKp isolates to animals and humans, as well as their potential colonization and/or infection, cannot be ruled out (Rocha et al., 2022).

Regarding virulence genes, the majority of *K. pneumoniae* isolates had a virulence score of 0, with 32.57% (94/286) showing a score greater than 0. The highest number of isolates with a virulence score of 1 (n=81) were predominantly from human clinical samples (n=47), followed by wastewater isolates (n=28). Interestingly, most isolates with a virulence score of 3 (presence of aerobactin) or 4 (presence of aerobactin and yersiniabactin) were found in diseased animals, particularly pigs, followed

by human clinical isolates. The precise combination of phenotypic and genotypic markers (e.g., mucoviscosity, siderophore concentration) that most accurately predict the hypervirulent phenotype remains unclear (Russo et al., 2024). Russo et al. developed a prediction model based on phenotypic and genotypic characteristics to better forecast the hypervirulent phenotype, which is crucial for surveillance, research, and clinical applications (Russo et al., 2024). One clinical human isolate exhibited both a virulence score of 4 and a resistance score of 3, indicating the occurence of a highly virulent and MDR *K. pneumoniae* strain. Such convergent strains, where a highly virulent strain acquires ARGs or an MDR strain acquires virulence factors, could significantly escalate the threat posed by *K. pneumoniae* (Wyres, Lam, et al., 2020). Hybrid vectors can arise through mechanisms like recombination of AMR and virulence plasmid backbones (Lam et al., 2019), insertion of AMR genes into common virulence plasmids (Dong et al., 2018; Shen et al., 2019), or the insertion of virulence loci into MDR plasmids (Turton et al., 2018). However, the presence of a hypervirulent phenotype can not be determined solely on the presence of virulence genes, and its associated virulence score. Russo et al. stated that the virulence score performed less well than the presence of five biomarkers *iucA, iroB, peg-344, rmpA,* and *rmpA2* for differentiating the hvKp and cKp strains (Russo et al., 2024). They reported a sensitivity of 44%, a specificity of 94% and an accuracy of 78% for the prediction of the hypervirulent phenotype. This may be due to the weight given to aerobactin in the virulence score by Kleborate and the fact that not all hvKp strains possess yersiniabactin or colibactin and some cKp strains also possess these genes (Russo et al., 2024).

Mapping the number of horizontally acquired AMR genes per sector revealed bimodal distributions. In the food chain and diseased animal samples, most strains either carried no acquired AMR genes or had between five and ten. In contrast, human and environmental strains typically carried between 10 to 15 acquired AMR genes. Additionally, a total of 1,146 plasmids were identified across our collection of 286 *K. pneumoniae* isolates, with the highest number of plasmids (n = 445) found in strains isolated from wastewater, highlighting the environment's significant role in the transmission of ARGs. A high proportion of the detected plasmids were putatively conjugative or mobilizable, increasing the risk of transmission. Notably, twelve isolates in our collection were plasmid-free. Since wastewater serves as a common convergence point for human, animal, and environmental waste, it may act as a mixing vessel where bacteria from different sources can exchange genetic material, including ARGs, via plasmids. Assessing the public health risks posed by environmental reservoirs of AMR remains challenging without large-scale sampling efforts combined with WGS (Holmes et al., 2016; Stanton et al., 2020; Thorpe et al., 2022). Wastewater-based epidemiology (WBE) has emerged as a valuable tool for monitoring public health and detecting pathogens. Although WBE currently focuses mainly on antibiotic-resistant *E. coli* (Huijbers et al., 2020; Hutinel et al., 2019; Pärnänen et al., 2019), it is still in the early stages for *K. pneumoniae* (Galarde-López et al., 2022; King et al., 2020; Radisic et al., 2023). Nevertheless, several studies have reported the presence of *K. pneumoniae* in sewage systems (Radisic et al., 2023), hospital effluents, and WWTPs (King et al., 2020; Thorpe et al., 2022). WBE

could potentially provide early warning signals for *K. pneumoniae* outbreaks and offer a non-invasive method to gather data on the entire population served by the wastewater treatment plants (Sims & Kasprzyk-Hordern, 2020).

Population structure analysis using MLST and cgMLST genotyping revealed significant genetic diversity among the *K. pneumoniae* isolates, with 141 different STs identified among 286 isolates. In our study, high-risk clonal groups (CG258, CG15, CG20, CG29, CG37, CG147, CG101, CG307) made up 32.9% (94/286) of the recovered isolates, with the majority of these (68.1%, or 64/94) belonging to ST11, ST15, ST147, and ST307. ST307 was found in both clinical and environmental isolates, while ST15 and ST147 were also observed in isolates from the food chain. *K. pneumoniae* ST11 was isolated from the environment, clinical strains and from a veterinary strain. Reports showed the presence of ST11 (Montenegro et al., 2023; Surleac et al., 2020), ST15 (Obasi et al., 2017), ST147 (Nüesch-Inderbinen et al., 2018) and ST307 (Savin, Bierbaum, Mutters, et al., 2022) in wastewater. Notably, global problem clones ST512 and ST258 were each observed only once, both as human clinical isolates, and carried the blaKPC-3 gene, consistent with findings from other studies (Campos-Madueno et al., 2022).

Furthermore, multiple clusters with these high-risk clones were detected in our collection, both within and across sectors, which should raise awareness of potential for transmission between sectors in a One Health approach. However, we were unable to obtain sufficient metadata in certain sectors, leading to data gaps which limits the interpretability of the results. For example, we were not able to obtain data regarding meat processing environments of the fresh meat samples, nor did we receive data regarding the location of isolation of the diseased animals.

Three potential clusters with the global problem clone *K. pneumoniae* ST15, all carrying the carbapenemase gene $bla_{\text{OXA-48}}$, were observed within the hospital sector. Within these clusters, a maximum difference of 14 SNPs was observed, suggesting clonal spread within patients of different hospitals. Similar events of clonal spread of *K. pneumoniae* ST15 was observed in a Vietnamese neonatal intensive care unit (Berglund et al., 2021). A cluster with the global problem clone *K. pneumoniae* ST307, carrying the carbapenemase gene $bla_{\text{OXA-48}}$, was observed in a hospital in Brussels involving two strains isolated about 1 month apart. A difference of only 3 SNPs was observed, suggesting that these were very likely linked. All these data suggest sporadic clonal transmission, however, we excluded human clinical strains that were part of a large hospital outbreak. No evidence was present for transmission across hospitals.

Several clusters of *K. pneumoniae* were identified among environmental isolates. Notably, a cluster of ST307 isolates showed only a 1 SNP difference, despite being collected from different wastewater treatment plants (WWTPs) in Ghent and Bruges (located about 50km from each other) isolated just one week apart. This could be explained by the persistence of a clone in a common source upfront of the WWTPs or by a clone that is highly circulating in the community, in animals or in the environment. Given that ST307 is a global problem clone, it is likely that these strains originated from

a hospital setting, although further analysis is needed to fully understand the transmission route. Another cluster, involving the high-risk clone ST11, was found from the same WWTP in Harelbeke, with isolates collected four months apart. This suggests that *K. pneumoniae* may have the ability to persist in aquatic environments over extended periods or the presence of a clone circulating in the community. For example, Carlsen et al. described the long term persistence of *K. pneumoniae* ST147 strains for over 7 years in a tertiary care hospital in Germany (Carlsen et al., 2023).

Evidence of clonal spread within the food chain (2 *K. pneumoniae* ST870 isolates from chicken meat isolated 5 months apart) and within the veterinary sector (3 *K. pneumoniae* ST870 isolates from pigs) could indicate that clonal transmission is not confined to clinical and environmental settings. Similar patterns have been observed in the poultry sector, where clonal transmission of colistin-resistant *K. pneumoniae* has been reported in chicken flocks (Mourão et al., 2023). Despite the overall high diversity of *K. pneumoniae*, the presence of closely related lineages across samples suggests that poultry could be a potential source of human exposure to this pathogen. This underscores the importance of continued surveillance and proactive farm-to-fork measures to mitigate public health risks (Mourão et al., 2023).

These clusters in our collections within sectors might represent sporadic clonal transmission, however we also observed evidence of cross-sector transmission, which is alarming. In our study, we identified a cluster of *K. pneumoniae* ST147 isolates consisting of two isolates from the food chain (poultry) and one from a wastewater sample in Leuven. Remarkably, no SNP differences were found between these three isolates. It is possible that the strains from the food chain contaminated wastewater in a meat processing factory, which then ended up in the wastewater treatment plant in Leuven, where it was detected two weeks after the *K. pneumoniae* was isolated from the chicken meat. However, due to the lack of data concerning the meat processing plants, we were unable to confirm this. Projahn et al. described the cross-contamination of carcasses with ESBL-producing Enterobacteriaceae during scalding and defeathering in slaughterhouses (Projahn et al., 2019). The authors described the presence of ESBL-*E. coli* transmission via scalding water (Projahn et al., 2019). Given that *K. pneumoniae* ST147 is considered a high-risk clinical clone (Wyres, Lam, et al., 2020), we hypothesize that the strain may have originated from a human working in the factory. A close relationship between retail meat and human urinary tract infections with *K. pneumoniae* has been reported before (Davis et al., 2015). However, further in-depth analysis is required to confirm this hypothesis, which is currently limited by the lack of information on the processing facilities. We advocate for including such information in mandatory monitoring data collection. Understanding these contamination sources is crucial for developing and implementing intervention measures to prevent the spread of ESBL-producers during processing and to limit their further dissemination into wastewater. A second cluster involving the environmental sector and the food chain (fresh beef meat) was observed with two *K. pneumoniae* (ST42) strains that differed by only eight SNPs. Remarkably, the strains were isolated 14 months apart. Due to

the lack of additional information regarding the meat samples, we are unable to speculate on the origin of relatedness between the isolates.

Several articles have documented the transmission of healthcare-associated clones between companion animals and humans, which warrants close attention (Garcia-Fierro et al., 2022; Marques, Menezes, et al., 2019; Røken et al., 2022). In this study, we observed a potentially similar event with a veterinary strain (ST11) from a companion animal (Debergh et al., 2022) that was closely related to two human clinical strains, isolated from different patients in the same hospital, suggesting possible exchange of strains between humans and companion animals. However, a direct epidemiological link between the dog and its possible owners could not be established due to the lack of additional information about the dog and possible contact between them. Lastly, an ST11 cluster was observed between two isolates from wastewater from a WWTP in Leuven and a clinical sample from a patient in Geel, with SNP differences of 10 to 15, suggesting that human- environmental transmission could occure.

Taken together, this study highlights the occurence of *K. pneumoniae* across various sectors, including the food chain, diseased animals, wastewater, and human clinical isolates, using a One Health approach. The results indicate high genetic diversity and resistance profiles among *K. pneumoniae*, particularly the global problem clones ST11, ST15, ST147, and ST307. A low detection rate of highly virulent strains was observed, with virulence scores of 3 and 4 detected in human clinical and diseased animal isolates. Although clusters within sectors suggest possible sporadic clonal transmission, cross-sector transmission of high-risk clones, such as ST147 between the food chain and wastewater, was also detected, raising concerns. The study underscores the potential for *K. pneumoniae* strains to persist in the environment and suggests wastewater as a significant reservoir for antimicrobial resistance genes (ARGs). Additionally, challenges related to the accuracy and the difference of isolation methods and the need for more detailed metadata were highlighted, calling for more comprehensive monitoring efforts across sectors to better understand transmission routes and mitigate public health risks.

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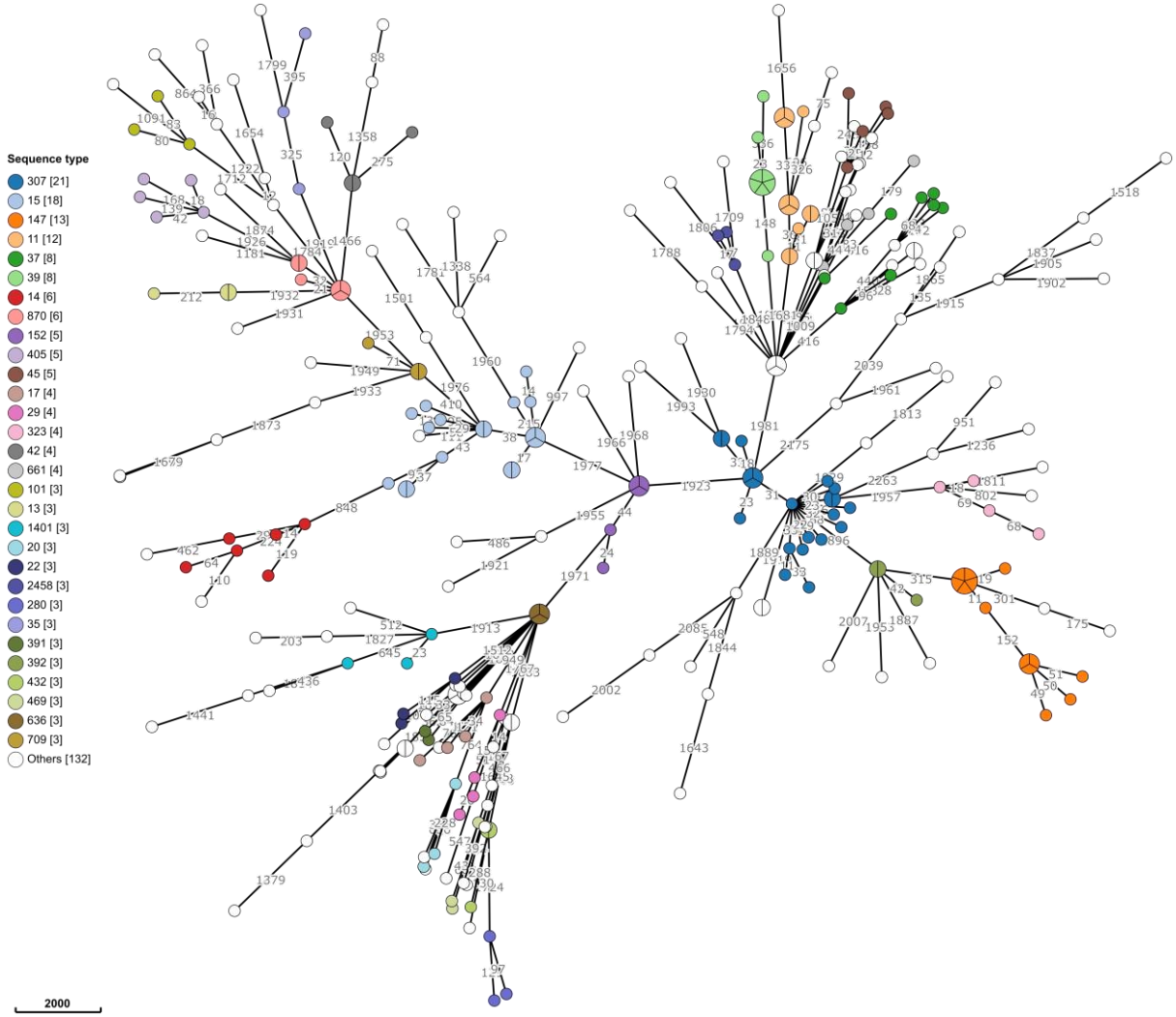
Supplemental material



Supplementary figure 1 - cgMLST-based minimum spanning tree of the 286 *K. pneumoniae* isolates. The legend displays the most frequently detected STs alongside their color code. Branches are collapsed at 10 alleles. Branch lengths and the scale bar represent the number of cgMLST allele differences on a logarithmic scale. The cgMLST scheme includes a total of 2,358 loci.

Supplementary table 1 - Ten wastewater treatment plants studied in this thesis and the population size covered by the WWTP

WWTP site	population covered by the WWTP
Brussels North	1100000
Brussels South	360000
Gent	221791
Brugge	181266
Oostende	136065
Leuven	121597
Harelbeke	108627
Grimbergen	107414
Liederkerke	101238
Aalst	98618

Supplementary table 2 - Selected reference genomes

Sequence type	**Reference genome RefSeq accession**
ST11	GCF_000445405.1
ST13	GCF_002951635.1
ST147	GCF_003031345.1
ST15	GCF_002209405.1
ST152	GCF_003957455.1
ST307	GCF_003934185.1
ST39	n/a*
ST392	GCF_001952875.1
ST42	GCF_036347675.1
ST4356	GCF_002257935.4
ST636	GCF_030845735.1
ST709	GCF_022353525.1
ST870	GCF_024918175.1
Novel ST[a]	GCF_900636455.1
Novel ST[b]	GCF_002752995.1

(*) The selected reference genome for ST39 was not available on RefSeq, but is available on the Intitut Pasteur Paris BIGSdb instance with ID 57203. This table lists the selected reference genomes for the SNP-based phylogenomic analysis. The first column lists the sequence type. The second column lists the RefSeq accession number for the selected reference genome.

Supplementary table 3 - Most commonly observed AMR genes/mutations according to matrix. Numbers indicate the number of strains

Gene symbol	Human	Wastewater	Diseased animal	Food chain	Total
fosA	91	89	32	56	268
oqxA	88	81	29	45	243
oqxB	50	37	13	36	136
$bla_{\text{CTX-M-15}}$	60	64	1	2	127
$bla_{\text{TEM-1}}$	48	52	7	15	122
aph(3'')-Ib	45	49	8	9	111
aph(6)-Id	45	49	7	9	110
sul2	43	46	9	11	109
sul1	45	29	14	18	106
qacEdelta1	42	28	14	16	100
tet(A)	28	35	7	22	92
$bla_{\text{SHV-11}}$	25	36	10	15	86
$bla_{\text{OXA-1}}$	47	36	2	0	85
catB3	48	36	1	0	85
dfrA14	32	43	2	6	83
aac(6')-Ib-cr5	39	38	1	0	78
$bla_{\text{SHV-1}}$	19	23	4	29	75
aac(3)-IIe	34	33	6	0	73
oqxB19	24	22	8	14	68
qnrS1	9	12	7	25	53
qnrB1	21	30	0	1	52

Gene symbol	Human	Wastewater	Diseased animal	Food chain	Total
aadA2	12	17	7	11	47
bla$_{\text{OXA-48}}$	39	7	0	0	46
mph(A)	22	12	8	4	46
aadA1	19	8	7	11	45
tet(D)	22	2	11	10	45
bla$_{\text{SHV-28}}$	32	8	0	0	40
dfrA12	13	10	7	5	35
aph(3')-Ia	13	11	9	1	34
oqxB25	6	14	8	4	32
arr-3	14	8	1	0	23
bla$_{\text{SHV}}$	1	10	6	3	20
dfrA1	3	4	4	9	20
catA1	10	6	0	3	19
oqxA10	0	10	0	9	19
bla$_{\text{LAP-2}}$	0	2	3	12	17
fosA10	3	9	0	4	16
fosA7	4	8	1	3	16
qacE	6	9	1	0	16
bla$_{\text{SHV-27}}$	0	5	4	6	15
lnu(F)	2	0	2	11	15
oqxB32	5	9	0	1	15
bla$_{\text{DHA-1}}$	6	6	1	0	13

Gene symbol	Human	Wastewater	Diseased animal	Food chain	Total
dfrA15	9	4	0	0	13
ble	12	0	0	0	12
qnrB4	6	5	1	0	12
bla_{TEM}	8	3	0	0	11
dfrA27	6	5	0	0	11
oqxA11	4	0	1	6	11
oqxB14	3	4	2	2	11
aadA22	2	0	4	4	10
lnu(G)	0	0	2	8	10
aac(6')-Ib	6	3	0	0	9
aadA16	5	4	0	0	9
$bla_{\text{NDM-1}}$	8	0	0	0	8
floR	1	1	4	2	8
oqxB5	1	3	1	3	8
sul3	0	0	4	4	8
ant(2'')-Ia	1	6	0	0	7
catA2	3	3	1	0	7
aac(3)-IId	2	4	0	0	6
$bla_{\text{CTX-M-9}}$	0	6	0	0	6
bla_{OXA}	4	2	0	0	6
mcr-1.1	1	1	2	2	6
qacL	0	1	2	3	6

Gene symbol	Human	Wastewater	Diseased animal	Food chain	Total
qnrB6	3	3	0	0	6
aph(3')-VI	5	0	0	0	5
bla$_{\text{KPC-3}}$	2	3	0	0	5
bla$_{\text{OXA-181}}$	5	0	0	0	5
bla$_{\text{OXA-9}}$	3	2	0	0	5
bla$_{\text{SHV-26}}$	1	2	0	2	5
bla$_{\text{SHV-76}}$	2	2	1	0	5
mph(E)	4	0	1	0	5
msr(E)	4	0	1	0	5
oqxB20	3	2	0	0	5
sat2	3	0	1	1	5
armA	4	0	0	0	4
bla$_{\text{CTX-M-1}}$	0	1	3	0	4
bla$_{\text{SHV-168}}$	2	2	0	0	4
bla$_{\text{SHV-187}}$	3	1	0	0	4
bla$_{\text{SHV-36}}$	1	3	0	0	4
erm(B)	1	0	3	0	4
qnrA1	0	4	0	0	4
tet(B)	1	0	1	2	4
tet(M)	0	0	4	0	4
aac(6')-Ib3	1	2	0	0	3
bla$_{\text{OXA-10}}$	2	1	0	0	3

Gene symbol	Human	Wastewater	Diseased animal	Food chain	Total
bla$_{\text{SCO-1}}$	2	0	1	0	3
blaSHV-60	1	0	1	1	3
cmlA1	0	0	1	2	3
oqxA3	1	0	2	0	3
aac(6')-Ib4	1	1	0	0	2
aadA13	0	0	0	2	2
ant(3'')-Ia	0	0	0	2	2
bla$_{\text{CTX-M-3}}$	0	2	0	0	2
bla$_{\text{NDM-5}}$	2	0	0	0	2
bla$_{\text{NDM-7}}$	2	0	0	0	2
bla$_{\text{OXA-232}}$	2	0	0	0	2
bla$_{\text{SHV-33}}$	1	0	1	0	2
bla$_{\text{SHV-40}}$	0	2	0	0	2
bla$_{\text{SHV-41}}$	0	0	0	2	2
bla$_{\text{VIM-1}}$	2	0	0	0	2
dfrA5	1	0	0	1	2
erm(42)	0	0	2	0	2
fosA5	1	1	0	0	2
mef(C)	0	0	2	0	2
mph(G)	0	0	2	0	2
qnrE1	1	0	1	0	2
aac(3)-IVa	0	0	0	1	1

Gene symbol	Human	Wastewater	Diseased animal	Food chain	Total
aac(3)-VIa	0	0	0	1	1
aac(6')-Ib-cr	0	1	0	0	1
aph(4)-Ia	0	0	0	1	1
arr	0	1	0	0	1
arr-2	1	0	0	0	1
$bla_{\text{CMY-6}}$	1	0	0	0	1
$bla_{\text{CTX-M-32}}$	0	0	1	0	1
$bla_{\text{CTX-M-65}}$	0	1	0	0	1
$bla_{\text{KPC-2}}$	0	1	0	0	1
$bla_{\text{SHV-115}}$	0	0	0	1	1
$bla_{\text{SHV-116}}$	0	0	1	0	1
$bla_{\text{SHV-119}}$	0	1	0	0	1
$bla_{\text{SHV-14}}$	0	1	0	0	1
$bla_{\text{SHV-196}}$	0	0	1	0	1
$bla_{\text{SHV-2}}$	1	0	0	0	1
$bla_{\text{SHV-217}}$	0	0	0	1	1
$bla_{\text{SHV-2A}}$	0	1	0	0	1
$bla_{\text{SHV-37}}$	1	0	0	0	1
$bla_{\text{SHV-77}}$	0	0	1	0	1
$bla_{\text{TEM-135}}$	0	0	0	1	1
$bla_{\text{TEM-30}}$	0	1	0	0	1
$bla_{\text{TEM-32}}$	0	1	0	0	1

Gene symbol	Human	Wastewater	Diseased animal	Food chain	Total
cmlA5	1	0	0	0	1
dfrA16	0	1	0	0	1
dfrA30	0	1	0	0	1
dfrA7	0	1	0	0	1
dfrA8	1	0	0	0	1
ere(A)	1	0	0	0	1
mcr-8.2	0	1	0	0	1
mph(B)	0	0	0	1	1
qacK	0	1	0	0	1
qnrB	1	0	0	0	1
qnrB2	0	1	0	0	1
qnrS2	0	1	0	0	1
rmtB1	1	0	0	0	1
rmtC	1	0	0	0	1

Supplementary table 4 - Gene combinations within columns with acquired genes, and the calculated number of acquired genes. n indicates the number of strains with this gene combination

acquiredgenes	N acquired	n
	0	46
aac(3)-IIa.v1^;aac(6')-Ib-cr.v2;strA.v1^;strB.v1;qnrB1.v2^;CatB4.v1?;sul2;tet(A).v1;dfrA14.v2;OXA-1;TEM-1D.v1^;CTX-M-15*	12	10
aac(3)-IIa.v1^;aac(6')-Ib-cr.v2;strA.v1^;strB.v1;qnrB1.v2^;CatB4.v1?;sul2;tet(A).v1;dfrA14.v2;OXA-1;TEM-1D.v1^;CTX-M-15;OXA-48*	13	8
tet(D)	1	8
strA.v1^;strB.v1;qnrB1.v2^;sul2;tet(A).v1;dfrA14.v2;TEM-1D.v1^;CTX-M-15*	8	5
aac(3)-IIa.v1^;aac(6')-Ib-cr.v2;aadA^;strA.v1^;strB.v1;CatB4.v1?;sul1^;sul2;tet(A).v1;dfrA15.v2;OXA-1;CTX-M-15;OXA-48	13	4
qnrS1;sul1;tet(A).v1;dfrA1.v1;LAP-2	5	4
TEM-1D.v1^;CTX-M-15	2	3
aac(3)-IIa.v1^;aac(6')-Ib-cr.v2;strA.v1^;strB.v1;qnrB1.v2^;CatB4.v1?;sul2;dfrA14.v2;OXA-1;TEM-1D.v1^;CTX-M-15*	11	3
aadA2;qnrS1;lnuF.v1?;sul2;tet(A).v1;LAP-2	6	3
aadA^;sul1;tet(D)	3	3
mcr-1.1	1	3
aac(3)-IIa.v1^;aac(6')-Ib'.v1;aadA;aadA2^;qnrB4;catA1^;sul1;dfrA12;dfrA14.v2*;DHA-1;OXA-9.v1;TEM-1D.v1^;CTX-M-15;OXA-48*	14	2
aac(3)-IIa.v1^;aac(6')-Ib-cr.v2;CatB4.v1?;dfrA14.v2;OXA-1;CTX-M-15*	6	2
aac(3)-IIa.v1^;aadA16;aph3-Ia.v1^;strA.v1^;strB.v1;mphA;CatB4.v1?;arr-3;sul1;sul2;tet(D);dfrA27;OXA-1;TEM-1D.v1^;CTX-M-15*	15	2
aac(3)-IIa.v1^;strA.v1^;strB.v1;qnrB1.v2^;sul2;dfrA14.v2;TEM-1D.v1^;CTX-M-15*	8	2
aac(6')-Ib-cr.v2;strA.v1^;strB.v1;CatB4.v1?;sul2;dfrA14.v2;OXA-1;TEM-1D.v1^;CTX-M-15*	9	2
aac(6')-Ib-cr.v2;strA.v1^;strB.v1;qnrB1.v2^;CatB4.v1?;sul2;dfrA14.v2;OXA-1;TEM-1D.v1^;CTX-M-15*	10	2
aadA17;qnrS1;lnuF.v1;sul1;tet(A).v1;dfrA1.v1;LAP-2*	7	2
*aadA22;qnrS1;lnuF.v1;tet(D);dfrA14.v2**	5	2
aadA22^;ermB.v1;mphA;tet(D);tetM.v1*;CTX-M-1*	6	2
aadA2^;aph3-Ia.v1^;strA.v1;strB.v1*;qnrS1;mphA;sul1;sul2;dfrA12;CTX-M-15*	10	2

acquiredgenes	N acquired	n
aadA2^;aph3-Ia.v1^;strA.v1;strB.v1*;qnrS1;mphA;sul1;sul2;tet(A).v1;dfrA12;CTX-M-15*	11	2
aadA2^;mphA;sul1;dfrA12	4	2
aadA^;strA.v1^;strB.v1;sul1^;sul2;tet(A).v1;dfrA15.v2;OXA-48	8	2
aph(3')-VI;qnrS1;TEM-1;CTX-M-15;NDM-1	5	2
*fosA7**	1	2
qnrS1;lnuG;TEM-1D.v1^*	3	2
strA.v1;strB.v1;qnrS1;floR.v1;sul2;tet(A).v2;TEM-1D.v1^*	7	2
strA.v1;strB.v1;tet(D)	3	2
strA.v1^;strB.v1;sul2;dfrA14.v2;TEM-1D.v1^;CTX-M-15*	6	2
CTX-M-15	1	1
CTX-M-3	1	1
OXA-232	1	1
aac(3)-IIa.v1;SCO-1;TEM-1D.v1^;CTX-M-15	4	1
aac(3)-IIa.v1;aac(6')-Ib-cr.v2;aadA16;aadA2;strB.v1;qnrB6^;catII.2*;arr-3;sul1;sul1;tet(D);dfrA16.v2;dfrA27;TEM-1D.v1^;CTX-M-15*	15	1
aac(3)-IIa.v1;aac(6')-Ib-cr.v2;aadA16;strA.v1;strB.v1;qnrB6^;arr-3;sul1;sul1;tet(A).v1;dfrA27;TEM-1D.v1^;CTX-M-15*	13	1
aac(3)-IIa.v1;aac(6')-Ib-cr.v2^;aadA;aadA2;qnrE1;mphA;catB3.v2;arr-3;sul1;tet(D);dfrA8;OXA-1;SCO-1;TEM-1D.v1^;SHV-2	15	1
*aac(3)-IIa.v1;aac(6')-Ib-cr.v2^;aadA^;aph3-Ia.v1^;qnrB4;qnrE1;mphA;catB3.v2;arr-3;sul1;sul1;tet(D);DHA-1;OXA-1;SCO-1;TEM-1D.v1^*	16	1
aac(3)-IIa.v1;aadA;aph(3')-Ia*;strB.v1;mcr-1.1;sul1;sul2*;sul3;tet(A).v1;OXA-1;TEM-1D.v1^*	11	1
aac(3)-IIa.v1;aadA1.v1^;aph(3')-Ia;strA.v1^;strB.v1;mcr-1.1;sul1;sul2*;sul3;tet(A).v1;dfrA1.v2;TEM-1D.v1^*	12	1
aac(3)-IIa.v1;aadA22;strA.v1^;strB.v1;qnrS1;lnuF.v1;floR.v1;sul2;tet(B).v2*;tet(D);tetR;dfrA14.v2*;TEM-1D.v1^*	13	1
*aac(3)-IIa.v1;aadA22^;qnrS1;lnuF.v1;TEM-1D.v1^*	5	1
aac(3)-IIa.v1;aadA2^;aph3-Ia.v1^;qnrB4;mphA;sul1^;tet(A).v1;dfrA12;DHA-1;TEM-1D.v1^;CTX-M-15	11	1
aac(3)-IIa.v1;aadA2^;aph3-Ia.v1^;strA.v1;qnrS1;mphA;catII.2*;floR.v1*;sul1;sul2;tet(D);dfrA12;TEM-1D.v1^*	13	1
aac(3)-IIa.v1;aadA2^;aph3-Ia.v1^;strA.v1^;erm(42);mphA;floR.v2*;sul1;tet(D);dfrA12;dfrA14.v2*;TEM-1D.v1^*	12	1
aac(3)-IIa.v1;aadA2^;strA.v1;qnrS1;floR.v1*;sul1;sul2;tet(A).v1;dfrA12;TEM-1D.v1^*	10	1

acquiredgenes	N acquired	n
aac(3)-IIa.v1;aadA^;qnrB4;qnrS1;sul1^;tet(A).v1;dfrA15.v2;DHA-1;TEM-1D.v1^;CTX-M-15;OXA-181	11	1
aac(3)-IIa.v1;aadA^;qnrB4;sul1^;tet(A).v1;dfrA15.v2;DHA-1;TEM-1D.v1^;CTX-M-15	9	1
aac(3)-IIa.v1;aph(3')-Ia;sul1;tet(A).v1;dfrA15.v2;TEM-1D.v1^;CTX-M-15	7	1
aac(3)-IIa.v1;aph(3')-Ia;tet(A).v1;TEM-1D.v1^;CTX-M-15	5	1
aac(3)-IIa.v1;strA.v1;strB.v1*;mphA;catII.2*;sul2;tet(D);dfrA14.v2*;TEM-1D.v1^;CTX-M-15*	10	1
aac(3)-IIa.v1^;CatB4.v1;sul1;dfrA12;dfrA14.v2;OXA-1;OXA-10;CTX-M-15;NDM-1;OXA-181*	10	1
aac(3)-IIa.v1^;CatB4.v1?;sul1;dfrA12;dfrA14.v2;OXA-1;OXA-10;CTX-M-15;NDM-1;OXA-181*	10	1
aac(3)-IIa.v1^;aac(3)-IIa.v1^;aac(6')-Ib-cr.v2;strA.v1^;strB.v1;CatB4.v1?;sul2;dfrA14.v2;OXA-1;TEM-1D.v1^;CTX-M-15*	11	1
aac(3)-IIa.v1^;aac(6')-Ib'.v1;aadA;tet(D);OXA-9.v1;TEM-1D.v1^;CTX-M-15;OXA-48*	8	1
aac(3)-IIa.v1^;aac(6')-Ib-cr.v1^;qnrB6^;CatB4.v1?;sul1;sul1;dfrA12;dfrA14.v2;OXA-1;CTX-M-15;NDM-1;OXA-181*	12	1
aac(3)-IIa.v1^;aac(6')-Ib-cr.v2;aadA16;aph3-Ia.v1^;strA.v1^;strB.v1;mphA;CatB4.v1?;arr-3;sul1;sul2;tet(D);dfrA27;OXA-1;TEM-1D.v1^;CTX-M-15*	16	1
aac(3)-IIa.v1^;aac(6')-Ib-cr.v2;aadA^;qnrS1;CatB4.v1?;dfrA15.v2;LAP-2;OXA-1;CTX-M-15	9	1
aac(3)-IIa.v1^;aac(6')-Ib-cr.v2;strA.v1;strB.v1^;qnrS1;CatB4.v1?;catA1^;sul1;tet(A).v1;dfrA1.v1;OXA-1;TEM-1D.v1^;CTX-M-15	13	1
aac(3)-IIa.v1^;aac(6')-Ib-cr.v2;strA.v1^;strB.v1;CatB4.v1?;sul2;dfrA14.v2;OXA-1;TEM-1D.v1^;CTX-M-15*	10	1
aac(3)-IIa.v1^;aac(6')-Ib-cr.v2;strA.v1^;strB.v1;CatB4.v1?;sul2;tet(A).v1;dfrA14.v2;OXA-1;TEM-1D.v1^;CTX-M-15;OXA-48*	12	1
aac(3)-IIa.v1^;aac(6')-Ib-cr.v2;strA.v1^;strB.v1;mcr-1.1;qnrB1.v2^;CatB4.v1?;sul2;tet(A).v1;dfrA14.v2;OXA-1;TEM-1D.v1^;CTX-M-15*	13	1
aac(3)-IIa.v1^;aac(6')-Ib-cr.v2;strA.v1^;strB.v1;qnrB1.v1;CatB4.v1?;sul2;tet(A).v1;dfrA14.v2;OXA-1;TEM-1D.v1^;CTX-M-15*	12	1
aac(3)-IIa.v1^;aac(6')-Ib-cr.v2;strA.v1^;strB.v1;qnrB1.v2^;CatB4.v1?;sul2;dfrA14.v2;OXA-1;TEM-1D.v1^;CTX-M-15;OXA-48*	12	1
aac(3)-IIa.v1^;aac(6')-Ib-cr.v2;strA.v1^;strB.v1;qnrB1.v2^;CatB4.v1?;sul2;dfrA14.v2;OXA-1;TEM-1D.v1^;CTX-M-15;SHV-12*;OXA-48*	13	1
aac(3)-IIa.v1^;aac(6')-Ib-cr.v2;strA.v1^;strB.v1;qnrB1.v2^;CatB4.v1?;sul2;tet(A).v1;dfrA14.v2;OXA-1;TEM-1D.v1^;CTX-M-15;SHV-2A^;OXA-48*	14	1
aac(3)-IIa.v1^;aac(6')-Ib-cr.v2;strA.v1^;strB.v1;qnrB1.v2^;CatB4.v1?;sul2;tet(A).v1;dfrA14.v2;OXA-1;TEM-1D.v1^;SHV-187* +69L;CTX-M-15*	13	1
aac(3)-IIa.v1^;aac(6')-Ib3^;rmtC;qnrB1.v2^;CatB4.v1?;sul1;dfrA14.v2;CMY-6;OXA-1;CTX-M-15;NDM-1*	11	1

acquiredgenes	N acquired	n
aac(3)-IIa.v1^;aadA1.v1^;aph3-Ia.v1^;strA.v1^;strB.v1;mphA;CatB4.v1?;catA1^;arr-3;sul1;sul2;tet(D);dfrA1.v2;dfrA27;OXA-1;TEM-1D.v1^;CTX-M-15	17	1
aac(3)-IIa.v1^;aph3-Ia.v1^;strA.v1^;strB.v1;mphA;CatB4.v1?;arr-3;sul1;sul2;tet(D);dfrA27;OXA-1;TEM-1D.v1^;CTX-M-15	14	1
aac(3)-IIa.v1^;aph3-Ia.v1^;strA.v1^;strB.v1;qnrB1.v2^;mphA;CatB4.v1?;catA1^;sul1;tet(A).v1;dfrA14.v2;OXA-1;TEM-1D.v1^;CTX-M-15;VIM-1*	15	1
aac(3)-IIa.v1^;strA.v1^;strB.v1;CatB4.v1?;sul2;dfrA14.v2;OXA-1;TEM-1D.v1^;CTX-M-15*	9	1
aac(3)-IIa.v1^;strA.v1^;strB.v1;qnrB1.v2^;mphA;arr-3;sul1;sul2;dfrA14.v2;TEM-1D.v1^;CTX-M-15;OXA-48*	12	1
aac(3)-IId^;aac(3)-IId^;CTX-M-15;CTX-M-15	4	1
aac(3)-IId^;aac(6')-Ib-cr.v2;arr-3;sul2;TEM-1D.v1^;CTX-M-3*	6	1
aac(3)-IId^;aac(6')-Ib-cr.v2;CatB4.v1?;dfrA30;OXA-1;TEM-122*;CTX-M-15;KPC-3*	8	1
aac(3)-IId^;aac(6')-Ib-cr.v2;aadA16;aadA2^;aadA^;aph3-Ia.v1^;strA.v1*;strB.v1*;mcr-8.1*;qnrB6^;qnrS1;mphA;floR.v1*;arr-3;sul1;sul1;sul2;tet(A).v1*;dfrA12;dfrA14.v2*;dfrA27;LAP-2;TEM-1D.v1^;CTX-M-65*	24	1
aac(3)-IId^;aadA;aadA2^;rmtB;ereA2*;mphA;catA1^;cmlA5;arr-2;sul1?;dfrA12;TEM-1D.v1^;CTX-M-15;NDM-5;OXA-181*	15	1
aac(3)-IId^;aadA16;strA.v1^;strB.v1;qnrB6^;CatB4.v1?;catII.2*;arr-3;sul1;sul1;sul2;tet(D);dfrA27;OXA-1;TEM-1D.v1^;CTX-M-15;NDM-7*	17	1
*aac(3)-IV;aadA;aadA2^;aph(4)-Ia;strA.v1;strB.v1;mphB.v1;sul1;sul2;tet(A).v1;tet(D);dfrA12;TEM-1D.v1^*	13	1
aac(3)-VIa;aadA2*;aadA^;lnuF.v1;sul1;tet(B).v2*;tetR*	7	1
aac(6')-Ib'.v1;KPC-3	2	1
aac(6')-Ib'.v1;aadA;aadA2^;catA1^;sul1;sul1;dfrA12;dfrA14.v2*;OXA-9.v1;TEM-1D.v1^;CTX-M-15;OXA-48*	12	1
aac(6')-Ib'.v1;aadA;aph(3')-VI;aph3-Ia.v1^;armA;qnrS1;ermB.v1*;mphA;mphE.v2;msrE;sul1;sul2;dfrA5;TEM-1;CTX-M-15;NDM-5*	16	1
aac(6')-Ib'.v1;aadA;strA.v1*;strA.v1*;strB.v1;strB.v1;sul2;sul2;tet(D);dfrA14.v2*;OXA-9.v1;TEM-1D.v1^;OXA-48*	13	1
aac(6')-Ib'.v1;aadA2^;mphA;catA1^;sul1;dfrA12;KPC-3	7	1
aac(6')-Ib-cr.v2;aac(6')-Ib-cr.v2;strA.v1^;strB.v1;qnrB1.v2^;CatB4.v1?;sul2;dfrA14.v2;OXA-1;OXA-1;TEM-1D.v1^;CTX-M-15*	12	1
aac(6')-Ib-cr.v2;aadA16;qnrB1.v2^;arr-3;sul1;dfrA27;CTX-M-15*	7	1
aac(6')-Ib-cr.v2;aadA2^;aph(3')-VI;armA;sat-2;mphE.v2^;msrE;CatB4.v1?;catA1^;sul1;dfrA1.v1;dfrA12;OXA-1;NDM-1;OXA-48	15	1
aac(6')-Ib-cr.v2;aadA2^;aph3-Ia.v1^;mphA;CatB4.v1?;sul1;tet(A).v1;dfrA12;OXA-1;TEM-1D.v1^;CTX-M-15	11	1
aac(6')-Ib-cr.v2;aadA2^;armA;sat-2;mphE.v2^;msrE;CatB4.v1?;sul1;tet(D);dfrA1.v1;dfrA12;OXA-1;TEM-1D.v1^;CTX-M-15;OXA-232*	15	1

acquiredgenes	N acquired	n
aac(6')-Ib-cr.v2;aadA2^;qnrB2.v1^;mphA;CatB4.v1;catII.2;sul1;sul1;dfrA12;dfrA14.v2*;OXA-1;CTX-M-15*	12	1
aac(6')-Ib-cr.v2;aadA2^;sat-2;strA.v1;strB.v1*;CatB4.v1?;sul1;sul2;dfrA1.v1;dfrA12;OXA-1;CTX-M-15;OXA-48*	13	1
aac(6')-Ib-cr.v2;aph3-Ia.v1^;CatB4.v1?;tet(A).v1;OXA-1;CTX-M-15;OXA-48	7	1
aac(6')-Ib-cr.v2;aph3-Ia.v1^;mphA;CatB4.v1?;OXA-1;CTX-M-15	6	1
aac(6')-Ib-cr.v2;aph3-Ia.v1^;strA.v1^;strB.v1;qnrB1.v2^;CatB4.v1?;sul1;sul2;tet(A).v1;dfrA14.v2*;dfrA7;OXA-1;CTX-M-15*	13	1
aac(6')-Ib-cr.v2;aph3-Ia.v1^;strA.v1^;strB.v1;qnrB1.v2^;CatB4.v1?;sul2;tet(A).v1;dfrA14.v2;OXA-1;TEM-1D.v1^;CTX-M-15*	12	1
aac(6')-Ib-cr.v2;qnrB1.v2^;CatB4.v1?;tet(A).v1;dfrA14.v2;OXA-1;TEM-104?;CTX-M-15*	8	1
aac(6')-Ib-cr.v2;strA.v1;strB.v1;qnrS1;mphA;CatB4.v1?;sul1;sul2;dfrA15.v2;OXA-1;TEM-1D.v1^;CTX-M-15;OXA-48	13	1
aac(6')-Ib-cr.v2;strA.v1^;strB.v1;CatB4.v1?;sul2;OXA-1;TEM-1D.v1^;CTX-M-15;OXA-48*	9	1
aac(6')-Ib-cr.v2;strA.v1^;strB.v1;CatB4.v1?;sul2;OXA-1;CTX-M-15;OXA-48	8	1
aac(6')-Ib-cr.v2;strA.v1^;strB.v1;qnrB1.v2^;CatB4.v1?;sul2;dfrA14.v2;OXA-1;TEM-1D.v1^;CTX-M-15;OXA-48*	11	1
aac(6')-Ib-cr.v2;strA.v1^;strB.v1;qnrB1.v2^;CatB4.v1?;sul2;tet(A).v1;dfrA14.v2;OXA-1;TEM-1D.v1^;CTX-M-15*	11	1
aac(6')-Ib-cr.v2;strA.v1^;strB.v1;qnrB1.v2^;sul2;dfrA14.v2;TEM-1D.v1^;CTX-M-15;OXA-48*	9	1
aac(6')-Ib-cr.v2;strA.v1^;strB.v1;sul2;dfrA14.v2;TEM-1D.v1^;CTX-M-15;OXA-48*	8	1
aac(6')-Ib-cr.v2^;aadA2;aph3-Ia.v1^;mphA;catB3.v2;arr-3;sul1;OXA-1	8	1
aac(6')-Ib-cr.v2^;aadA2^;qnrB4;mphA;catB3.v2;sul1;dfrA12;DHA-1;OXA-1	9	1
aac(6')-Ib-cr.v2^;mphA;catB3.v2;arr-3;sul1;OXA-1;KPC-2	7	1
aac(6')-Ib-cr.v2^;mphA;catB3.v2;arr-3;sul1;OXA-1;OXA-48	7	1
aac(6')-Ib-cr.v2^;mphA;catB3.v2;arr-3;sul1;tet(A).v1;OXA-1;OXA-48*	8	1
aac(6')-Ib-cr.v2^;qnrB4;mphA;catB3.v2;arr-3;sul1;sul1;DHA-1;OXA-1;OXA-48	10	1
aac(6')-Ib-cr.v2^;qnrB4;mphA;catB3.v2;arr-3;sul1;sul1;tet(A).v1;DHA-1;OXA-1;OXA-48*	11	1
aac(6')-Ib-cr.v2^;qnrB4;qnrS2;mphA;catB3.v2;arr-3;sul1;sul1;DHA-1;OXA-1	10	1
aac(6')-Ib-cr.v2^;strA.v1;strB.v1;qnrB4;catB3.v2;arr-3;sul1;sul1;tet(A).v1;DHA-1;OXA-1	11	1
aac(6')-Ib4^;aadA1.v1^;sul1;CTX-M-15;VIM-1	5	1
aadA;ant(2'')-Ia;armA;mphE.v2;msrE;catA1^;sul1;KPC-3*	8	1

acquiredgenes	N acquired	n
aadA1.v1^;aadA2;mphG;cmlA1;sul1?;sul3^;dfrA12*	7	1
aadA13;qnrS1;sul2;tet(A).v1;dfrA14.v2*;LAP-2*	6	1
aadA13;strB.v1;sul2;tet(A).v1;dfrA14.v2;TEM-1D.v1^*	6	1
aadA16;qnrB6^;CatB4.v1?;catII.2*;arr-3;sul1;sul1;tet(D);dfrA27;OXA-1*	10	1
aadA17;qnrS1;lnuF.v1;sul1;tet(A).v1;dfrA1.v1;LAP-2;TEM-1D.v1*	8	1
aadA22;lnuG;sul3;tet(B).v2*;tetR;dfrA5;TEM-1D.v1^*	7	1
aadA22^;ermB.v1;mphA;tetM.v1*;CTX-M-1*	5	1
*aadA22^;qnrS1;lnuF.v1;tet(D);dfrA14.v2**	5	1
aadA2;ant(2'')-Ia;mcr-9.1?;qnrA1^;catA1;sul1;TEM-30^;CTX-M-9*	8	1
aadA2;ant(2'')-Ia;mcr-9.1?;qnrA1^;sul1;CTX-M-9	6	1
aadA2;ant(2'')-Ia;mcr-9.1?;qnrA1^;sul1;sul1;TEM-135.v2;CTX-M-9*	8	1
aadA2;ant(2'')-Ia;mcr-9.1?;sul1;CTX-M-9	5	1
aadA2;ant(2'')-Ia;mcr-9.1?;sul1^;CTX-M-9	5	1
aadA2;ant(2'')-Ia;qnrA1^;sul1;sul1;CTX-M-9	6	1
*aadA2^;aph3-Ia.v1^;lnuG;mphA;catA1^;sul1;tet(A).v1;tet(D);dfrA12;TEM-1D.v1^*	10	1
aadA2^;aph3-Ia.v1^;mphA;CatB4.v1?;sul1;dfrA12;OXA-1;TEM-1D.v1^;CTX-M-15;OXA-48	10	1
aadA2^;aph3-Ia.v1^;mphA;CatB4.v1?;sul1;tet(A).v1;dfrA12;OXA-1;TEM-1D.v1^;CTX-M-15;OXA-48	11	1
aadA2^;aph3-Ia.v1^;qnrS1;lnuF.v1;mphA;sul1;sul2;tet(D);dfrA12	9	1
aadA2^;aph3-Ia.v1^;strA.v1;strB.v1*;qnrS1;mphA;catII.2*;sul1;sul2;tet(A).v1;dfrA12;CTX-M-15*	12	1
aadA2^;mphA;sul1;dfrA12;TEM-1D.v1^;CTX-M-15	6	1
aadA2^;strA.v1;strB.v1*;qnrS1;lnuG;mphA;sul1;sul2;tet(A).v1;dfrA12;TEM-1D.v1^;CTX-M-15*	12	1
aadA;sul1;tet(A).v1;dfrA1.v2	4	1
aadA^;catA1^;sul1;tet(D)	4	1
*aadA^;qnrS1;lnuF.v1;sul1;sul3;tet(A).v1;dfrA1.v1;LAP-2;TEM-1D.v1^*	9	1
aadA^;qnrS1^;cmlA1;sul3*	4	1

acquiredgenes	N acquired	n
aadA^;qnrS1^;cmlA1;sul3;tet(A).v1*;TEM-135.v2*	6	1
aadA^;strA.v1^;strB.v1;catA1;sul1^;sul2;tet(A).v1;dfrA15.v2;TEM-1D.v1^;OXA-48*	10	1
aadA^;sul1^;tet(A).v1;dfrA15.v2;CTX-M-15	5	1
aph(3')-VI;qnrS1;CTX-M-15;NDM-1	4	1
aph3-Ia.v1^;strA.v1;strB.v1*;qnrS1;sul2;CTX-M-15*	6	1
aph3-Ia.v1^;strA.v1;strB.v1;qnrS1;sul1;tet(A).v1;dfrA1.v1;LAP-2	8	1
catA1;tet(D);TEM-1D.v1^;OXA-48*	4	1
*catA1^;dfrA14.v2**	2	1
dfrA14.v2;CTX-M-15;OXA-48*	3	1
erm(42);mphG;sul1;sul2;tetM.v1*;dfrA12*	6	1
*lnuG;catA1^;sul1;tet(A).v1;tet(D);TEM-1D.v1^*	6	1
*lnuG;tet(D);TEM-1D.v1^*	3	1
mphA;CTX-M-1	2	1
qnrB1.v2^;TEM-122;KPC-3*	3	1
qnrS1	1	1
*qnrS1;TEM-1D.v1^*	2	1
qnrS1;lnuF.v1;sul2;tet(A).v1;LAP-2	5	1
*qnrS1;lnuG**	2	1
qnrS1;sul1;sul1;tet(A).v1;dfrA1.v1;DHA-1;OXA-10;SHV-187 +69L*	8	1
qnrS1;sul2;tet(A).v1;dfrA14.v2;LAP-2*	5	1
*sat-2;strA.v1;strB.v1;lnuG;catA1^;sul2;dfrA1.v1;TEM-1D.v1^*	8	1
sat-2;strA.v1;strB.v1;lnuG;mphE.v2;msrE;floR.v1;sul1;tet(B).v2*;tetR;dfrA1.v1;CTX-M-32*	12	1
strA.v1;strB.v1*;qnrS1;sul2;CTX-M-15*	5	1
strA.v1;strB.v1	2	1
strA.v1;strB.v1;OXA-48	3	1

acquiredgenes	N acquired	n
strA.v1;strB.v1;qnrB4;sul1;DHA-1	5	1
strA.v1^;strB.v1;qnrB1.v2^;sul2;dfrA14.v2;TEM-1D.v1^;CTX-M-15*	7	1
strA.v1^;strB.v1;qnrB1.v2^;sul2;dfrA14.v2;TEM-1D.v1^;OXA-48*	7	1
strA.v1^;strB.v1;qnrB1.v2^;sul2;tet(A).v1;dfrA14.v2;CTX-M-15;OXA-48*	8	1
strA.v1^;strB.v1;qnrS1;sul2;dfrA14.v2;TEM-1D.v1^;CTX-M-15*	7	1
strA.v1^;strB.v1;qnrS1;sul2;tet(A).v1;dfrA14.v2*;TEM-1D.v1^;CTX-M-15*	8	1
strA.v1^;strB.v1;qnrS1;sul2;tet(A).v1;TEM-1D.v1^;CTX-M-15	7	1
strA.v1^;strB.v1;sul2;TEM-1D.v1^;CTX-M-15;OXA-48	6	1
strB.v1;qnrB1.v2^;sul2;dfrA14.v2;TEM-1D.v1^;CTX-M-15*	6	1
sul1;tet(D);dfrA1.v2	3	1
tet(A).v1	1	1
*tet(D)^*	1	1

Annotations indicate aspects of the hit: ^ (inexact nucleotide but exact amino acid match), * (inexact nucleotide and inexact amino acid match), ? (incomplete match), -X% (truncated amino acid sequence), $ (mutated start codon, translation may be disrupted).

Experimental section

Study 2

First Belgian Report of Ertapenem Resistance in an ST11 *Klebsiella pneumoniae* Strain Isolated from a Dog Carrying $bla_{\text{SCO-1}}$ and $bla_{\text{DHA-1}}$ Combined with Permeability Defects

Preamble

In Belgium, the use of carbapenem antibiotics is prohibited in veterinary medicine. However, studies have reported the presence of carbapenem-resistant isolates in companion animals. In this study, we identified a *Klebsiella pneumoniae* isolate exhibiting an AmpC phenotype and resistance to ertapenem, but not to imipenem or meropenem. Hybrid sequencing was conducted to examine the antibiotic resistance markers, associated mobile genetic elements, and their transmissibility. Three plasmids were reconstructed: the conjugative IncFIB(K), the non-mobilizable IncR, and the mobilizable but non-conjugative ColRNAI. The IncFIB(K) plasmid carried the bla_{SCO-1} gene, while the IncR plasmid harbored the bla_{DHA-1} gene, both along with several other antimicrobial resistance genes.

Our findings suggest that ertapenem resistance coupled with imipenem and meropenem susceptibility in *K. pneumoniae* may be attributed to the presence of bla_{SCO-1} and bla_{DHA-1} genes, combined with permeability defects caused by point mutations in an outer membrane porin (*OmpK37*). The detection of the bla_{SCO-1} gene on a conjugative IncFIB(K) plasmid is particularly concerning, as it heightens the risk of transmission to humans, animals, and the environment.

In this work, data analyisis of hybrid sequencing data was performed by Dr. Margaux Maex.

Experimental section

Study 2

First Belgian Report of Ertapenem Resistance in an ST11 *Klebsiella pneumoniae* Strain Isolated from a Dog Carrying bla_{SCO-1} and bla_{DHA-1} Combined with Permeability Defects

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Abstract

Klebsiella pneumoniae of sequence type (ST) 11 is a hyper-epidemic nosocomial clone, which is spreading worldwide among humans and emerging in pets. This is the first report, to the best of our knowledge, of multidrug-resistant (MDR) *K. pneumoniae* ST11 carrying *bla*$_{SCO-1}$ and *bla*$_{DHA-1}$, isolated from a four-month-old dog in Belgium. Antimicrobial susceptibility testing (AST) of the isolate, performed via broth microdilution following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines, revealed resistance to eight different classes of antimicrobials, including carbapenems, in particular ertapenem, third-generation cephalosporins and fluoroquinolones. A hybrid approach, combining long- and short-read sequencing, was employed for in silico plasmid characterization, multi-locus sequence typing (MLST) and the identification and localization of antimicrobial resistance (AMR) and virulence-associated genes. Three plasmids were reconstructed from the whole-genome sequence (WGS) data: the conjugative IncFIB(K), the non-mobilizable IncR and the mobilizable but unconjugative ColRNAI. The IncFIB(K) plasmid carried the *bla*$_{SCO-1}$ gene, whereas IncR carried *bla*$_{DHA-1}$, both alongside several other antimicrobial resistance genes (ARGs). No virulence genes could be detected. Here, we suggest that the resistance to ertapenem associated with susceptibility to imipenem and meropenem in *K. pneumoniae* could be related to the presence of *bla*$_{SCO-1}$ and *bla*$_{DHA-1}$, combined with permeability defects caused by point mutations in an outer membrane porin (*OmpK37*). The presence of the *bla*$_{SCO-1}$ gene on a conjugative IncFIB(K) plasmid is worrisome as it can increase the risk of transmission to humans, to animals and to the environment.

Introduction

The opportunistic bacterium *K. pneumoniae* is a common source of antimicrobial-resistant hospital-acquired infection and the increase in antimicrobial resistance (AMR) in general is one of the greatest global public health challenges of this time (Wyres, Lam, et al., 2020). The six leading pathogens for deaths attributed to AMR, i.e. *Escherichia coli*, *Staphylococcus aureus*, *K. pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, caused an estimated 929 000 (660 000–1 270 000) deaths in 2019 (Murray et al., 2022).

The World Health Organization (WHO) has taken up *Enterobacteriaceae* resistant to third-generation cephalosporins and/or carbapenems in the list of urgent threats, each causing 50.000-100.000 deaths in 2019 (Murray et al., 2022; Tacconelli et al., 2018). *K. pneumoniae* is naturally resistant to penicillins and is a known trafficker of acquired resistance genes to multiple antimicrobials, such as to β-lactams and carbapenems (Wyres, Lam, et al., 2020; Wyres & Holt, 2018). Multi-drug resistance (MDR), which is defined as resistance to more than 3 antimicrobial classes, in addition to resistance to ampicillin for *K. pneumoniae*, has evolved many times leading to hundreds of distinct lineages (Wyres, Lam, et al., 2020). Many of them will persist locally, but a subset of lineages have evolved into global problem clones with wide dissemination worldwide (Navon-Venezia et al., 2017; Wyres, Lam, et al., 2020). These include, amongst others, the well-studied clonal groups CG258, CG101 (CG43), CG15, and CG307 (Navon-Venezia et al., 2017; Wyres, Lam, et al., 2020). CG258 includes sequence types (ST) ST11, ST258, and ST512, which have often been reported as resistant to third-generation cephalosporins and carbapenems (Wyres, Lam, et al., 2020). Different mechanisms of resistance against carbapenems exist and carbapenem resistance may be caused by the production of carbapenemases, such as KPC, VIM, NDM and OXA-48 (Wyres, Lam, et al., 2020). On the other hand, the co-occurrence of alterations in the expression or lesions of outer membrane porins (OMPs) combined with the production of β-lactamases that possess sparse carbapenemase activity, may also lead to reduced carbapenem susceptibility, most often to ertapenem (Pitout et al., 2015).

There are limited data on clones found in non-human niches, however, some overlap is seen between clinical isolates and other sources. Global problem clones, such as ST15 and ST11, are increasingly being reported in companion animals (Hidalgo et al., 2013; Marques, Menezes, et al., 2019; Sellera et al., 2021). In humans, CRKp may leave serious infections such as urinary tract infections (UTIs), pneumonia and septicemia, incurable and fatal due to limited therapeutic options (R. M. Martin et al., 2016; R. M. Martin & Bachman, 2018). In companion animals, *K. pneumoniae* has been described to cause UTIs, upper respiratory tract infections, pyometra and bloodstream infections (septicemia) (K. Harada et al., 2016; Z. Zhang et al., 2022). Although carbapenems are not standard of care in companion animals, infections with carbapenem-resistant K. *pneumoniae* are increasingly being reported (Silva et al., 2022). Due to the close contact of companion animals with humans, they may constitute an important reservoir of carbapenem-resistant Enterobacterales (CRE), as such raising a public health concern

(Marques, Belas, et al., 2019; Røken et al., 2022; Silva et al., 2022). Therefore, it is crucial to understand the molecular mechanisms involved in the resistance. This study aimed to investigate the genotypic mechanisms related to the ertapenem resistance in a *K. pneumoniae* isolated from a companion animal with fatal sepsis.

Results and discussion

1 Phenotypic resistance testing

K. pneumoniae strain Kpn1695 was screened for ESBL production and carbapenem resistance using McConkey + CTX (1 mg/L) and CHROMID® CARBA SMART Agar. Growth was observed on McConkey + CTX and on the CARB side of CHROMID® CARBA SMART Agar. Further phenotypic resistance testing was performed by broth microdilution.

K. pneumoniae strain Kpn1695 was resistant to ampicillin (minimal inhibitory concentration [MIC] > 32 mg/L), azithromycin (MIC > 64 mg/L), cefepime (MIC = 2 mg/L), cefotaxime (MIC = 8 mg/L), cefotaxime/clavulanic acid (MIC = 8 mg/L), cefoxitin (MIC > 64 mg/L), ceftazidime (MIC >8 mg/L), ceftazidime/clavulanic acid (MIC = 32 mg/L), chloramphenicol (MIC = 64 mg/L), ciprofloxacin (MIC >8 mg/L), ertapenem (MIC = 0.25 mg/L), gentamicin (MIC >16 mg/L), nalidixic acid (MIC > 64 mg/L), sulfamethoxazole (MIC > 512 mg/L), temocillin (MIC = 16 mg/L), tetracycline (MIC > 32 mg/L) and trimethoprim (MIC = 8 mg/L). However, this strain was susceptible to amikacin (MIC ≤ 4 mg/L), colistin (MIC ≤ 1 mg/L), imipenem (MIC = 0.25mg/L), meropenem (MIC = 0.06 mg/L) and tigecycline (MIC = 1 mg/L).

Epidemiological cutoff (ECOFF) values are used to distuinguish microorganisms with (non-wild type) and without (wild-type) phenotypically detectable acquired resistance genes to a certain antibiotic agent. EUCAST does not provide ECOFFs for all antibiotics included in the sensititre™ plates EUVSEC 3 and EUVSEC 2 (Supplementary table 5,Supplementary table 6). In the absence of ECOFF for *K. pneumoniae*, those for *E. Coli*, the indicator bacterium for *Enterobacterales* were applied (Table 8)

Table 8 - Minimal inhibitory concentrations (MIC, mg/L) of Kpn1695 using sensititre™ plates EUVSEC 3 and EUVSEC 2.

EUVSEC 3		**EUVSEC 2**	
Antibiotic	**MIC (mg/L)**	**Antibiotic**	**MIC (mg/L)**
Amikacin	<= 4	Cefepime*	2**
Ampicillin*	> 32**	Cefotaxime	8**
Azithromycin*	> 64**	Cefotaxime/clavulanic acid*	8**
Cefotaxime	> 4**	Cefoxitin	> 64**
Ceftazidime	> 8**	Ceftazidime	8**
Chloramphenicol*	64**	Ceftazidime/clavulanic acid*	32**
Ciprofloxacin	> 8**	Ertapenem*	0.25**
Colistin	<= 1	Imipenem	0.25
Gentamicin	> 16**	Meropenem	0.03
Meropenem	0.06	Temocillin	16**
Nalidixic Acid*	> 64**		
Sulfamethoxazole*	> 512**		
Tetracycline	> 32**		
Tigecycline	1		
Trimethoprim*	8**		

ECOFF values of *K. pneumoniae* or *E. Coli* (*, in the absence of ECOFF for *K. pneumoniae*) were used to interpret the obtained MIC values. Resistance is shown by '**'.

2 Genotypic characterization

In silico multi-locus sequence typing (MLST) determination classified K. *pneumoniae* Kpn1695 as ST11 (allelic profile: gapA, 3; infB, 3; mdh, 1; pgi, 1; phoE, 1; rpoB, 1; and tonB, 4). This sequence type was one of the most detected sequence types related to third-generation cephalosporin resistant and/or carbapenem resistant *K. pneumoniae* (CRKp) in the Europe-wide EuSCAPE study (Wyres, Lam, et al., 2020). The clinical high-risk ST11 clones, which are part of the clonal group (CG) 258, have become global problem strains due to their wide dissemination and their multiple drug resistance (MDR) characteristics (Wyres, Lam, et al., 2020). ST11, which derived from ST258 by recombination, represents about 12% of CRKp in Europe and is much more widely distributed than other STs from clonal group 258 (CG258), consisting of ST258 and ST512 (Wyres et al., 2015; Wyres, Lam, et al., 2020). In China, ST11 represents the single dominant cause of CRKp human infections (Wyres, Lam, et al., 2020). It has also been isolated from non-human niches such as from poultry in China (R. Zhang

et al., 2019a) and from dogs and cats in Spain and Switzerland (Brilhante et al., 2021; Hidalgo et al., 2013).

Besides, the polysaccharide capsule of *K. pneumoniae* plays a major role in its virulence and can, with over 138 distinct combinations identified to date (Follador et al., 2016; Wyres et al., 2016; Wyres, Lam, et al., 2020), be used as an epidemiological marker (Wyres et al., 2016). This strain was typed as a KL105, a clade which has been described before in clinical ST11 *K. pneumoniae* strains isolated in Europe and Latin America (Garcia-Fulgueiras et al., 2020; Novais et al., 2022). Furthermore, this ST11 KL105 clade has succesfully disseminated in Europe, bearing variable β-lactamases. It is however most often related to ESBL strains or $bla_{\text{DHA-1}}$ producers with presence of different plasmid types (Novais et al., 2022). Genotypic characterization identified Kpn1695 as O2v2 (Table 9). Currently 12 distinct O-loci have been described (Follador et al., 2016; Wick et al., 2018). Clinical strains are most often typed as serotypes O1 and O2 (Follador et al., 2016). No clear association has been established between K-locus or O-locus and niche or host specialization (Mostowy & Holt, 2018).

3.7 Plasmid analysis

A total of 27 ARGs were found in Kpn1695 (Table 9, Figure 38, Figure 39, Supplementary figure 1). Following hybrid assembly from long and short reads, analysis of the assembly with ABRicate and PlasmidFinder, revealed the presence of 3 plasmids: IncFIB(K), IncR and ColRNAI (Carattoli et al., 2014; Seemann, 2014). A nucleotide BLAST search of the plasmid sequences against the GenBank database showed highest similarity for the IncFIB(K) plasmid with a *K. pneumoniae* isolate bearing plasmid tig00000000 (GenBank accession no. CP021713.1) with 81% query coverage and 99.78% identity. A query coverage of 100% and 99.97% identity was seen for the IncR type plasmid with pMBR_DHA-1_1CO (GenBank accession no. CP049718.1). This *K. pneumoniae* strain was isolated from the veterinary setting in Switzerland. The ColRNAI type plasmid displayed 100% query coverage and 99.387% identity with pC17KP0039-3 (GenBank accession no. CP052402.1), which was retrieved from a human *K. pneumoniae* sample.

The IncFIB(K) plasmid, named pKPn1695_SCO-1, is a multidrug resistence conjugative plasmid (length = 192683bp) carrying the following resistance genes: the CARB-type β-lactamase $bla_{\text{SCO-1}}$ gene, *tet(D)*, $bla_{\text{SHV-2}}$, *aac(3)IIa*, $bla_{\text{TEM-1A}}$, *sul1*, *aadA1*, *qnrE1* and *emrE* (Figure 38). The IncFIB(K) type plasmid has previously been described in clinical *K. pneumoniae* ST11 from humans and is amongst the most detected plasmid replicon types in *K. pneumoniae* (de Oliveira et al., 2020; Paskova et al., 2018). The *emrE* gene, encoding a multidrug exporter, is associated with resistance to a wide range of toxic cationic hydrophobis compounds, such as the disinfectant quaternary ammonium compounds (QACs). It is a known disinfectant resistance gene and belongs to the small multidrug resistance (SMR) family. This β-lactamase has been detected in *Pseudomonas aeruginosa, Acinetobacter baumannii* and *Vibrio cholera* but occur in low frequencies in Enterobacteriaceae (Papagiannitsis et al., 2007). It has been described before in *E. coli* and *K. pneumoniae* (Papagiannitsis et al., 2007; Venditti et al., 2020). The conjugative plasmid pKPn1695_SCO-1 alose carries the β-

lactamase *bla*$_{SCO-1}$ gene (Figure 38) which has previously been described as having carbenicillinase activity (Papagiannitsis et al., 2007).

A circular plasmid diagram was generated using BRIG and the *bla*$_{SCO-1}$ carrying plasmid was compared to a selection of its top BLAST hits: plasmid tig00000000 (strain AR_0129, accession nr. CP021713), pBK13043_1 (accession nr. CP020838) and plasmid tig00000002 (strain AR_0115, accession nr. CP020072) (Figure 38). Strikingly, high similarity is seen between these plasmids in the backbone, but little coverage is seen in the MDR region and *bla*$_{SCO-1}$ was not present in any of these plasmids. The lack of homology in the MDR region of pKPn1695_SCO-1 to its top BLAST hits suggests transmission of the MDR region from other plasmids. Likely this occurred in several recombination events through insertion sequences (IS), as several IS are flanking the MDR region without similarity to the other plasmids. The *bla*$_{SCO-1}$ gene on pKPn1695_SCO-1 displayed the highest identity percentage to a *bla*$_{SCO-1}$ gene originating from *Acinetobacter baumannii* (accession nr. EF063111), which might suggest a recombination event. The fact that this gene is lying on a conjugative plasmid highlights the risk of its spread to other *K. pneumoniae* strains or other potential hazardous species.

The non-mobilizable IncR plasmid (length = 56132), named pKpn1695_DHA-1, carried *bla*$_{DHA-1}$, bla$_{OXA-1}$, *aac(6')Ib-cr5, aph(3')-Ia*, *qnrB4*, *mph(A)*, *catB3*, *arr3*, *sul1* and *emrE* (Figure 39). IncR plasmids have been linked before to the spread of *bla*$_{DHA-1}$ in ST11 *K. pneumoniae* strains (Hennequin, Chlilek, et al., 2018). The *bla*$_{DHA-1}$ cephalosporinase is currently reported worldwide in *K. pneumoniae*. The *bla*$_{DHA-1}$ β-lactamase is usually co-expressed with plenty of other ARGs such as extended-spectrum β-lactamases (*bla*$_{CTX-M-}$, *bla*$_{SHV}$-types), oxacillinases (*bla*$_{OXA-1}$), penicillinases (*bla*$_{TEM}$-type), carbapenemases (*bla*$_{OXA48}$, *bla*$_{KPC-2}$), aminoglycosides (*aacA*, *aadA*, *armA*), fluoroquinolones (*qnrB4*, *aac6'-1b-cr*), and sulfonamide (*sul1*) resistance genes of which some are also present in the current isolate Kpn1695 (Table 9) (Hennequin, Ravet, et al., 2018). The presence of *bla*$_{DHA-1}$ β-lactamase in combination with *OmpK35/OmpK36* porin loss has been linked to carbapenem resistance in *K. pneumoniae* before (Shin et al., 2012).

The mobilizable but unconjugative plasmid ColRNAI did not carry antibiotic resistance genes (ARGs).

Upon comparing phenotypic properties with genotypic data, a complete match was observed for this isolate, except for rifampicin and fosfomycin. No phenotypic data was collected for these antibiotics as they are not included in the EUCAST panel for the AMR monitoring of zoonotic and indicator bacteria following the European Decision (EUVSEC 3 and EUVSEC 2) (Commission Implementing Decision (EU) 2020/1729 of 17 November 2020 on the Monitoring and Reporting of Antimicrobial Resistance in Zoonotic and Commensal Bacteria and Repealing Implementing Decision 2013/652/EU, 2020). However, *arr-3* and *fosA* were detected by genotypic means and are known to confer resistance to rifampicin and fosfomycin, respectively (Table 9 and Supplementary figure 1). On the contrary, phenotypic resistance was observed for trimethoprim without the presence of known resistance genes.

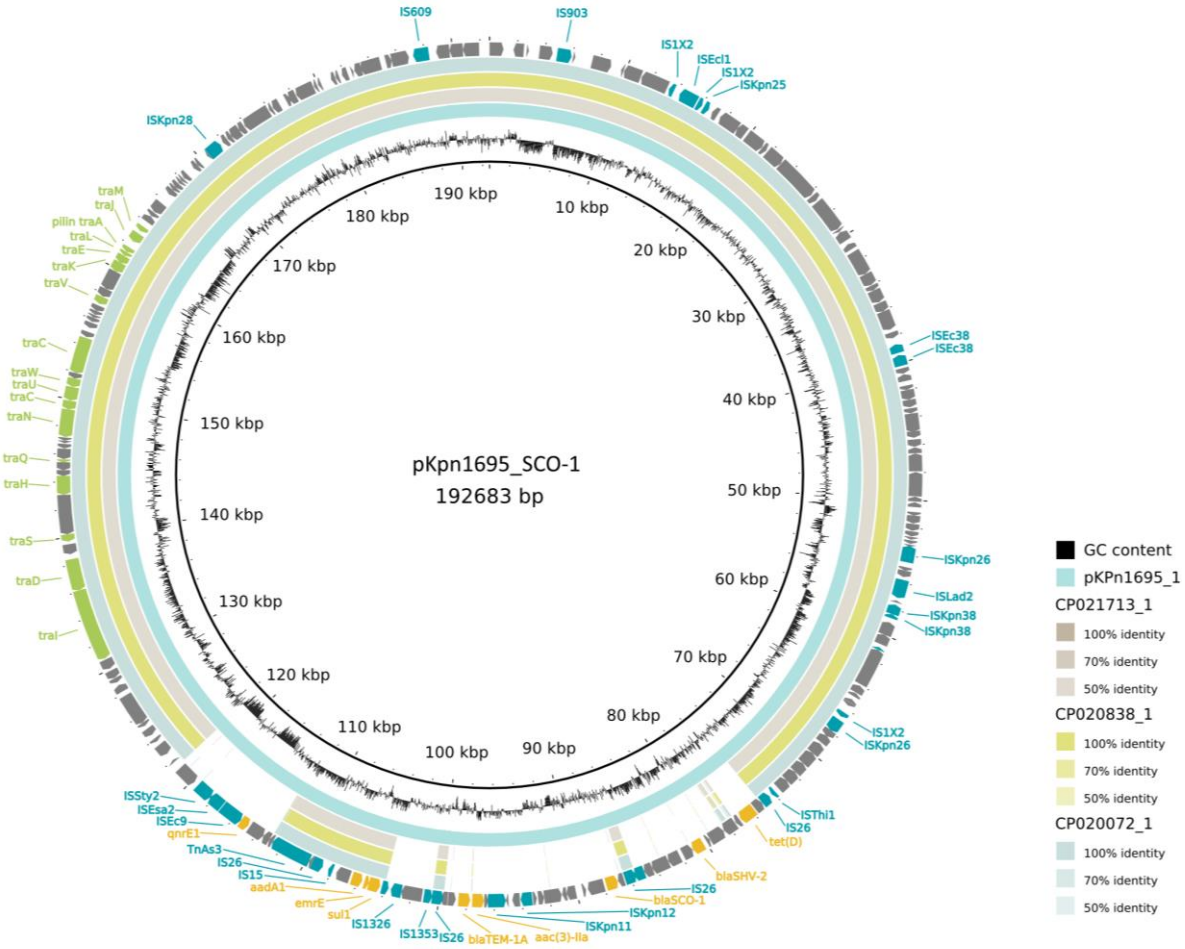
Table 9 - Phenotypic antimicrobial susceptibility testing by micro broth dilution method and genotypic characterization through whole genome sequencing of *K. pneumoniae* Kpn1695

Parameter	**Characterization**
MLST[1]	11
K locus	KL 105
O locus	O2V2
IncFIB(K)	β-lactams (*bla*$_{SCO-1}$, *bla*$_{TEM-1}$, *bla*$_{SHV-2}$), Aminoglycosides (*aac(3)-IIa*, *aadA1*) Sulfonamide (*sul1*) (Fluoro)quinolones (*qnrE1*) Tetracycline (*tet(D)*)
IncR	β-lactams (*bla*$_{DHA-1}$, *bla*$_{OXA-1}$) Aminoglycosides (*aac(6')-Ib-cr5*, *aph(3')-Ia*) Phenicols (*catB3*) Sulfonamide (*sul1*) Rifamycin (*arr-3*)* Quinolones (*qnrB4*) Macrolide (*mph(A)*)
ColRNAI	No antibiotic resistance genes found
Chromosome	Fosfomycin (*fosA6*)* Quinolones (*GyrA-83L*, *ParC-80I*) Polymyxins (*arnT*, *eptAB*, *phoPQ*)** Macrolide (*mdfA*) Phenicol (*oqxA*, *oqxB*) β-lactams (*bla*$_{SHV-11}$) Outer membrane porins (*OmpK37*, *OmpA*)

Legend: [1] MLST = multi locus sequence typing, [2]AMR = antimicrobial resistance, * no phenotypic data available to test for phenotypic rifamycin resistance, No phenotypic resistance was observed for colistin.

Phenotypic screening tests on CHROMID® CARBA SMART Agar showed growth on the CARB side of the plate. The latter is usually indicative of the presence of *bla*$_{KPC}$ and *bla*$_{NDM-1}$ carbapenemase genes. However, no phenotypic resistance was seen for imipenem and meropenem and, similarly, whole genome sequencing did not identify the presence of such genes. Growth on the CARB side of the selective plate, could be explained by the presence of the bla$_{SCO-1}$ gene, which has weak carbenicillinase activity (Papagiannitsis et al., 2007). Phenotypic screening tests that show growth on the CARB side of CARBASMART and resistance to ertapenem but susceptibility to imipenem and

meropenem, could be used for epidemiological purposes to screen for the presence of the *bla*$_{SCO-1}$ gene in other *Enterobacterales*. Further investigations are required to verify this hypothesis by screening Enterobacteriaceae bearing the *bla*$_{SCO-1}$ gene for growth on CARBASMART and phenotypic resistance to ertapenem.



1.

Figure 38 - Genomic map and BRIG comparison of the *bla*$_{SCO-1}$ carrying conjugative IncFIB(K) plasmid and three genetically closely related plasmids retrieved from GenBank. The inner light blue ring represents pKpn1695_SCO-1 and the gray (*K. pneumoniae* of unknown origin), green (clinical *K. pneumoniae*) and light blue (*K. pneumoniae* of unknown origin) ring represent three plasmids with overall high sequence similarities (accession nrs. CP021713_1, CP020838_1, CP020072_1). The outer ring depicts antimicrobial resistance genes in yellow, insertion sequences in blue and transfer genes in green. The circular map was generated with BLAST Ring Image Generator (BRIG) software (Alikhan et al., 2011).

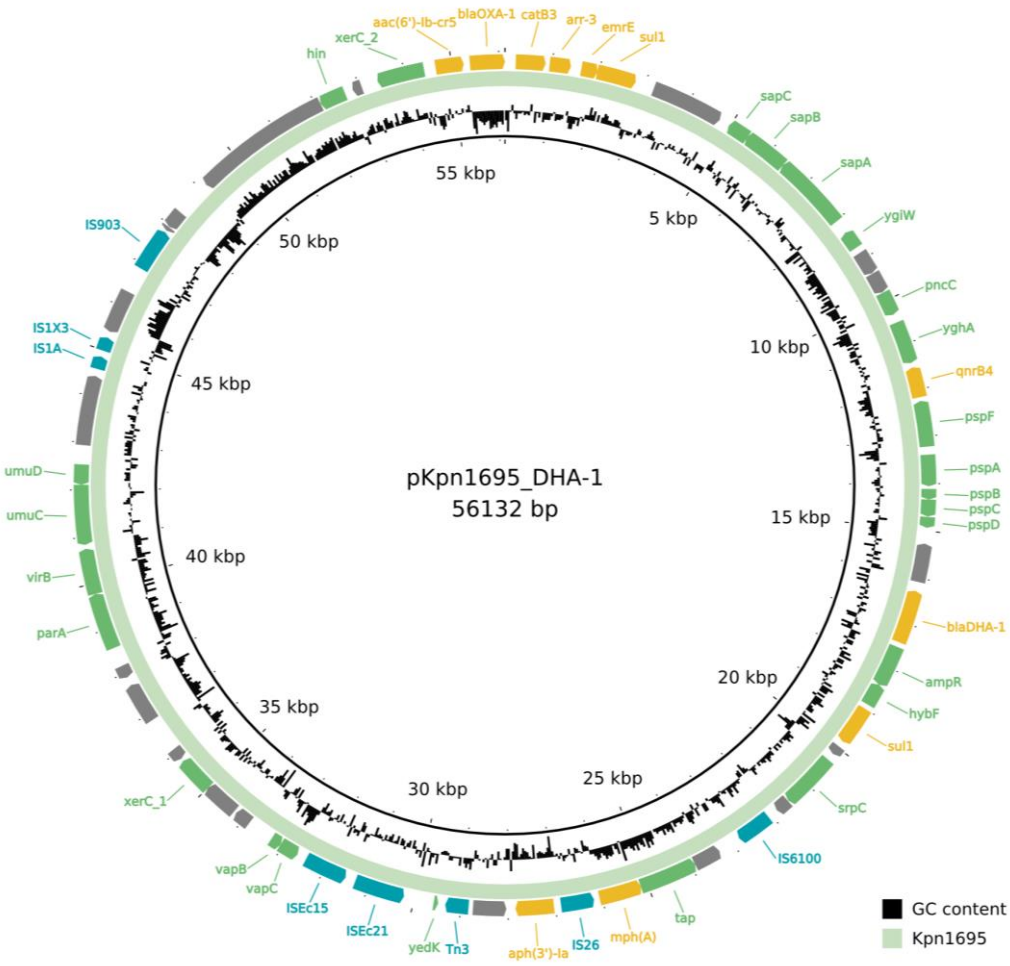


Figure 39 - Circular map of the *bla*$_{DHA-1}$ carrying IncR plasmid. The green ring represents pKpn1695_DHA-1. The outer ring indicates antimicrobial resistance genes in yellow, insertion sequences in blue and green genes with various functions. The circular map was generated with BLAST Ring Image Generator (BRIG) software (Alikhan et al., 2011).

An increasing number of studies have reported a role for AmpC enzymes, such as *bla*$_{DHA-1}$, in carbapenem resistance in clinical ST11 *K. pneumoniae* strains (Shin et al., 2012). All of them report a combination of AmpC cephalosporinase expression and outer membrane porin gene alterations, such as in *OmpK35*/*OmpK36* (Chudáčková et al., 2010; Shin et al., 2012). In contrast to an earlier finding linking mutations in *OmpK37* to lower carbapenem MICs (Bulman et al., 2021), we observed elevated MIC values for ertapenem. Furthermore, plasmid pKpn1695_DHA-1 contains the gene *ampR*, upstream regulatory gene governing *bla*$_{DHA-1}$ synthesis (Hennequin et al., 2012). This gene is also involved in the expression of virulence factors in *Pseudomonas aeruginosa* (Hennequin et al., 2012) and plays a pleiotropic role in the pathogenic process of *K. pneumoniae* since it is involved in biofilm formation and type 3 fimbrial gene expression (Hennequin et al., 2012). The loss of function of *ampR* has been linked to decreased MIC values of β-lactams, whereas presence of a functional *ampR* gene showed high resistance rates to β-lactams and higher β-lactams activity. Nakano et. al suggested that higher β-

lactamase activity of *bla*$_{CFE-1,}$ a plasmid-encoded AmpC β-lactamase, depends on the function of *ampR* rather than on its own high hydrolyzing activity (Nakano et al., 2017). More research is needed to examine whether *ampR* can play a similar role in regulating *bla*$_{DHA-1}$ expression and thus affect β-lactam and carbapenem resistance.

No virulence factors were found using Kleborate (Wick et al., 2018).

The recovery of an ST11 strain from a companion animal carrying several antimicrobial resistance genes (ARGs) of critical importance on a conjugative plasmid is worrisome as this could potentially be transferred to other animals, its owner or other members of the family, or the environment (excreta) as such threatening public health. The dog could potentially have obtained the strain from its owner or from other sources, we could however not obtain a sample from the owner to confirm this. However, a correlation of ARGs between dogs and their owners has already been demonstrated (R. Zhao, 2022).

3.8 Chromosome analysis

Using the CARD database, a match was found for the outer membrane protein *OmpK37*, located on the genome (Supplementary figure 1), which had 18 SNPs and 43 holes and showed 95.50% coverage compared to accession nr. AJ011502.1. In depth analysis of the outer membrane protein revealed 12 synonymous mutations, 6 missense mutations and one in-frame insertion. Reduced carbapenem susceptibility in the presence of *bla*$_{SCO-1}$ combined with porin deficiency related to outer membrane porins *OmpK35/OmpK36* has been previously described (Shin et al., 2012; Venditti et al., 2020). Also two chromosomal mutations contributing to antimicrobial resistance to quinolones, gyrA-83L and parC-80I, were detected. In the same database, a match was observed for a*rnT* and *eptAB*, which are related to chromosomal polymyxins resistance. In Gram-negative bacteria, polymyxins resistance usually occurs via the addition of positively charged moieties [e.g. pEtN and 4-amino-4-deoxy-L-arabinose (L-Ara4N)] to the lipid A component of LPS (Nang et al., 2019). These lipid A modifications alter the net negative charge with a decreased electrostatic interaction to polymyxins as a result. Lipid A modifications in pEtN can arise by either a chromosomally encoded pEtN transferase gene, *eptAB* or a plasmid-encoded *mcr* gene. In contrast, L-Ara4N modification of lipid A is activated by a transferase encoded by *arnT* that is exclusively encoded on the chromosome (Baron et al., 2016; Olaitan et al., 2014). However, phenotypic data did not show resistance against colistin (Table 8). Protein analysis of *arnT* showed an amino acid similarity of 99.82% and 100% query coverage to *arnT* from *K. pneumoniae* (accession nr. FO834906.1) and *eptB* showed 99.30% amino acid similarity and 100% query coverage to a *K. pneumoniae* entry in the GenBank protein database with accession nr. FO203501.1. On the other hand, *phoP* showed 100% amino acid similarity with 95% query coverage to a *Klebsiella* spp. entry in the GenBank reference sequence protein database with accession nr. WP_004151175.1 and *phoQ* showed an amino acid similarity of 100% and 100% query coverage to *phoQ* from *K. pneumoniae* (accession nr. WP_004147969.1). Further studies could help to decipher if such variants in *arnT* and

eptB could affect the functionality of the genes resulting in colistin susceptibility in *K. pneumoniae* Kpn1695.

Material and methods

1 Bacterial isolate

A four-month old female dog with bacteremia was admitted to a veterinary clinic in Belgium in June 2020 where she died of fatal sepsis. Post-mortem biopsies of the liver and brain were individually inoculated on both Gassner (Oxoid, UK) and Columbia medium (Oxoid, UK) and were incubated at 37 ± 2 °C for 24 h in aerobic conditions and 24h in aerobic conditions at 37°C with 5% CO2, respectively. Suspected K. *pneumoniae* colonies were purified on Columbia agar (Oxoid, UK) and species confirmation was performed by MALDI-TOF mass spectroscopy (Bruker Daltonics, Bremen, Germany). The isolate identified as *K. pneumoniae* (strain Kpn1695) was screened for ESBL production and carbapenem resistance using McConkey + CTX (1 mg/L, Biorad, CA, USA) and CHROMID® CARBA SMART Agar (Biomérieux, Marcy-l'Étoile, Frankrijk), respectively (Commission Implementing Decision (EU) 2020/1729 of 17 November 2020 on the Monitoring and Reporting of Antimicrobial Resistance in Zoonotic and Commensal Bacteria and Repealing Implementing Decision 2013/652/EU, 2020; Silva et al., 2022).

2 Phenotypic resistance testing

K. pneumoniae Kpn1695 was subjected to antimicrobial susceptibility testing using the broth microdilution method, according to ISO 20776-1:2019 using EUVSEC3 and EUVSEC2 sensititre™ plates (Trek Diagnostic Systems; Thermo Scientific, MA, USA). E. coli ATCC 25922 was used for quality control. The antibiotic panel EUVSEC3 sensititre™ plate included amikacin, ampicillin, azithromycin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, colistin, gentamicin, meropenem, nalidixic acid, sulphamethoxazole, tetracycline, tigecycline and trimethoprim. The antibiotic panel EUVSEC2 sensititre™ plate included cefepime, cefotaxime, cefotaxime/clavulanic acid, cefoxitin, ceftazidime, ceftazidime/clavulanic acid, ertapenem, imipenem, meropenem and temocillin.

Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) using epidemiological cutoff values (ECOFF) as described in the Commission Implementing Decision 2020/1729 (Commission Implementing Decision (EU) 2020/1729 of 17 November 2020 on the Monitoring and Reporting of Antimicrobial Resistance in Zoonotic and Commensal Bacteria and Repealing Implementing Decision 2013/652/EU, 2020). In the absence of epidemiological cutoff values for K. *pneumoniae*, those for E. coli, the indicator Enterobacteriaceae bacterium were applied (Supplementary table 5 and Supplementary table 6).

3 Genotypic resistance testing using whole genome sequencing (WGS)

Genomic DNA of K. *pneumoniae* Kpn1695 was extracted from 10 ml of pure culture using the genomic Tip 20/G kit (Qiagen, Benelux B.V., Venlo, The Netherlands) following the manufacturer's instructions. The purity of the DNA was evaluated with a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific) and DNA was determined with a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Nextera XT DNA sample preparation kit (Illumina, San Diego, CA) was used for library preparation and sequencing was performed on an Illumina MiSeq instrument (Illumina, San Diego, CA) using the MiSeq V3 chemistry, as described by the manufacturer's protocol, for the production of 2 x 300 bp paired-end reads. Raw MiSeq sequencing reads were trimmed using Trimmomatic (v0.38) with the 'SLIDINGWINDOW:4:20' option (Bolger et al., 2014b). The leading and trailing bases of a read were removed when the Phred dropped below a score of 3 (Bolger et al., 2014b). The trimmed MiSeq reads were used in a de novo assembly generated with SPAdes v3.15.4 (Bankevich et al., 2012). Raw sequencing data were submitted to NCBI Genbank under BioProject PRJNA870711, with accession nrs. CP103301, CP103302, CP103303 and CP103304.

Long-read library preparation was performed using the Rapid Barcoding Sequencing kit (SQK-RBK004) according to the standard protocol provided by the manufacturer Oxford Nanopore Technologies (ONT, Oxford, UK). The constructed library was loaded onto an R9.4.1 MinION flow cell (FLO-MIN106) and sequenced for 48h on a MinION Mk1C. Base-calling and demultiplexing were performed with Guppy (v6.0.1). Subsequently, long reads were checked with NanoStat and NanoPlot (v1.32.0) to have a general overview of the read quality. The adapter and barcode sequences were removed with Qcat (v1.1.0). Low quality (< q8) and short (< 10000 bp) reads were removed with NanoFilt (v2.0.0)(De Coster et al., 2018). Complete genome sequences were obtained by a hybrid de novo assembly with Unicycler (v0.4.7) with default settings (Wick et al., 2017). Short-read assembly and hybrid assembly data were annotated using Prokka and manually curated (version 1.14.6) (Seemann, 2014).

Acquired antibiotic resistance-encoding gene prediction was done using NCBI AMRFinder (version 3.10.18), ResFinder v4.0 (Florensa et al., 2022), ARG-ANNOT (Gupta et al., 2014)and CARD using default settings (Alcock et al., 2020). Mobile genetic elements were analysed with MobileElementFinder using default settings (Johansson et al., 2021). Analyses of the plasmid replicon type was performed by PlasmidFinder 1.3 (Carattoli et al., 2014). BIGSdb was used for the determination of the sequence type. K locus, O locus and the presence of virulence factors were analyzed using Kleborate with default settings (Version v2.0.4) (Lam et al., 2021a). To determine the position of the acquired resistance genes and/or mutations on either chromosome or plasmid, the four contigs obtained in the hybrid assembly were analyzed separately with ABRicate with default settings (v1.0.1). PLASCAD was used to locate genes associated with transfer. Additional annotation of plasmid sequences was performed using PROKKA version 1.14.6 (Seemann, 2014). Protein analysis was

performed with BLASTP with the RefSeq Select proteins database. Visualization of in depth plasmid analysis was performed using Blast ring Image Generator (BRIG) (Alikhan et al., 2011).

Conclusions

In this work, we investigated the molecular mechanism causing ertapenem resistance in a *K. pneumoniae* strain isolated from a four-month old dog using hybrid sequencing analysis. We described ertapenem resistance that could be related to the presence of bla_{SCO-1} and bla_{DHA-1} combined with porin lesions in the outer membrane porin *OmpK37*. It is the first report describing the combination of bla_{SCO-1} and bla_{DHA-1} in *K. pneumoniae.* In depth plasmid analysis via hybrid sequencing technologies showed the presence of the bla_{SCO-1} gene on a conjugative IncFIB(K) plasmid, possibly increasing the risk of its transmission to humans, to animals and to the environment. Our research highlights the importance of combining long and short-read sequencing in AMR research as it overcomes common challenges in plasmid reconstruction. Investigation of other *Enterobacteriaceae* that also harbor bla_{SCO-1} carrying plasmids through phenotypic screening tests, with growth on the CARB side of CARBASMART, and phenotypic resistance to ertapenem but susceptibility to imipenem and meropenem, could be of epidemiological importance, as the extent of its spread in veterinary and clinical isolates is not fully understood.

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Data Availability Statement:

The data that support the findings of this study are available from the corresponding author upon request. Raw sequencing data were submitted to NCBI Genbank under BioProject PRJNA870711.

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Supplemental material

Supplementary table 5 - Epidemiological cut-off values (ECOFF) used in the EUVSEC 3 sensititre™ plates for the interpre-tation of minimal inhibitory concentration values of *K. pneumoniae*

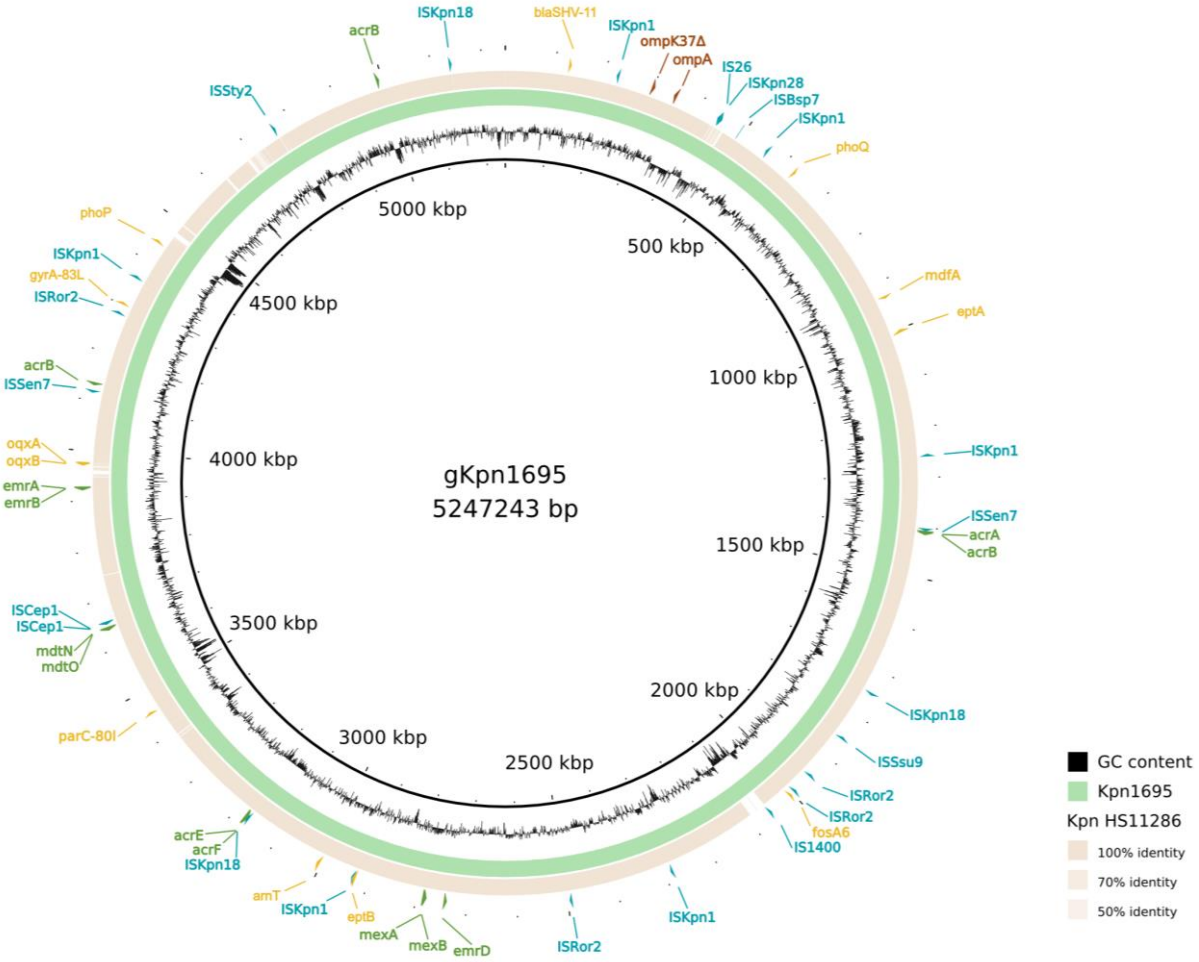
Antibiotic	ECOFF (mg/L)
Amikacin	8
Ampicillin	8*
Azithromycin	16*
Cefotaxime	0.25
Ceftazidime	0.5
Chloramphenicol	16*
Ciprofloxacin	0.125
Colistin	2
Gentamicin	2
Meropenem	0.125
Nalidixic Acid	8*
Sulfamethoxazole	64*
Tetracycline	8
Tigecycline	2
Trimethoprim	2*

*ECOFF of *E. coli* was used since no ECOFF is available for *K. pneumoniae* for this antibiotic.

Supplementary table 6 - Epidemiological cut-off values (ECOFF) used in the EUVSEC 2 sensititre™ plates for the inter-pre-tation of minimal inhibitory concentration values of *K. pneumoniae*

Antibiotic	ECOFF (mg/L)
Cefepime	0.125*
Cefotaxime	0.25
Cefotaxime/clavulanic acid	0.25*
Cefoxitin	8
Ceftazidime	0.5
Ceftazidime/clavulanic acid	0.5*
Ertapenem	0.03*
Imipenem	1
Meropenem	0.125
Temocillin	8

*ECOFF of *E. coli* was used since no ECOFF is available for *K. pneumoniae* for this antibiotic



Supplementary figure 1 - Genomic map of the genome of strain Kpn1695 compared with *K. pneumoniae* reference genome HS11286. The circular map was generated with BLAST Ring Image Generator (BRIG) (Alikhan et al., 2011).

Experimental section

Study 3

Detection of pan-azole resistant *Aspergillus fumigatus* in horticulture and a composting facility in Belgium

Preamble

Azole resistance in *A. fumigatus* is increasingly prevalent globally, primarily driven by the use of agricultural azoles for crop protection. Cross-resistance between agricultural and medical azoles is seen, attributable to their similar chemical structures, limiting the treatment options in the clinics. The first report, by Snelders et. al, highlighting the potential implications of pesticide use in agriculture on azole resistance appeared in 2009. Since then, reports on the occurrence of azole-resistant *Aspergillus fumigatus* (ARAf) have surged worldwide. In Belgium, data on environmental ARAf prevalence were previously unavailable.

This study aimed to determine the prevalence of ARAf in Belgian agriculture, horticulture, and composting facilities. Air, compost, and soil samples were collected from multiple composting facilities and across various time points in agricultural and horticultural settings. Azole susceptibility patterns were assessed, and the *cyp51A* gene along with its promoter region were sequenced in *A. fumigatus* isolates exhibiting phenotypic azole resistance. This investigation documented the first TR34/L98H and TR46/Y121F/T289A mutations isolated from composting facilities and horticulture in Belgium.

The findings underscore the need for standardized environmental surveillance of *A. fumigatus* across Europe to facilitate international comparisons and the implementation of continent-wide measures.

Experimental section

Study 3 :

Detection of pan-azole resistant *Aspergillus fumigatus* in horticulture and a composting facility in Belgium

Medical mycology : 62(7) ; myae055

Debergh Hanne, Castelain Philippe, Goens Karine, lefevere Paulien, Claessens Jessie, De Vits Elien, Vissers Marc, Blindeman Liesbet, Bataille Charlotte, Saegerman Claude, Packeu Ann

Abstract

Azole resistance in *A. fumigatus* (ARAf) is becoming a worldwide health threat due to increasing occurrence in the environment. However, environmental surveillance programs are not commonly in place and are lacking in Belgium. Since no data on the occurrence of ARAf and the presence of hotspots for the selection of azole resistance is available in Belgium, a first study on the prevalence of ARAf in the environment was conducted. A total of 232 air and compost or soil samples were taken from two composting facilities, and from horticultural and agricultural crops. The azole susceptibility pattern was determined using the EUCAST method (E. Def. 9.4) and the c*yp*51A gene and its promotor region were sequenced in *A. fumigatus* isolates with phenotypic azole resistance. Six pan-azole resistant *A. fumigatus* isolates were identified, originating from compost and horticultural crops. Four isolates carried the TR_{34}/L98H mutation and one isolate the TR_{46}/Y121F/T289A mutation. However, we did not observe any ARAf isolates from agricultural crops. In conclusion, this study reported the first TR_{34}/L98H and TR_{46}/Y121F/T289A mutation isolated from a composting facility and horticulture in Belgium. The implementation of standardisation in environmental surveillance of *A. fumigatus* on a European level would be beneficial in order to identify hotspots.

Lay summary

The ubiquitous fungus *Aspergillus fumigatus* can cause serious invasive diseases in humans. Due to the extensive use of environmental azoles, an increase of clinical infections with azole resistant *Aspergillus fumigatus* is seen. This pilot study aimed to estimate the prevalence of azole-resistant *A. fumigatus* in environmental reservoirs in Belgium.

Introduction

Aspergillus fumigatus is a widespread saprophytic mold which naturally lives on decaying plant material and in soil (J. P. Latgé, 1999). This highly sporulating mold is commonly found in compost from household and green waste, moldy hay and woodchips (Jeanvoine et al., 2017b; Shelton et al., 2022). Due to its thermotolerance, *A. fumigatus* is able to proliferate at high temperatures up to 60°C during the composting process (Bhabhra & Askew, 2005). It is widespread in the air microflora and can also be isolated from water and soil (Burks et al., 2021; Caggiano et al., 2020; Gómez Londoño & Brewer, 2023; Góralska et al., 2020; Snelders et al., 2009). This opportunistic human pathogen can cause a variety of diseases in both immunocompromised and immunocompetent patients, from allergic conditions to acute and chronic angio-invasive pulmonary aspergillosis (IA) or chronic bronchopulmonary aspergillosis (CPA) with high morbidity and mortality (Resendiz Sharpe et al., 2018; Verweij, Chowdhary, et al., 2016). Studies have shown that azole-resistance in both clinical and environmental isolates has increased since the late 1990s in Europe and is currently considered as a growing global health threat due to lack of treatment options (Berger et al., 2017; Gonçalves et al., 2020; Howard et al., 2009; Jeanvoine et al., 2017b; Resendiz Sharpe et al., 2018; Snelders et al., 2008; Wiederhold & Verweij, 2020). Like any other antibiotic resistance, the development of azole resistant *A. fumigatus* (ARAf) can occur via the patient route, which takes place when resistance develops through prolonged azole treatment. In 2009, Snelders *et al.* introduced the possible environmental origin of azole resistance in *A. fumigatus* due to the use of environmental azole fungicides (Snelders et al., 2009). Since then, it was demonstrated that the majority of cases of azole-resistant disease arise when an azole-naive patient is infected with an azole-resistant strain from the environment, which is frequently found in air and soil (Resendiz Sharpe et al., 2018; Snelders et al., 2008, 2009; Verweij, Snelders, et al., 2009; J. Zhang et al., 2015).

Nowadays, the widespread use of azole fungicides in the environment is considered one of the cornerstones in the development of azole resistance in *A. fumigatus*.

In agriculture, azole fungicides, such as tebuconazole (107534-96-3), epoxiconazole (133855-98-8), propiconazole (60207-90-1) and bromuconazole (116255-48-2) are or have been intensively used for crop protection (Godeau et al., 2023; Schoustra et al., 2019; Snelders et al., 2009). In 2019, these were, respectively, the second, fourth, seventh and tenth most sold triazoles in agriculture in Belgium (*Sold Volumes of Plant Protection Products for Agriculture in Belgium*, 2019). Both epoxiconazole and propiconazole are now banned from use at the EU-level under Regulation (EC) No 1107/2009, because of suspected fertility, developmental and endocrine perturbation properties (European commission, 2009). The largest volumes of azoles are sold in Asia and Europe, accounting for more than two-thirds of all azoles used worldwide (Jørgensen & Heick, 2021).

Since all azoles target the enzyme lanosterol-14-alpha-demethylase (*cyp51*), which inhibits the biosynthesis of ergosterol, cross resistance is seen between the medical and agricultural azoles (Gómez Londoño & Brewer, 2023; Pérez-Cantero et al., 2020; Snelders et al., 2012). *A. fumigatus* has two *cyp*51 isoforms, namely c*yp51A* and *cyp51B*. Most azole-resistant strains display mutations in the *cyp51A* gene, although other resistance mechanisms have been described (Camps, Dutilh, et al., 2012; Fraczek Mg et al., 2013; Hagiwara et al., n.d.; Resendiz-Sharpe et al., 2020; Wei et al., 2017). *Cyp51A* gene mutations can be either tandem repeats in the gene promoter, single-nucleotide polymorphisms (SNPs) or both (Resendiz Sharpe et al., 2018). The most frequently detected tandem repeat mutations in the promotor region are TR34/L98H and TR46/Y121F/T289A (Burks et al., 2021; Pérez-Cantero et al., 2020; Resendiz Sharpe et al., 2018).

Subsequently, multiple articles have described the presence of so called hotspots for azole resistance in different environments across Europe, namely flower bulb waste, green waste and woodchips (Burks et al., 2021; Doughty et al., 2021; Fraaije et al., 2020; Schoustra et al., 2019). Hotspots can be defined as settings that support the growth, the reproduction and the dispersal of a population of *A. fumigatus.* Furthermore, the conditions must be as such that selection of resistant phenotypes occurs within a mixed population of susceptible and resistant strains and the substrate must contain residues of demethylation inhibitors (DMIs) at sufficient concentrations (Doughty et al., 2021). To date, limited data is available on the presence of azole resistant *A. fumigatus* in the environment in Belgium.

The use of these azoles in ornamental plants for manipulating the shape, size, and aesthetic quality, in countries such as the Netherlands and Denmark, is significant and is linked to their market value (Jørgensen & Heick, 2021). Consequently, waste materials from agriculture and horticulture, along with input material from wood, forestry, paper, household and garden waste, are likely to contain fungicide residues. These waste streams are generally composted by industrial composting facilities, where compost is processed in different stages and the rows are frequently turned to improve oxygenation and to control heat and moisture levels. Research from the Netherlands(Schoustra et al., 2019; J. Zhang, Lopez Jimenez, et al., 2021) and the United States (Hurst et al., 2017) showed that composting material is a hotspot for the development and release of a large number of ARAf spores.

In a One Health perspective, the essential role of azole fungicides to secure the food supply should be balanced with the need to preserve the activity of structurally related azoles in the clinical practice (FPS Health, Food Chain Safety and Environment, 2021; Verweij et al., 2020). Therefore, it is needed to identify the drivers behind the resistance development against azole fungicides released in the environment. In this context, the European Centre for Disease Prevention and Control (ECDC) advocates active environmental surveillance in all its member states (European Centre for Disease Prevention and Control., 2013). To assess the Belgian situation, a pilot study was carried out in the

environment to map the Belgian reservoirs of ARAf. This was addressed by assessing the susceptibility pattern and associated genetic mutations of potential ARAf isolates from air, soil and/or compost samples from an agricultural field, a horticultural site and two industrial composting facilities. We describe the need for harmonization and standardization of environmental surveillance methods in Europe and recommend the development of a European surveillance network.

Materials and methods

1 Environmental sampling

Selection of the sampling sites was performed based on a literature search for hotspots of ARAf. Soil, air and compost were collected from 7 different sampling locations comprising two commercial compost processing facilities of green waste and manure (Antwerp region and Flemish Brabant), horticultural (East-Flanders) and agricultural crops (Walloon Brabant) (Table 10). Both the horticultural and agricultural sampling campaigns were performed in experimental sites where known concentrations of different azoles and growth inhibitors were applied in the framework of experimental efficacy trials under the Good Agricultural Practice. A blank sample, in the absence of fungicide application, has been included in the horticultural (only hibiscus) and the agricultural sampling campaign. The level of azoles or plant growth regulators (PGRs) in the compost samples have not been determined in this pilot project but will be included in future projects.

Table 10 - Environmental sampling locations and sampling approaches

Environmental sampling locations		Sampling dates	Sampling approach	Air impaction method
Compost	Facility 1	August 2020	Air and compost	RCS®
	Facility 2	April 2021	Air and compost	RCS®
Agriculture	Wheat	May – July 2022[1]	Air and soil	MAS-100NT®
Horticulture	Roses	June 2020[2]	Air and substrate	MAS-100NT®
	Hibiscus	June 2020[3]	Air and substrate	MAS-100NT®
	Primula	January 2021[4]	Air and substrate	MAS-100NT®
	Residue heap[5]	June 2020	Soil	NA

[1]four sampling campaigns were performed: before treatment (11/05/2022), 6 days after treatment (18/05/2022), 3 weeks after treatment (2/06/2022) and during harvest (28/07/2022); [2] sampling was performed 3 days after last treatment; [3] sampling was performed 28 days after last treatment; [4] sampling was performed 41 days after last treatment; [5] The residue heap acts as a bioremediation location where left-overs of plant protection product (PPP) spray dilutions are poured in order to eliminate PPP waste; MAS-100NT® = Microbial Air Sampler; RCS® = Reuter Centrifugal Sampler; NA = not applicable.

1.1 Compost sampling

The first compost company treated household waste, green waste and animal manure. The compost was processed in five stadia going from fresh material to mature compost over the course of approximately two weeks. Temperature and moist content is monitored to ensure a good composting environment. Compost heaps were turned every few days to ensure enough aeration. A total of eight samples from the five different maturing stages of composting were taken (Table 11).

The second company processed green waste derived from diverse waste streams, as well as from food products deemed unsuitable for human or animal consumption, such as rejected, mold-infested, expired, or improperly packaged items. These waste materials were initially subjected to depackaging within the packaging facility, where they were stored for a short period of time resulting in partial fermentation. While the utilization of azoles remained not documented, it primarily encompassed

products from conventional agriculture, wherein the application of fungicides is prevalent, and the presence of residues of active substances (a.s.) and/or their metabolites was likely. A total of six samples were taken: starting material, compost, crude digestate, solid digestate fraction, liquid digestate fraction and concentrated liquid digestate fraction.

Table 11 - Moisture measurements in composting facility one from the 8 sampling locations

	Street 1	Street 2	Days of maturity
1	Moisture 45%		0-3
2	Moisture 46%	Moisture 41%	3-7
3		Moisture 36%	7-11
4	Moisture 34%	Moisture 35%	11-14
5	Moisture 36%	Moisture 31%	14-17

Compost heaps were turned every few days to ensure enough oxygenation. Stage 1 shows the fresh material and stage 5 the mature compost.

1.2 Horticulture and agriculture soil sampling

Experimental wheat crops were treated with combinations of various fungicides and at different concentrations, by foliar spraying. The trials were part of a single-timepoint fungicide management program. A total of 28 plots with different treatment programs were sampled (Supplementary table 7). One was a control plot, where no antifungal was applied, and one plot was treated with a non-azole PGR only. All other 26 plots received an azole or a combination of azoles.

Four sampling campaigns were carried out: before treatment (11/05/2022), 6 days after treatment (12/05/2022), 21 days after treatment (2/06/2022) and during harvest (28/7/2022). Approximately five gram was collected from the topsoil (0-3cm)(Y. Chen et al., 2020), consisting of a pooled sample from three replicates (sampling locations were 30 cm apart within the same experimental plot). Each experimental plot was 30 cm separated from the next.

Experimental horticultural crops (primula, hibiscus, roses) were treated with combinations of various fungicides and/or PGRs, and at different concentrations, by foliar spraying or pour-on application. The foliar sprays were part of a multiple timepoint-based disease and growth inhibitor management program (Supplementary table 8, Supplementary table 9, Supplementary table 10). Approximately five gram was collected from the substrate. The residue heap, which acts as a bioremediation location where left-overs of plant protection product (PPP) spray dilutions are poured in order to eliminate PPP waste, was also sampled. Approximately five gram of soil was collected from the residue heap.

1.3 Air sampling in field trials and compost facilities

The Microbial Air Sampler (MAS-100NT®) (Merck, Darmstadt, Germany) was used for air sampling on agricultural fields and in horticultural greenhouses, whereas in composting facilities, given the high humidity inside, the Reuter Centrifugal Sampler (RCS®) was employed (Table 10). The residue heap was located in a greenhouse where doors and windows were open. One general outdoor air sample was taken that represented the outdoor situation, including the air from the horticultural residue heap.

2 Isolation of *A. fumigatus*

Air sampling inside and outside of the composting facilities was performed using the RCS®, where a standardized volume of 40L (24s at 100L/min) of air was captured inside and 80L outside (48s at 100 L/min). The airborne spores were captured on a Rose Bengal Chloramphenicol Agar (RBCA) medium (Biorad, California, USA) and incubated at 45°C±1°C for 48h±2h. Isolated colonies were transferred afterwards onto malt extract agar supplemented with chloramphenicol (0.5g/L, MC) and MC supplemented with 4 mg/L of tebuconazole (MC+T, Sigma Aldrich, Saint-Louis, MO, USA) and incubated at 48°C ±1°C for 48h±2h. Using the MAS-100NT® in agricultural and horticultural sites, a standardized volume of 1000L of air was captured (10 min at 100L/min) and airborne spores were collected on MC and MC+T and incubated at 48°C ±1°C for 48h±2h. The different air sampling methods were applied because of the high moisture content in the composting facilities, which is not favorable for the MAS100-NT®.

To isolate *A. fumigatus* from soil or compost, 1g of material was added to 9 mL of 0.85% NaCl + 0.01% Tween 20 solution. After thorough vortexing, 100 µL of the supernatant was seeded onto MC and MC+T agar. Colonies of *A. fumigatus* were recovered from the MC+T plates of both soil and air samples after 48h ± 2h of incubation at 48°C ± 1°C. Single colonies were subcultured in MC tubes and stored at 4 °C until further analysis. *A. fumigatus* colonies were identified based on their microscopic and macroscopic characteristics. Matrix-assisted laser desorption/ionization- time of flight mass spectrometry (MALDI-TOF MS) was used to confirm their identity as *Aspergillus* section *Fumigati* using a microflex LT MALDI-TOF MS instrument (Bruker Daltonics) with the default settings and as described by Cassagne *et al*. (Cassagne et al., 2011). Their identification was performed with the software Biotyper 4.1 (Bruker Daltonics) using the MSI 2.0 database. The latter is shared online through a free web application (https://msi.happydev.fr) (A.-C. Normand et al., 2021a).

The selection of colonies isolated from MC+T were considered as representative for any ARAf and were subjected for further analysis.

3 Minimal inhibitory concentration analysis and *cyp51A* sequencing

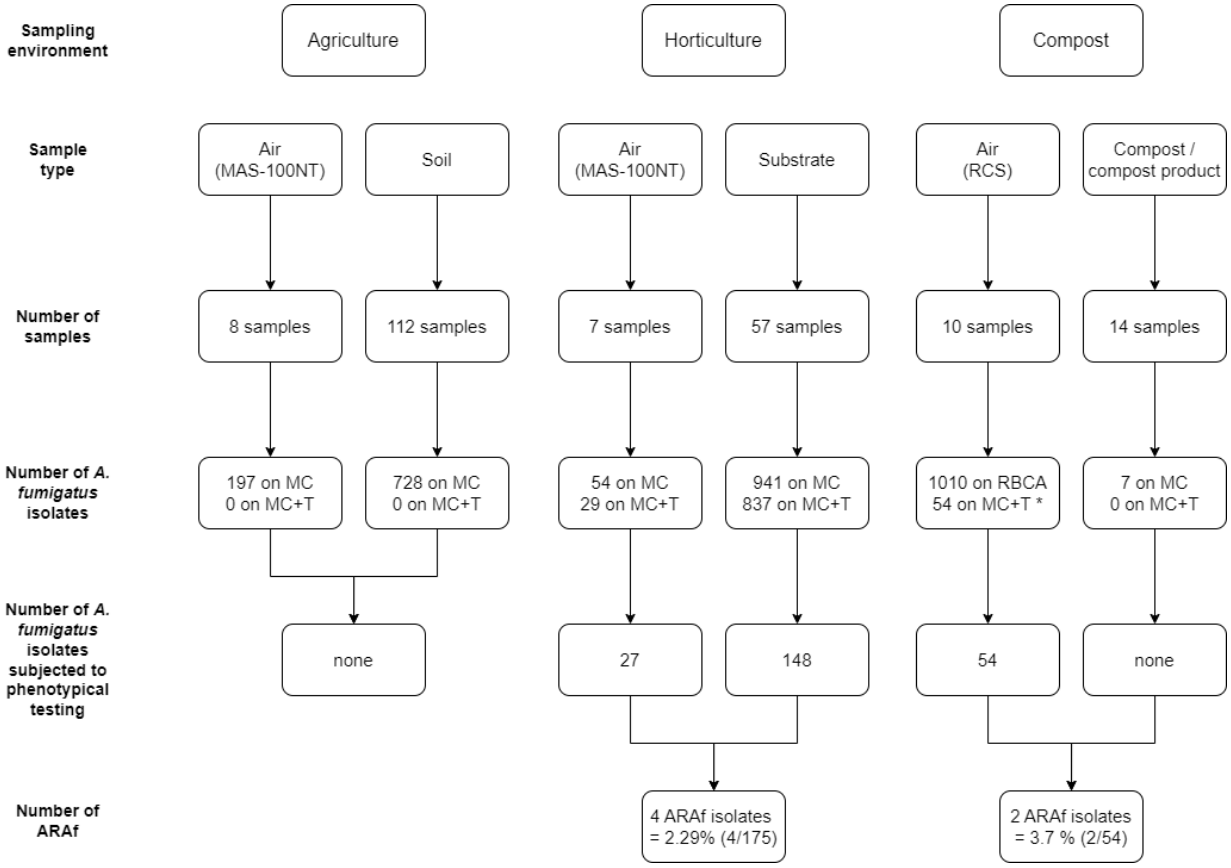
All colonies isolated from MC+T were tested by the broth microdilution method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (E. Def. 9.4) (Guinea et al., 2022). Briefly, a cell suspension of 1-5 x 10^6 CFUs (colony-forming units) per mL was prepared in 10 mL of saline water (8.5g/L NaCl) from a 5-day-old subculture on Sabouraud chloramphenicol agar tube. Subsequently, 1 mL of the cell suspension was added and mixed with 10 mL of RPMI-1640 medium (Sigma-Aldrich, Saint-Louis, MO, USA). A total of 100 µL of this cell suspension was added to each well of a 96-well plate containing 100 µL of serial dilutions of the antifungals and a control. The plates were incubated at 35°C ± 1°C for 48 h. The minimal inhibitory concentration (MIC) of four medical azoles (itraconazole (ITC), voriconazole (VOR), posaconazole (POSA) and isavuconazole (ISA)) was determined on colonies isolated from the MC+T medium. The MIC was determined visually as the lowest concentration of antifungal drugs causing complete inhibition of fungal growth. *Pichia kudriazevii* (IHEM 9560 = ATCC 6258), *Candida parapsilosis* (IHEM 3270 = ATCC 22019) and *A. fumigatus* (IHEM 28944 = ATCC 204305) were used as quality control strains. Azole resistance was defined according to the EUCAST clinical breakpoints (v10.0)(EUCAST, 2020), for ITC and VOR with MIC >1 mg/L, POSA with MIC >0.25 mg/L and ISA with MIC >2 mg/L) .

All colonies displaying phenotypic resistance were subjected to c*yp51A* sequencing. DNA extraction was performed using the ZR Fungal/Bacterial DNA kit (Zymo research, Freiburg im Breisgau, Germany) following the manufacturer's instructions. The *cyp51A* gene and its promotor region were amplified and sequenced using five primer pairs (Supplementary table 5) and the BigDye-Terminator-v3.1 cycle-sequencing kit (Applied-Biosystems, Lithuania). Reaction products were purified using magnetic beads (CleanSeq Agencourt®, Beckman Coulter life sciences, California, USA) according to the manufacturer's instructions and run on an ABI3500 Genetic Analyzer (Applied biosystems, Lithuania). The obtained sequences were compared to the reference *cyp*51A sequence of wild-type *A. fumigatus* strain ATCC36607 (GenBank accession number: AF338659.1) using the FunResDb database (Weber et al., 2018).

Results

A total of 232 environmental samples were collected between June 2020 and July 2022 in Belgium: 136 obtained from agriculture (58.6%), 67 from horticulture (28.9%) and 29 from composting facilities (12.5%) (Figure 40).

Figure 40 - Sampling plan for the detection of (azole-resistant) *Aspergillus fumigatus* in the Belgian environment



MC = malt chloramphenicol; MC+T = malt chloramphenicol + tebuconazole; * *A. fumigatus* colonies were transferred from the Rose Bengal Chloramphenicol Agar (RBCA) medium to the MC+T medium. Phenotypical resistance testing was performed to determine the MIC value using the EUCAST guidelines (E. Def. 9.4) (EUCAST, 2020; Guinea et al., 2022)

3.1 Compost

Aspergillus fumigatus was isolated from two composting facilities in Belgium in both air and compost samples. A high number of airborne spores were captured on the RBCA medium from the first facility (Table 12). However, due to contamination by other fungi, only a subset was analyzed on MC+T medium, resulting in the growth of 24 *A. fumigatus* isolates. A total of 16 colonies were isolated from indoors, whereas 8 were from outside. In addition, a total of 8 compost samples were collected from this first composting facility. In the second composting facility, a lower number of colonies (170) were isolated from air samples on the RBCA medium, of which single, non-contaminated colonies were subcultured onto the MC+T medium, resulting in 30 *A. fumigatus* colonies. A total of 29 colonies were isolated from air samples taken indoors. No *A. fumigatus* colonies were detected in the compost samples from both facilities. In total, 54 *A. fumigatus* colonies were subjected to antifungal susceptibility testing. Two isolates displayed phenotypic resistance against at least one medical azole (Table 13).

3.2 Horticulture and agriculture

Air sampling on agricultural and horticultural sampling sites was performed with the MAS-100NT® air sampler, allowing sampling directly on both MC and MC+T from both air and soil or substrate. A total of 29 *A. fumigatus* colonies were isolated from the MC+T medium in air samples from greenhouses in horticulture (Table 14). Regarding the soil/substrate sampling in horticulture, *A. fumigatus* was not recovered from the substrate of roses, nevertheless, *A. fumigatus* was isolated from the substrate of hibiscus plants and in the soil of the residue heap. The highest number of colonies were retrieved from the substrate of primula plants (Table 14). Five *A. fumigatus* colonies were isolated from an air sample and three soil samples from the non-treated hibiscus plants (negative control), however non were azole-resistant.

A high number of airborne spores (n = 197) were captured on the MC medium from agriculture (Table 14). However, no *A. fumigatus* was isolated from the MC+T medium. This was also observed in the soil samples, with a high *A. fumigatus* spore count on the MC medium (n = 728), nonetheless, no *A. fumigatus* was isolated from the MC+T medium (Table 14).

3.3 Phenotypic and genotypic resistance testing

Five samples (5/232, 2.16%) contained *A. fumigatus* isolates that showed phenotypical azole resistance against at least one medical azole (Table 13). A total of 6 isolates that showed phenotypic azole resistance according to the EUCAST clinical breakpoints were detected, resulting in a prevalence of ARAf of 2.62% (6/229 *A. fumigatus* isolates). Sequencing the *cyp*51A gene for those *A. fumigatus* isolates revealed the TR34/L98H mutation in 4 out of 6 (66.7%) isolates and 1 (16.7%) isolate carried the TR46/Y121F/T289A (Table 14). One isolate did not display any known resistance mutations in the *cyp*51A gene. The remaining isolates presented low azole MIC values typical for susceptible isolates.

Table 12 - Total number of CFU of *Aspergillus fumigatus* per sampling type and per medium in two composting facilities

Sampling site	Air[1]			Compost[2]		
	Number of samples	RBCA	MC+T[3]	Number of samples	MC	MC+T
Facility 1 - inside	1	121	16	8*	7	0
outside	4	692	8			
Facility 2 - inside	3	196	29	6**	0	0
outside	2	1	1			

RBCA: Rose Bengal Chloramphenicol Agar; MC: Malt + chloramphenicol; MC+T : Malt + chloramphenicol + tebuconazole; [1] Air sampling was performed using the RCS® sampler; [2] The sample was directly seeded on both media; [3] single colonies were subcultured onto MC+T medium from the RBCA medium. * A total of eight samples were taken from 5 different maturing stages of the compost; ** One sample per type of product was analysed: starting material (green waste and/or animal manure), compost, crude digestate, solid digestate fraction, liquid digestate fraction and concentrated liquid digestate fraction.

Table 13 – Minimal inhibitory concentration results and *cyp*51A mechanism

Sampling site	Sample type	Accession nr*	MIC (mg/L)[1]				*cyp*51A mutations
			VOR	ITC	ISA	POSA	
Compost (F1)	Air	28546	8	>16	8	1	TR34/L98H
	Air	28547	4	>16	8	1	TR34/L98H
Horticulture	Air (hibiscus)	28954	8	8	2	0,5	TR34/L98H
Horticulture	Soil (residue heap)	28947	>16	0,5	>64	0,5	TR46/Y121F/T289A
Horticulture	Substrate (Primula)	28553	4	>16	8	1	TR34/L98H
Horticulture	Substrate (Primula)	28554	>16	4	8	2	No mutation detected

MIC = Minimal inhibitory concentration; VOR = Voriconazole; ITC = Itraconazole; ISA = Isavuconazole; POSA = Posaconazole; F1 = Facility 1; 1 Phenotypical resistance testing was performed to determine the MIC value using the EUCAST guidelines (E. Def. 10.2) (EUCAST, 2020; Guinea et al., 2022); * The isolates were deposited in the BCCM/IHEM collection under the mentioned accession numbers (https://bccm.belspo.be/about-us/bccm-ihem).

Table 14 - Total number of CFU of *A. fumigatus* per sampling type and per medium in agricultural and horticultural sites

Sampling site	Air (CFU/1000L)			Soil (CFU/10mg)		
	# of samples	MC	MC+T	# of samples	MC	MC+T
Horticulture						
Roses	1	4	5	4	0	0
Hibiscus	4	8	10	14	30	23
Primula	2	42	14	18	646	706
Residue heap[1]	0	NA	NA	6	265	108
Agriculture (wheat)	8	197	0	112	728	0

MC: Malt + chloramphenicol; MC+T : Malt + chloramphenicol + tebuconazole; NA: not applicable; [1] The residue heap acts as a bioremediation location where left-overs of plant protection product (PPP) spray dilutions are poured in order to eliminate PPP waste.

Discussion

Agriculture, horticulture and composting facilities have been the subject of research on hotspots for azole resistance selection in Europe (Barber, Riedel, et al., 2020; Burks et al., 2021; Rocchi et al., 2021; Schoustra et al., 2019). However, the possible presence of ARAf from these environments has not yet been investigated in Belgium. In this pilot study, a total of 6 isolates, originating from horticulture and composting facilities, displayed phenotypic resistance against at least one medical azole and genetic mutations were present in the *cyp*51A gene. As such, we observed a prevalence of 2.62% (6/229) of ARAf. The isolates were deposited in the BCCM/IHEM collection under the following accession numbers: 28546, 28547, 28553, 28554, 28947, 28954 (https://bccm.belspo.be/about-us/bccm-ihem). We observed a prevalence of 66.7% (4/6) of the TR34/L98H mutation and 16.7% (1/6) of the TR46/Y121F/T289A mutation, which is comparable to what is reported in clinical cases in Belgium, with 83% and 13.87%, respectively (Resendiz-Sharpe et al., 2020). However, due care must be exercised with these values given the low number of isolates. Burks *et al*. reported a prevalence of 75% of the TR34/L98H mutation among resistant isolates worldwide and in Europe(Burks et al., 2021). One isolate did not display known resistance mutations in the *cyp*51A gene, which can be an indication of the presence of other resistance mechanisms (Camps, Dutilh, et al., 2012; Fraczek Mg et al., 2013; Hagiwara et al., n.d.; Resendiz-Sharpe et al., 2020; Wei et al., 2017).

The use of fungicides is essential to secure the food supply, as plant pathogenic fungi can cause crop loss of up to 30% (Jørgensen & Heick, 2021; Oerke, 2006; Savary et al., 2019). However, the development of medical azole resistance has been linked to the use of fungicides, amongst others in agriculture (Snelders et al., 2009; Verweij, Snelders, et al., 2009). Different classes of fungicides are used in cropland and in ornamentals, and azole fungicides display a very similar chemical structure and cause cross-resistance with medical azoles. In Germany, Barber *et al.* estimated the prevalence of ARAf in the environment and in the clinics to be 1.3% and 3.2% respectively (Barber, Riedel, et al., 2020). The environmental resistance rates reported vary greatly between European countries. Some studies reported almost no resistance, whereas others reported frequencies approaching 14% (Jeanvoine et al., 2017b; Mortensen et al., 2010; Sewell, Zhang, et al., 2019). These differences could be explained by the different nature of the samples. While they are all environmental samples, there is a difference in urban or rural settings, or a location with ornamental plants. This is supported by results published by Sewell *et al*. where rural areas displayed much lower resistance rates (1.1%) as compared to urban areas (13.8%) (Sewell, Zhang, et al., 2019).

In recent years there has been a growing interest in identifying hotspots for the development of ARAf. Compost of green waste and flower bulb waste have been identified as hotspots (Schoustra et al., 2019). In this pilot study, two commercial composting facilities were sampled. It was not possible to

determine neither the exact nature of the input material of the compost nor the level of azole residues or PGRs in it. Air samples inside the first composting facility did result in many *A. fumigatus* colonies, and two resistant strains carrying the TR34/L98H mutation were detected from the first composting facility. The obtained prevalence of *A. fumigatus* from the composting facilities, generally representing high-load environments, are highly likely to be underestimated in this study; the isolation of *A. fumigatus* was challenged by the presence of other fast-growing species such as *Mucorales* spp, which has been documented before (Viegas et al., 2022). These results of the prevalence rates of (azole resistant) *A. fumigatus* from the composting facilities thus need to be interpreted with caution. The use of the selective flamingo medium for the isolation of *A. fumigatus* could limit the risk of contamination (J. Zhang, Debets, et al., 2021).

Regarding the experimental cropland trials in this study, 128 soil samples and 8 air samples have been collected, resulting in 925 *A. fumigatus* colonies on the MC medium. However, no resistant *A. fumigatus* isolates were found, which is similar to the low prevalence of ARAf isolates described by other researchers in Germany, the United Kingdom, France and Italy (Barber, Riedel, et al., 2020; Godeau et al., 2023; Prigitano et al., 2014; Sewell, Zhang, et al., 2019). The absence of ARAf in the soil of agricultural crops has also been confirmed in root vegetables (van der Torre et al., 2020).The absence of ARAf in our samples may in part be due to the narrow timeframe of sampling from May to July 2022 covering only spring and the beginning of summer. Other researchers did observe ARAf in samples from cereal soils, but in low concentrations (Rocchi et al., 2020; Tsitsopoulou et al., 2018). Fraaije *et a*l. stated that even long-term azole-based foliar fungicide applications did not result in the selection of ARAf strains (Fraaije et al., 2020).

Fungicides, including azoles, are used in horticultural crops to decrease crop losses, but also to increase appearance and longevity (Jørgensen & Heick, 2021). Most research on ARAf has focused on flower bulb waste from DMI treated flower bulbs (Rocchi et al., 2020; Schoustra et al., 2019). A number of studies have found that compost made from flower bulb waste treated with DMIs represent a hotspot of ARAf (Rocchi et al., 2021; Schoustra et al., 2019). In the Netherlands, it has been reported that these waste piles produce a high number of spores and a prevalence of up to 24.5% ARAf has been detected in flower bulb waste (Schoustra et al., 2019). Although we did not study compost heaps of the sampled material that was treated with fungicides and growth inhibitors, we did sample the soil where all residues of the spraying liquid of fungicides was gradually released, thus representing a source of high concentrations of fungicides. Pan-azole resistant isolates bearing the TR34/L98H mutation were found in this soil. No *A. fumigatus* was detected in the substrate from the roses, which was explained by the different nature of the substrate for the roses, which was a coconut substrate rather than a soil substrate.

We are aware that this pilot study might have some limitations. The most important one is the inconsistency in sampling methods that complicated comparison of prevalence of ARAf in the different environmental settings. This is enforced by the disproportion of sample numbers in agriculture vs horticulture and composting facilities. These limitations highlight the need for clear guidelines on sampling methods in the surveillance of environmental (azole-resistant) *A. fumigatus*. Secondly, this pilot study lacks concentration measurements of azole residues in the soil and compost samples. Future research projects will include more elaborate sampling in horticulture and composting facilities of green waste alongside measurements of azole residues. Additionally, in the present study we focused on the agricultural and ornamental crops itself, rather than on their waste products. As we found several ARAf originating from ornamental crops, it would be of value to investigate the presence of ARAf in compost heaps originating from ornamental crops.

The World Health Organization's fungal priority pathogens list ranks *A. fumigatus* as a critical pathogen to guide research (World Health Organization, 2022). At EU-level (regulatory PPP approval under Reg. no (EC) 1107/2009), the potential of resistance forming as regards to efficacy is already a data requirement, and the issue of ARAf has recently been extended to the safety assessment of human health (Authority (EFSA) et al., 2023). A more general EFSA mandate to investigate the impact of the use of the azole fungicides, other than as human medicines, on the development of ARAf was formulated accordingly (European commission, 2022).

To fill existing knowledge gaps in this field, coordinated actions at national and international levels are essential. European standardised guidelines and protocols could contribute to harmonize environmental surveillance, resulting in a better understanding of the epidemiology of *A. fumigatus* resistance. We believe it is necessary to have a clear definition of a hotspot, as well as guidelines on how to measure them. For good management and surveillance, the waste streams within agriculture and horticulture, especially of fungicide-treated crops, must be mapped. These regulations should include a list of all environmental fungicide residues to quantify, including SDHIs, Q_oIs and DMIs such as azoles. Furthermore, studies at locations other than the agricultural and horticultural setting should be performed, e.g. sampling of residential gardens and in the hospital environment (Gonzalez-Jimenez et al., 2021; Shelton et al., 2022). Studies on ARAf in retail products are also scarce (Burks et al., 2021).

In conclusion, our pilot study reveals that azole-resistant *A. fumigatus* with an environmental background exists in Belgium, highlighting the significance of the One Health perspective to track the development of resistance and prevent its impact in both animal and human health. We report a prevalence of 2.62% of ARAf and the first occurrence of the TR34/L98H and the TR46/Y121F/T289A mutations in the c*yp*51A gene in isolates from horticulture and compost in Belgium. Future work should include more extensive sampling in compost and horticulture with azole residue measurements. Moreover,

standardization of environmental surveillance of *A. fumigatus*, is lacking and requires urgent developments in collaboration *e.g.* with public health authorities. Further work should include the identification and monitoring of the environmental hotspots and their drivers, mapping of the fungicide (not only azoles) use and their waste streams in agricultural and horticultural environments, as well as fungicide residue measurements.

Author contributions

Hanne Debergh (conceptualization, investigation, writing – original draft), Philippe Castelain (conceptualization, Writing – review & editing), Karine Goens (investigation), Paulien Lefevere (investigation), Jessie Claessens (investigation), Elien De Vits (investigation), Marc Vissers (Resources), Liesbet Blindeman (Resources), Charlotte Bataille (Resources), Claude Saegerman (PhD supervision, Writing – review & editing), Ann Packeu (conceptualization, funding acquisition, supervision, Writing – review & editing).

Conflict of interest

The authors declare no conflicts of interest.

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Supplemental material

Supplementary table 7 - All plant protection products, their concentrations and the quantity applied on the experimental wheat cropland

			Quantity			Quantity			Quantity	
N°	**Trade name**	**Active substance**	**(g/L)**	**(g/ha)**	**Active substance**	**(g/L)**	**(g/ha)**	**Active substance**	**(g/L)**	**(g/ha)**
1	Control1									
2	Eminent	tetraconazole	125.0	125.0						
3	Proline	prothioconazole	250.0	200.0						
4	Lenvyor	mefentrifluconazole	100.0	150.0						
5	Tebucur	tebuconazole	250.0	250.0						
6	Simveris	metconazole	90.0	90.0						
7	Narita	difenoconazole	250.0	125.0						
8	Soleil	tebuconazole	107.0	128.4	bromuconazole	167.0	200.4			
9	Kestrel	prothioconazole	160.0	200.0	tebuconazole	80.0	100.0			
10	Fandango	prothioconazole	100.0	150.0	fluoxastrobine	100.0	150.0			
11	Delaro	prothioconazole	175.0	175.0	trifloxystrobine	150.0	150.0			
12	Balaya	mefentrifluconazole	100.0	150.0	pyraclostrobine	100.0	150.0			
13	Simveris	metconazole	90.0	90.0						
	Flosul	sulfur	800.0	2400.0						
14	Simveris	metconazole	90.0	90.0						
	Vertipin	sulfur	700.0	2450.0						
15	Simveris	metconazole	90.0	90.0						
	Stavento	folpet	500.0	750.0						
16	Valpura Xpro	bixafen	125.0	125.0						
17	Revystar Gold	mefentrifluconazole	100.0	150.0	fluxapyroxad	50.0	75.0			
18	Revytrex	mefentrifluconazole	66.7	100.1	fluxapyroxad	66.7	100.1			
19	Librax	metconazole	45.0	90.0	fluxapyroxad	62.5	125.0			

		Quantity			Quantity			Quantity		
N°	**Trade name**	**Active substance**	**(g/L)**	**(g/ha)**	**Active substance**	**(g/L)**	**(g/ha)**	**Active substance**	**(g/L)**	**(g/ha)**
20	Aviator Xpro	prothioconazole	150.0	187.5	bixafen	75.0	93.8			
21	Siltra Xpro	prothioconazole	200.0	200.0	bixafen	60.0	60.0			
22	Velogy Era	prothioconazole	150.0	150.0	benzovindiflupyr	75.0	75.0			
23	Univoq	prothioconazole	100.0	150.0	fenpicoxamid	50.0	75.0			
24	Gigant	prothioconazole	150.0	150.0	isopyrazam	125.0	125.0			
25	Skyway Xpro	prothioconazole	100.0	125.0	bixafen	75.0	93.8	tebuconazole	100,0	125,0
26	Priaxor	fluxapyroxad	75.0	75.0	pyraclostrobine	150.0	150.0			
	Lenvyor	mefentrifluconazole	100.0	100.0						
27	Variano Xpro	prothioconazole	100.0	175.0	bixafen	40.0	70.0	fluoxastrobine	50,0	87,5
28	Ascra Xpro	prothioconazole	130.0	195.0	bixafen	65.0	97.5	fluopyram	65,0	97,5

Supplementary table 8 - All plant protection products and/or plant growth regulators, their concentrations and the quantity applied on hibiscus plants

	Trade name	Active substance	Concentration (mL/L)	Method	Quantity (= water volume)	Administration days
1	Vidi parva	75% seaweed extract	10.0	Pour on	100 mL/pot	A, B
			10.0	Foliar spray	10 L/are	C
2	Tebuphyt	250 g/l tebuconazole	8.0	Foliar spray	10 L/are	A, B
3	Fungaflash	10 g/l imazalil	20.0	Foliar spray	10 L/are	A
4	Eminent	125 g/l tetraconazole	7.5	Foliar spray	10 L/are	A, B, C
5	Geyser	250 g/l difenoconazole	5.0	Foliar spray	10 L/are	A, B, C
6	Geyser	250 g/l difenoconazole	5.0	Foliar spray	10 L/are	A, B, C
	Siltac SF	<75% silicone, siloxanes, polymers	1.0			
7	Geyser	250 g/l difenoconazole	5.0	Foliar spray	10 L/are	A, B, C
	Actirob B	92,8% esterified rapeseed oil	1.0			
8	Geyser	250 g/l difenoconazole	5.0	Foliar spray	10 L/are	A, B, C
	Bond	450 g/l synthetic latex, 100 g/l non-ionic wetting agent	1.0			
9,10,11,12	Water	none	none	Foliar spray	10 L/are	A, B, C

A = 7/05/2020, B = 15/05/2020 , C = 25/05/2020

Supplementary table 9 - All plant protection products and/or plant growth regulators, their concentrations and the quantity applied on primula plants

			Application days + concentration									
	Trade name	Active substance	A	B	C	D	E	F	G	H	I	J
1	Control		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2	Bumper 25 EC	250.0 g/L propiconazole	0.3 mL	0.5 mL	0.5 mL	0.5 mL	0.5 mL	0.5 mL	0.5 mL	0.5 mL	NA	0.5 mL
3	Alar	850.0 g/kg daminozide	3.0 g/L	3.0 g/L	3.0 g/L	3.0 g/L	3.0 g/L	3.0 g/L	3.0 g/L	3.0 g/L	NA	3.0 g/L
	Cycocel	750.0 g/l chlormequat	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	NA	1.0 mL
4	Caramba	60.0 g/kg metconazole	NA	0.5 mL/L	0.5 mL/L	0.5 mL/L	0.5 mL/L	0.5 mL/L	0.5 mL/L	0.5 mL/L	0.5 mL/L	0.5 mL/L
5	Alar	850.0 g/kg daminozide	3.0 g/L	3.0 g/L	3.0 g/L	3.0 g/L	3.0 g/L	3.0 g/L	NA	NA	NA	NA
	Cycocel	100.0 g/l chlormequat	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	NA	NA	NA	NA
	Caryx	30.0 g/l metconazole + 210.0 g/l chlormepiquat	NA	NA	NA	NA	NA	NA	1.5 mL	1.5 mL	NA	1.5 mL
6	Fungaflash	100.0 g/l imazalil	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	2.0 mL	2.0 mL	2.0 mL
	Siltac	polymers	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL
7	Medax top	300.0 g/l mepiquatchloride + 50.0 g/l prohexadione	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL
8	Tebuphyt	250.0 g/l tebuconazole	0.8 mL	0.8 mL	0.8 mL	0.8 mL	0.8 mL	0.8 mL	0.8 mL	0.8 mL	0.8 mL	0.8 mL
	Siltac	polymers	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL	1.0 mL
9	Terpal	305.0 g/l chlormepiquat + 155.0 g/l ethephon	3.5 mL	3.5 mL	3.5 mL	3.5 mL	3.5 mL	3.5 mL	3.5 mL	NA	NA	NA

A = 24/09/2020; B= 08/10/2020, C = 14/10/2020; D = 22/10/2020; E = 28/10/2020; F = 05/11/2020; G = 13/11/2020; H = 19/11/2020; I = 26/11/2020; J = 04/12/2020; NA = not applicable. All plants were treated using foliar sprays with a concentration of 10 L/are (water volume).

Supplementary table 10 - All plant protection and/or plant growth regulators, their concentrations and the quantity applied on roses

	Trade name	Active substance	Concentration (mL/L)	Method	Quantity (= water volume)
1	Fungaflash	100 g/L Imazalil	2.0	Foliar spray	10 L/are
2	Closer	120 g/l sulfoxaflor	0.2	Foliar spray	10 L/are

Supplementary table 11 - Primer sequences for the c*yp*51A gene and its promotor region of *Aspergillus fumigatus*

Primer	sequence
CYP 1F	TCATATGTTGCTCAGCGG
CYP 1R	TCTCTGCACGCAAAGAAGAAC
CYP 2F	CACTGCAACTCTAATCCTCG
CYP 2R	TAACGCAGACTGAGTCAAGC
CYP 3F	TTCGGATCGGACGTGGTGT
CYP 3R	CGCTGATGGACGAAGACGAA
CYP 4F	TGACGGTGACAAGGACTCTC
CYP 4R	ACAACCTCGTCGTTCTCCTG
CYP 5F	AGTCTTCCTCCGCTCCAGTA
CYP 5R	ACACCTATTCCGATCACACC

F = Forward primer; R = Reverse primer

Experimental section

Study 4

Pan-azole resistance in clinical *Aspergillus fumigatus* isolates carrying TR34/L98H from birds and mammals in Belgium

Preamble

Aspergillosis, caused by *Aspergillus fumigatus*, poses significant health risks to birds and mammals, leading to illness and death, particularly in immunocompromised animals. The study investigated *A. fumigatus* isolates from 152 cases of aspergillosis in birds and mammals in Belgium, with a focus on azole resistance. Among the isolates, 3.3% showed phenotypical resistance to medical azoles, with three pan-azole resistant strains carrying the TR34/L98H mutation, indicating environmental exposure as the primary route for resistance development. The findings highlight the need for enhanced surveillance and preventive measures in veterinary medicine to address azole resistance, which is a growing concern due to environmental exposure. The study underscores the importance of the One Health approach in managing fungal resistance across human, animal, and environmental health sectors.

Experimental section

Study 4

Pan-azole resistance in clinical *Aspergillus fumigatus* isolates carrying TR34/L98H from birds and mammals in Belgium

One Health 19 (2024) 100907

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Abstract

Aspergillosis causes significant health risks to both birds and mammals. The outcome of these infections is often poor due to delayed diagnosis and treatment failure. We investigated 152 cases of aspergillosis from birds and mammals in Belgium. Most samples originated from the taxonomic orders Artiodactyla (40.1 %) and Columbiformes (19.7 %). Five isolates (3.3 %) showed phenotypical resistance against at least one medical azole. Three of these isolates were pan-azole resistant bearing the TR34/L98H mutation. The predominance of this resistance mutation supports an environmental route for exposure and resistance selection, highlighting the importance of the One Health concept.

Introduction

Aspergillosis, primarily caused by the fungus *Aspergillus fumigatus*, poses a significant health risk to both birds and mammals, leading to illness and death. Aspergillosis in birds is quite common and affects birds of all ages and environments. In comparison, aspergillosis is less prevalent in mammals, regardless of the increasing number of immunocompromised animals(Tell, 2005). Exceptions include canine sinonasal aspergillosis, equine guttural pouch mycosis and bovine mycotic abortion caused by *Aspergillus* spp (Dobesova et al., 2012; Elad & Segal, 2018; Tell, 2005). Predisposing factors for the development of aspergillosis in birds and mammals consists of immunosuppression, previous debilitating illnesses, stress factors and environmental factors (Higgins & Pusterla, 2006; U. P. Melo et al., 2024; Tell, 2005). These latter factors can consist of contaminated feed, soil and bedding or poor husbandry such as poor ventilation, high humidity, and warm temperatures (Tell, 2005)**.** The mode of entry is presumed to be oropharyngeal during inhalation, as such the most frequently affected areas are the head region for mammals, and lungs and air sacs in birds (Tell, 2005). Diagnosis often occurs late and diagnostic tools are scarce, inaccurate or expensive, which limits the chance of timely treatment (Desoubeaux et al., 2022). Additionally, because of the concurrent severe underlying diseases in mammals, treatment or prophylaxis is challenging (U. P. Melo et al., 2024). The emergence of azole resistance in *A. fumigatus* in the clinics and the environment has become a major concern. This emergence of resistance also creates additional difficulties for the treatment of aspergillosis in veterinary medicine (Beernaert et al., 2009; Debergh et al., 2023; Martinez et al., 2022; Ziołkowska et al., 2014). The pathogen *A. fumigatus* is a perfect example of the interconnectedness of human, animal, and environmental health that are taken into account in the framework of the OneHealth approach (Panel (OHHLEP) et al., 2022). In this study we aimed to contribute to the understanding of the occurrence of azole resistance in veterinary aspergillosis caused by in *A. fumigatus* in Belgium in the OneHealth perspective.

Materials and methods

Between 01/2020 and 01/2024, 152 cases of animal aspergillosis caused by *A. fumigatus* were identified by Zoolyx (n = 70), Animal health care Flanders (n = 60), Ghent University (n = 15), the Regional Association for Animal Health and Identification Wallonia (ARSIA) (n = 4) and the Scientific Department of Avian Virology and Immunology of Sciensano (n = 3). Diagnosis of aspergillosis was based on macroscopic lesions suspected of aspergillosis, detection of fungal hyphae during histological examination, and isolation and identification via culture of the fungus of the affected organs. Necropsy was performed on 131 animals (86.2%). Cases were identified based on fungal morphology after three to five days of growth on malt chloramphenicol (0.5%; MC) at 37°C and confirmed using MALDI-TOF MS identification (A. C. Normand et al., 2017).

Data on antibiotic and antifungal treatment were available for 70 animals (46.05 %), however no details on duration or which treatment was used was available. Out of these, 44 (62.86 %) received antibiotic treatment, whereas only 2 (2.86 %) were treated with antifungals. No data were available for 82 animals (53.9 %).

Azole susceptibility testing was performed on all isolates (n = 152) using the broth microdilution method following EUCAST guidelines (E. Def. 9.4) to determine the minimum inhibitory concentrations (MICs) to the medical azoles voriconazole, itraconazole, isavuconazole and posaconazole. In the absence of veterinary breakpoints for predicting the clinical response to therapy in mammals and birds, human clinical breakpoints were used (EUCAST v10.0). Sanger sequencing of the *cyp51A* gene, the target gene of the azoles, was performed on the isolates showing phenotypical azole resistance.

Results

All isolates were confirmed as *A. fumigatus* by MALDI-TOF MS. *A. fumigatus* isolates were cultured from various animal species and infection sites. Among the avian and mammalian species, the taxonomic orders Artiodactyla (n = 61, 40.1%) and Columbiformes (n = 30; 19.7%) were respectively the most frequently observed, followed by Perissodactyla (n = 13, 8.6%), Charadriiformes (n = 12, 7.9%), Psittaciformes (n = 7, 4.6%), Carnivora (n = 7, 4.6%) and Passeriformes (n = 6, 3.9%) (Table 15). Most individuals were production animals (n = 62, 40.8%), all belonging to Artiodactyla, while 60 other animals were pets (39.5%), including 29 pigeons, 13 horses, 6 dogs, 3 grey parrots, 2 macaws, 1 Rosella parrot and one cat. The cases also included 18 wild birds (11.8%) consisting of 10 common guillemots (*Uria aalge*), one razorbill (*Alca torda*), one Eurasian collared dove (*Streptopelia decaocto*), one Red-throated loon (*Gavia stellata*), one great crested grebe (*Podiceps cristatus*), one northern gannet (*Morus bassanus*) and two other *Passeriformes.* Twelve birds (7.9%) were sent from zoos and included four Humboldt penguins (*Spheniscus humboldti*), common scoter (*Melanitta nigra),* one freckled duck *(Stictonetta naevosa)* and a tree duck (subfamily Dendrocygninae), one swift parrot (*Lathamus discolor*), one squacco heron (*Ardeola ralloides*), one Bird-of-paradise (family *Paradisaeidae*), one cinereous vulture (*Aegypius monachus*) and one crested oropendola (*Psarocolius decumanus*) (Table 15).

In total, 88 respiratory samples (57.9 %) and 50 samples from the digestive system (32.9 %) were taken. Out of the 88 respiratory samples, 64 (72.7%) originated from avian species, whereas 48/50 samples (96.0 %) from the digestive tract were obtained from cattle. Additionally, six samples were obtained from the reproductive system (four from the uterus and two from the placenta) and eight originated from various other sources: abscess, ear, skin, kidney, pericardium swab, ear canal, tongue swab and a foot abscess (Table 15).

Table 15 – Number and taxonomy of animals with veterinary aspergillosis according to their origin and sample type

Taxonomic order		Origin				Sample type				Necropsy
		Pet	Production	Wild	Zoo	Digestive system	Reproduction system	Respiratory system	Other*	
Avian (n = 71)	Accipitriformes (n = 1)	0	0	0	1	0	0	1	0	1
	Anseriformes (n = 4)	1	0	0	3	0	0	3	1	3
	Charadriiformes (n = 12)	0	0	12	0	0	0	12	0	12
	Columbiformes (n = 30)	29	0	1	0	0	0	27	3	30
	Galliformes (n = 3)	2	1	0	0	0	0	2	1	3
	Gaviiformes (n = 1)	0	0	1	0	0	0	1	0	1
	Passeriformes (n = 6)	2	0	2	2	2	0	4	0	6
	Pelecaniformes(n = 1)	0	0	0	1	0	0	1	0	1
	Podicipediformes (n = 1)	0	0	1	0	0	0	1	0	1
	Psittaciformes (n = 7)	6	0	0	1	0	0	7	0	7
	Sphenisciformes (n = 4)	0	0	0	4	0	0	4	0	3
	Suliformes (n = 1)	0	0	1	0	0	0	1	0	0
Mammal (n= 81)	Artiodactyla (n = 61)	0	61	0	0	48	3	9	1	60
	Carnivora (n = 7)	7	0	0	0	0	0	5	2	1
	Perissodactyla (n = 13)	13	0	0	0	0	3	10	0	2
Total (n = 152)		60 (39.5 %)	62 (40.8 %)	18 (11.8 %)	12 (7.9 %)	50 (32.9 %)	6 (3.9 %)	88 (57.9 %)	8* (5.3 %)	131 (86.2%)

* other sample types include: abscess, ear, skin, kidney, pericardium swab, ear canal, tongue swab and foot abscess.

MIC testing identified five isolates (3.3 %) that showed phenotypical resistance against at least one medical azole. No elevated MIC values were present in the other isolates (n = 147) for all four medical azoles, showing no evidence of acquired resistance in avian or mammalian populations (Figure 41). Azole resistance was *cyp51A* mediated in 80% of resistant isolates.

Table 16 – Minimal inhibitory concentration against medical azoles data and their related *cyp51A* gene sequencing result.

Accession number	Species	Sample type	Year of isolation	MIC (mg/L)[1]				*Cyp51A* mutations
				VOR	ITC	ISA	POS	
28429	Cow	Digestive	2020	**2**	0,5	2	**0,5**	F46Y, M172V, E427K, N248T, D255E
28550	Cow	Digestive	2020	**4**	**>16**	**8**	**1**	TR34/L98H
28552	Cat	Ear	2021	**8**	1	2	**2**	no mutation
29030	Pigeon	Respiratory	2023	**4**	**>16**	**8**	**1**	TR34/L98H
29031	Pigeon	Respiratory	2023	**4**	**>16**	**8**	**1**	TR34/L98H

MIC = Minimal inhibitory concentration; VOR = voriconazole; ITC = itraconazole, ISA = isavuconazole ; POS = posaconazole. MIC was determined following the EUCAST method for susceptibility testing of moulds (version 9.4). Numbers in bold represents phenotypical resistance according to EUCAST clinical breakpoints for fungi v10.0 (EUCAST, 2020; Guinea et al., 2022). The isolates were deposited in the BCCM/IHEM collection under the mentioned accession numbers **(https://bccm.belspo.be/about-us/bccm-ihem).**

Among the avian isolates, two azole resistant *A. fumigatus* (ARAf) isolates (2/71, 2.8 %) were isolated from birds living in captivity (pigeons). Both birds received antibiotic treatment but no antifungal treatment. No details were provided about the treatment regimen. They displayed the pan-azole resistant phenotype and carried the TR34/L98H mutation in the *cyp51A* gene (Table 16).

The remaining three ARAf isolates (3/81, 3.7 %) were isolated from mammals: 2 cows and one cat. The cat (IHEM 28552) received antibiotic and antifungal treatment and displayed resistance to voriconazole, isavuconazole and posaconazole. This *A. fumigatus* strain was isolated from an infected ear of a cat. One isolate (IHEM 28550) originating from cattle displayed the pan-azole resistant phenotype and carried the TR34/L98H mutation. The other isolate (IHEM 28429) displayed resistance to posaconazole but was susceptible to itraconazole. Regarding voriconazole and isavuconazole, the MIC value was in the area of technical uncertainty (ATU). The isolate displayed the F46Y, M172V, E427K, N248T, D255E polymorphism. These polymorphisms have been detected in both azole resistant as azole susceptible isolates (Chowdhary et al., 2017).

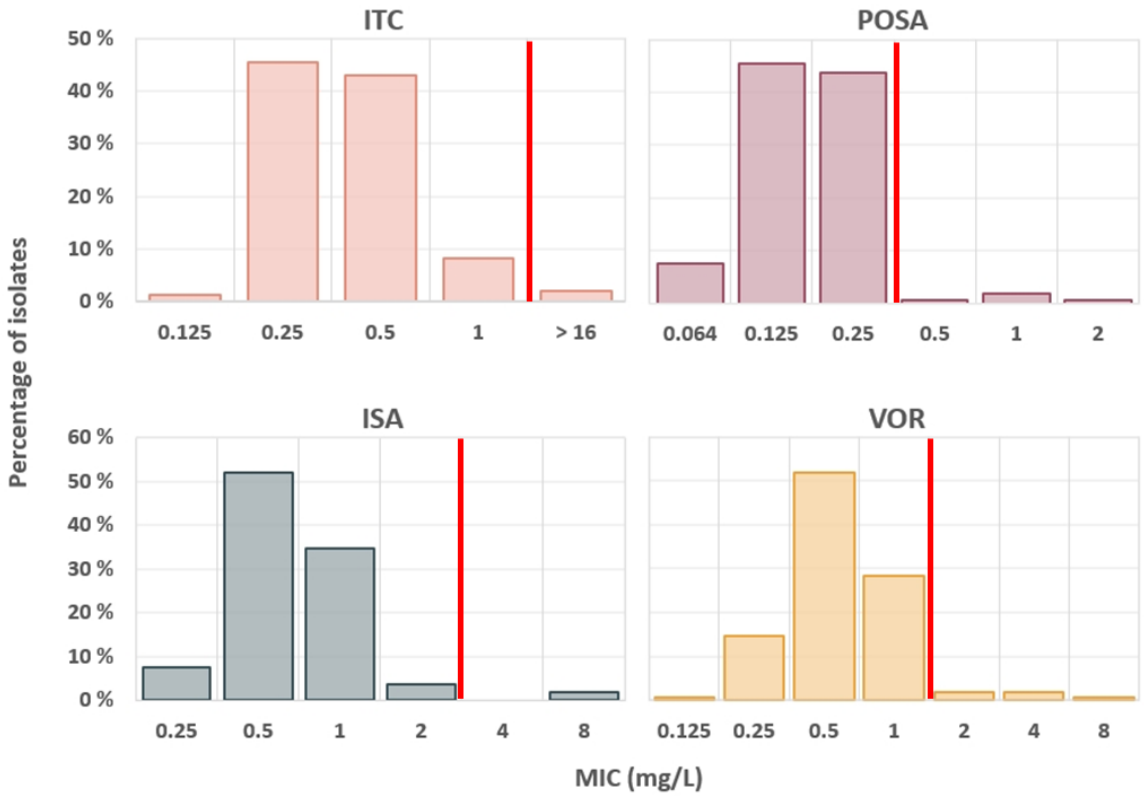

Figure 41 - Azole susceptibility in avian and mammalian *Aspergillus fumigatus* isolates (n = 152). Minimal inhibitory concentration (MIC) values for itraconazole (ITC), posaconazole (POSA), isavuconazole (ISA), and voriconazole (VOR) were determined using broth microdilution following EUCAST protocol E.DEF 9.3.2 (Arendrup, Meletiadis, et al., 2020, p. 9). No isolates displayed itraconazole MIC values of 2, 4, or 8; therefore, these values are not shown in the graph. The red line represents the clinical breakpoint (EUCAST v10.0).

Discussion

This study showed the presence of pan-azole resistant *A. fumigatus* isolates in veterinary cases. Scarce data is available on azole resistance frequency in *A. fumigatus* from animals. However, azole resistance in veterinary medicine might be an increasing concern due to the rise of azole resistance in the environment, since infection generally has an environmental source in veterinary aspergillosis (Tell, 2005). Here, we observed an overall resistance rate of 3.3%, with resistant isolates found in birds (2.8 %) and in mammals (3.7 %). The prevalence of resistance in isolates from birds was higher than in other studies on avian aspergillosis (Barber, Scheufen, et al., 2020; Nawrot et al., 2019), but lower than described in Humboldt penguins in Belgium (Debergh et al., 2023). An overall prevalence of ARAf of 11.3% was observed in veterinary clinical isolates in the Netherlands, which is similar to the frequency observed in humans in the Netherlands (van Dijk et al., 2024). The difference in ARAf prevalence in animals between Belgium and the Netherlands might be explained by the presence of hotspots and the consequently higher environmental prevalence of ARAf in the Netherlands (Schoustra et al., 2019), compared to Belgium's 2.6 %(Debergh et al., 2024). A Belgian surveillance program at the tertiary care center, University Hospitals Leuven, assessed the prevalence of triazole resistance in *A. fumigatus* from complex culture-positive patients in clinical isolates from 2016 to 2020. The surveillance revealed triazole resistance prevalence rates of 8.3%, 6.7%, 7.0%, 7.1%, and 7.4% for the years 2016 – 2020 respectively (Resendiz-Sharpe et al., 2021). In comparison, in the same period, a Dutch national surveillance program reported significant higher triazole resistance prevalence rates of 12.9%, 14.7%, 10.5%, 9.1% and 8.2% from 2016 to 2020 respectively (two-sample Wilcoxon rank-sum test; p-value = 0.016 (van Dijk et al., 2024).

The observed overall occurrence of resistance was lower than the prevalence reported in human cases in Belgium (Resendiz-Sharpe et al., 2021). Caution is however needed when interpreting the prevalence of resistance data, as these samples were not derived from a systematic monitoring program, potentially introducing bias. Additionally, no clinical breakpoints currently exist for *A. fumigatus* in veterinary medicine. Clinical breakpoints used in human medicine are often applied to interpret susceptibility patterns in animals, however, the predictive value of susceptibility data might be low due to the different anatomical and physiological characteristics (Elad & Segal, 2018). Nevertheless, applying these clinical breakpoints can help indicate the potential presence of *cyp51A* mutations. In this study, 80% of the resistance mutations was related to the *cyp51A* gene, which is in line with the Belgian human population (Resendiz-Sharpe et al., 2021). One isolate did not display any mutation in the *cyp51A* gene, but other mechanisms that confer azole resistance in *A. fumigatus* exist and are known to circulate in the environment at low frequencies (Sharma et al., 2019). The presence of the TR34/L98H mutation in the veterinary isolates, which knows its origin in the environment, suggests a possible link with the environment from which the animals could have inhaled the resistant spores (Snelders et al., 2009). Here, we did not observe single nucleotide polymorphisms (SNPs) in the *cyp51A* gene, which are

generally related to development of resistance through prolonged treatment. Although the current study was restricted by the limited data provided concerning the antibiotic or antifungal treatment regimens, this suggests that, similar to human medicine, the environmental route is dominant in veterinary medicine.

In this study, all isolates were identified as *A. fumigatus* by MALDI-TOF MS. We acknowledge the limitation that species-level identification was not further confirmed by sequencing. However, several studies indicate a very low prevalence of cryptic species, with *A. fumigatus* sensu stricto remaining the predominant causal agent within the *Fumigati* section in birds (A. M. Melo, Silva-Filho, et al., 2020; Sabino et al., 2019). For instance, Berber et al. and Sabino et al. reported no cryptic species (Barber, Scheufen, et al., 2020; Sabino et al., 2019), while Cateau et al. identified only one cryptic species (*A. nishimurae*) (Cateau et al., 2022).

Generally, treating aspergillosis in animals presents challenges and is associated with poor outcomes (Dobesova et al., 2012; Tell, 2005). The treatment of aspergillosis in animals is often prolonged, and assessing its effectiveness can be challenging. Moreover, in some cases, treatment is no longer viable due to delayed diagnosis. If infection with an azole-resistant *A. fumigatus* strain is suspected, alternative antifungal options such as liposomal amphotericin B or caspofungin may be considered (Olson et al., 2010). Considering the increasing rates of azole resistance in the environment and the risk of animal infections through the inhalation of airborne conidia, raising awareness among veterinarians is essential. Prevention through measures such as avoiding the inhalation of conidia by maintaining mold-free husbandry practices or feed is crucial. Do Nascimento et al. found that introducing *A. fumigatus* into waste piles from horse stables can facilitate composting and decrease total coliforms (do Nascimento et al., 2024). However, while the transmission of *A. fumigatus* from infected animals to humans is improbable, the potential for a high density of environmental *Aspergillus* conidia in animal housing due to this practice may elevate the risk of inhalation for both horses and humans, posing a significant threat of invasive infections, particularly in hematological and immunocompromised patients (Cavallo et al., 2013; Seyedmousavi et al., 2015).

Our study demonstrates that azole resistance is present in clinical *A. fumigatus* isolates from birds and mammals in Belgium at a similar frequency as observed in the environment. The predominance of *cyp51A* TR34/L98H resistance mutation supports an environmental route for resistance selection. With the growing concern of azole resistance in both the environment and human medicine, further investigation into azole resistance in veterinary medicine is imperative for implementing effective control measures and maintaining the efficacy of antifungal treatments in veterinary practice.

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Author contributions:

Hanne Debergh (conceptualization, investigation, writing – original draft), Roel Haesendonck (Resources), Nadine Botteldoorn (Resources), An Martel (Resources), Frank Pasmans (Resources), Claude Saegerman (PhD supervision, Writing – review & editing), Ann Packeu (conceptualization, funding acquisition, supervision, Writing – review & editing). All authors read and approved the final manuscript.

Conflict of interests

The authors declare no conflicts of interest.

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Experimental section

Study 5

Pulmonary Aspergillosis in Humboldt Penguins—Susceptibility Patterns and Molecular Epidemiology of Clinical and Environmental *Aspergillus fumigatus* Isolates from a Belgian Zoo, 2017–2022

Preamble

The study investigated the prevalence of *A. fumigatus* contamination and azole-resistant strains in the environment of Humboldt penguins at a Belgian zoo. Environmental samples (air, water, sand, and nests) and clinical samples from deceased penguins and other birds were analyzed for fungal contamination and azole resistance.

Between 2017 and 2022, pulmonary aspergillosis was confirmed in 51 of 75 necropsied penguins, resulting in a high mortality rate of 68%. In 2017 and 2018, mortality from aspergillosis in Humboldt penguins was exceptionally high, reaching 86.7% and 76.5%, with most deaths occurring during the hot summer of 2018. Overcrowding and an active breeding program likely contributed to the increased incidence. Measures such as improved ventilation, monitoring water temperatures, and pausing the breeding program were implemented in 2019 to reduce mortality. However, when the breeding program resumed, aspergillosis cases rose again, particularly affecting adult penguins rearing young, suggesting that immune suppression may play a role in the chronic form of the disease.

The study revealed that environmental contamination with *A. fumigatus* was consistent throughout the seasons, though slightly higher in spring and summer. Azole resistance, largely driven by environmental exposure to agricultural azoles, presents a growing threat to both animal and human health under the One Health framework. Of the samples collected between 2021 and 2022, 14 azole-resistant *A. fumigatus* (ARAF) isolates were detected, with the TR34/L98H mutation found in both environmental and veterinary isolates, marking the first report of this mutation in penguins.

Microsatellite genotyping demonstrated significant genetic diversity among environmental and veterinary strains, but some clustering indicated potential transmission between penguins and their environment. However, no direct evidence of bird-to-bird transmission was found. This study highlights the importance of monitoring *A. fumigatus* and azole resistance in captive environments, especially under changing global conditions such as climate change.

Experimental section

Study 5

Pulmonary Aspergillosis in Humboldt Penguins—Susceptibility Patterns and Molecular Epidemiology of Clinical and Environmental *Aspergillus fumigatus* Isolates from a Belgian Zoo, 2017–2022

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Abstract

Aspergillus fumigatus is the main causative agent of avian aspergillosis and results in significant health problems in birds, especially those living in captivity. The fungal contamination by *A. fumigatus* in the environment of Humboldt penguins (*Spheniscus humboldti*), located in a Belgian zoo, was assessed through the analysis of air, water, sand and nests samples during four non-consecutive days in 2021-2022. From these samples, potential azole-resistant *A. fumigatus* (ARAF) isolates were detected using a selective culture medium. A total of 28 veterinary isolates obtained after necropsy of Humboldt penguins and other avian species from the zoo were also included. All veterinary and suspected ARAF isolates from the environment were characterized for their azole-resistance profile by broth microdilution. Isolates displaying phenotypic resistance against at least one medical azole were systematically screened for mutations in the *cyp51A* gene. A total of 14 (13.6%) ARAF isolates were identified, from the environment (n = 8) and from Humboldt penguins (n = 6). The TR34/L98H mutation was observed in all resistant environmental strains, and in 2 resistant veterinary strains. To the best of our knowledge, this is the first description of this mutation in *A. fumigatus* isolates from Humboldt penguins. During the period 2017-2022, pulmonary aspergillosis was confirmed in 51 necropsied penguins, which reflects a death rate due to aspergillosis of 68.0%, mostly affecting adults. Microsatellite polymorphism analysis revealed a high level of diversity among environmental and veterinary *A. fumigatus* isolates. However, a cluster was observed between 1 veterinary isolate and 6 environmental strains, all resistant to medical azoles. In conclusion, the environment of the Humboldt penguins is a potential contamination source of ARAF, making their management even more complex.

Keywords: *Aspergillus fumigatus,* Avian aspergillosis; *Spheniscus humboldti*; Antifungal susceptibility testing, MIC, azole resistance, *cyp51A*, microsatellite typing, genotyping, One Health

Introduction

The saprophytic fungus *Aspergillus fumigatus* is responsible for opportunistic infections affecting birds and mammals, including humans (Seyedmousavi et al., 2015). It can affect a wide variety of species such as domestic, free-ranging or captive wild animals (Arné et al., 2021). Aspergillosis is the most common fungal infectious disease affecting penguins in zoos, with up to 99% of cases attributed to *Aspergillus* section *Fumigati*. It represents a major limiting factor for the rehabilitation of penguins in captivity (Beernaert et al., 2010; Seyedmousavi et al., 2015; Silva Filho et al., 2015b; Stidworthy, n.d.; Xavier et al., 2007). On the contrary, aspergillosis in free-ranging birds is only rarely described (Ewbank et al., 2021; Graczyk & Cockrem, 1995).

The most common route of infection is by inhalation of conidia present in the environment (Arné et al., 2021; A. M. Melo, Stevens, et al., 2020). Due to their small size, the spores can bypass the mucociliary clearance by the upper airways (Arné et al., 2021; A. M. Melo, Stevens, et al., 2020). As such, they disseminate first in the posterior air sacs where ideal temperature and oxygen availability conditions are present to allow their germination and the production of hyphae (Arné et al., 2021). The important susceptibility of birds, and especially penguins, to aspergillosis also results from the scarcity of immune surveillance cells in the air sac system, and the lack of an epiglottis or diaphragm to block the inhalation of the spores (Lofgren et al., 2022; Tell, 2005). In addition to these predisposing factors, external factors including thermal discomfort, overcrowding or stress also contribute to the high incidence of avian aspergillosis in birds in captivity (Arné et al., 2021; Cateau et al., 2022; Ewbank et al., 2021; A. M. Melo et al., 2021; Reed et al., 2020), as compared to free-ranging birds (Smith et al., 2008). The exposure to high concentrations of *Aspergillus* conidia are presumed to further enhance the occurrence of aspergillosis. High loads of conidia are considered seasonal and influenced by climatic parameters such as temperature, humidity and wind speed (Arné et al., 2021; Sautour et al., 2009; van Rhijn et al., 2021). Furthermore, avian aspergillosis can be acute or chronic, with the former primarily related to young birds and the latter mostly associated with adult animals displaying various levels of immunosuppression, due to external stressors or poor husbandry (Lofgren et al., 2022).

Avian aspergillosis can act as a primary infection, affecting mainly the respiratory system. However, infections of the eyes, liver, kidneys, heart, joints and bones have also been described (Beernaert et al., 2010; Leishangthem et al., 2015). Symptoms associated with aspergillosis in birds are non-specific and include lethargy, weight-loss and anorexia, open-beak breathing and dyspnea, coughing, altered vocalization and self-isolation. Moreover, diagnostic tools are not highly reliable and ante mortem diagnosis is thus difficult to make (Arné et al., 2021; Cabana et al., 2019; Cray et al., 2009; Desoubeaux et al., 2018; German et al., 2002). Therefore, a confirmed diagnosis is generally obtained post mortem (Fischer & Lierz, 2015; Savelieff et al., 2018). Treatments often have a poor outcome due to the late diagnosis and the resulting advanced stage of the disease (Beernaert et al., 2010). However, if the disease is suspected, treatment can be initiated with oral administration of itraconazole (ITC)

(Bunting et al., 2009). Prophylaxis with ITC is usually applied for susceptible individuals, e.g. following transfers or antibiotic treatments (Arné et al., 2021; Bunting et al., 2009). The One Health concept aims to balance and optimize the health of people, animals and the environment by unifying the topics (*One Health | CDC*, 2023). Increasing rates of azole resistance in *A. fumigatus* in human medicine are observed (Resendiz Sharpe et al., 2018). Considering the One Health concept, animals can also be infected by resistant strains. Antifungal treatments in veterinary medicine should be used with care and monitoring of antifungal resistance profiles of veterinary *A. fumigatus* isolates should be adopted (A. M. Melo, Stevens, et al., 2020; Verweij et al., 2020).

A. fumigatus is considered as an emerging health threat and was placed in the fungal priority pathogens list by the World Health Organization (WHO) (World Health Organization, 2022), highlighting the importance of its monitoring in human medicine, but also in veterinary health. In a recent five-year study from a tertiary care center in Belgium, an overall prevalence of 7.1% azole-resistance in *A. fumigatus* was detected (Resendiz-Sharpe et al., 2021). Acquired azole resistance is mostly associated with mutations in the *cyp51A* gene, encoding for lanosterol-C14-α-demethylase (Resendiz Sharpe et al., 2018; Snelders et al., 2009). Other resistance mechanisms exist, such as mutations in *hmg1*, *cdr1B*, *HapE* or overexpression of efflux pumps (Camps, Dutilh, et al., 2012; da Silva Ferreira et al., 2006; Fraczek Mg et al., 2013; Resendiz-Sharpe et al., 2020, p. 1; Sharma et al., 2019). The intensive use of agricultural azole antifungals has been linked to the development of azole resistance against medical antifungals (Burks et al., 2021; Snelders et al., 2009). The surveillance of azole resistance in *A. fumigatus* in clinical strains is a common practice, however, this is not the case for avian aspergillosis with only few epidemiological studies published (Barber, Scheufen, et al., 2020; Beernaert et al., 2010; Cateau et al., 2022; A. M. Melo et al., 2021). In this respect, the study of the genetic diversity in *A. fumigatus* is a valuable tool to better understand the transmission routes involved. This study aimed to assess the impact of environmental *A. fumigatus* contamination on the clinical incidence of avian aspergillosis in Humboldt penguins in a Belgian zoo. The susceptibility pattern towards medical azoles and the mutations in the *cyp51A* gene were studied, alongside the genotyping of the strains, in order to estimate the epidemiology of the infections.

Results

1 Environmental *Aspergillus fumigatus* sampling inside the penguin enclosure

Cultures obtained on malt extract + chloramphenicol (MC) medium from environmental air sampling at four pre-defined locations (Figure 42) revealed a mean *A. fumigatus* burden in spring and summer of 41.5 and 56.5 CFU/1000 L, respectively, and 15.75 and 22.75 CFU/1000 L in autumn and winter, respectively . These differences were however not significant and the overall A. *fumigatus* contamination (log_{10} CFU/1000L) statistically similar between the different study time points for all four study locations (p-value = 0.27) (Figure 43). All *A. fumigatus* colonies isolated from malt extract + chloramphenicol + 4 mg/L tebuconazole (MC+T) medium (n = 68) were stored and further analyzed in this study. All isolates were confirmed as *A. fumigatus* using MALDI-TOF MS with a score ≥ 2.0. The samples from sand, water and nest swabs were negative for *A. fumigatus*.

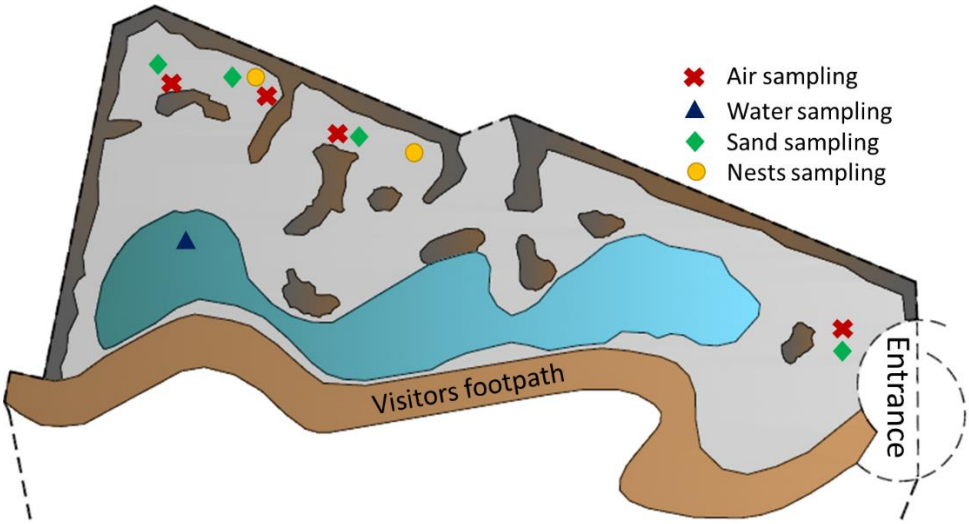

Figure 42 - Map of the Humboldt penguin enclosure. Dark brown represents artificial rock formations with 25 built-in nests in total. Light brown represents the visitors pathway. Gray represents the sand. Four sample types were taken: red cross = air sample; blue triangle = water; green diamond = sand; yellow circle = nest swab.

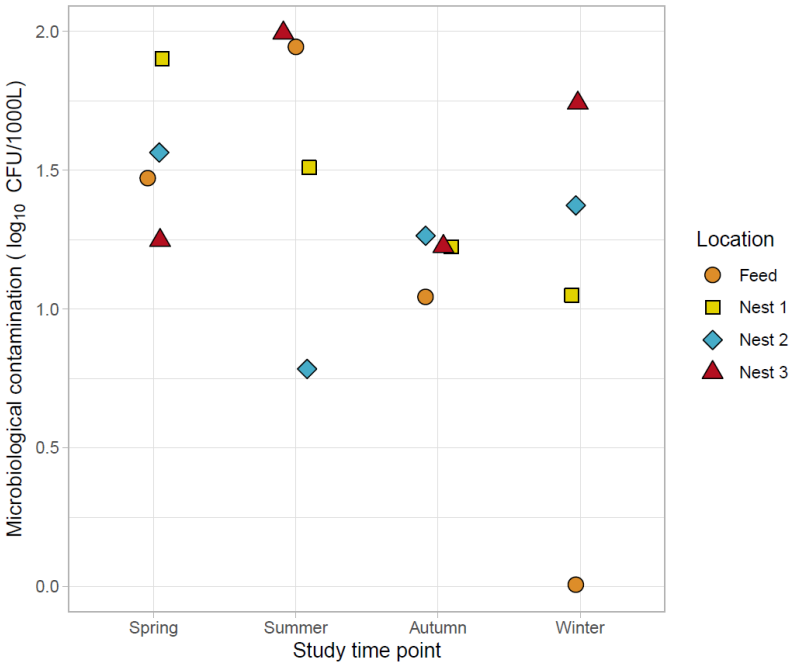

Figure 43 - Environmental analysis in the enclosure of the Humboldt penguins. Scatter plot showing the microbiological contamination of *A. fumigatus* in air samples (log_{10} CFU/1000L air) grown on MC medium. Samples were taken during four non-consecutive study days covering all seasons, in four different locations (feeding stage and 3 locations near the nests).

2 Clinical incidence of avian aspergillosis in Humboldt penguins in a Belgian zoo

Since the start of the Humboldt penguin program in 2013, a total of 214 animals were counted, of which 189 have died (88.3%) until December 2022. Clinical samples from 2017-2022 were included in the study. During this 6-year period, 96 Humboldt penguins died and necropsy was performed on 75 carcasses (78.1%) (Table 1). Necropsy was not performed on the remaining animals due to alternative causes of death (rotten in nests at few days old or trampled). In 2018, the highest death rate was observed, with 37 dead animals (37/78; 47.4%) (Table 1). Out of them, 34 were necropsied and in 26 cases, pulmonary aspergillosis was confirmed as the cause of death (26/34; 76.5%). Over the course of 6 years, pulmonary aspergillosis was confirmed in 51 necropsied penguins, which reflects a death rate due to aspergillosis of 68.0% (51/75). Five of the penguins included in the study received ITC at 20mg/kg per day as treatment for an extended period of time (Table 18). The amount of breeding couples in 2017 and 2018 was not known. In 2019 and 2022 no breeding program was initiated, whereas in 2020 and 2021, respectively, four and eight breeding couples were present.

Table 17 - Population information of the Humboldt penguins in a Belgian zoo from 2017 to 2022.

Year	Living animals	Dead animals	Death < 2 months old	Mortality rate (%)	Necropsy	# confirmed aspergillosis	Aspergillosis (%)*	Incidence (%)**
2017	99	27	11	27/99 (27.3)	15	13	86.7	13.1
2018	78	37	3	37/78 (47.4)	34	26	76.5	33.3
2019	41	2	0	2/41 (4.9)	2	1	50.0	2.4
2020	45	6	2	6/45 (13.3)	4	1	25.0	2.2
2021	52	20	4	20/52 (38.5)	16	7	43.8	13.5
2022	28	4	0	4/28 (14.3)	4	3	75.0	10.7

*Percentage of aspergillosis was calculated as number of confirmed pulmonary aspergillosis cases per number of necropsies.
**Mortality incidence directly attributable to aspergillosis was calculated as number of confirmed aspergillosis cases per number of living animals.

A. fumigatus isolates (n = 35) were obtained from Humboldt penguins (n = 29), South African penguin (*Spheniscus demersus*) (n = 1), Red-billed blue magpie (*Urocissa erythrorhyncha*) (n = 1), Chilean flamingo (*Phoenicopterus chilensis*) (n = 1), Chestnut-backed thrush (*Geokichlia dohertyi*) (n = 1), Crested oropendola (*Psarocolius decumanus*) (n = 1) and Crested partridge (*Rollulus rouloul*) (n = 1) that died between 2017 and 2022. A total of 66% of those isolates were obtained from 2018 (23/35). Other isolates were obtained in 2017 (n = 2) , 2019 (n = 1), 2020 (n = 2), 2021 (n = 6) and 2022 (n = 1).

Table 18 - Demography of the bird specimens included in the study, and minimum inhibitory concentration towards antifungal drugs of the *A. fumigatus* strains isolated from the autopsied bodies. 1

ID *A. fumigatus* strain	VOR	ITC	ISA	POSA	Host species	Date of death	Age	Treatment since**
21-0659	1	0.5	0.5	0.125	Humboldt penguin	28/12/2017	16y10m5d	N/A
21-0660	1	0.25	1	0.25	Humboldt penguin	29/12/2017	20y7d	N/A
21-0029	0.25	0.25	0.25	0.125	Humboldt penguin	12/11/2018	11y 5m 18d	N/A
21-0030	0.5	0.25	0,5	0.125	Humboldt penguin	12/12/2018	11y 5m 18d	N/A
21-0661	1	0.5	1	0.25	Humboldt penguin	1/01/2018	20y9m23d	N/A
21-0662	4*	>16*	4*	0.5*	Humboldt penguin	26/02/2018	8y9m27d	16/02/2017
21-0663	1	0.5	0.5	0.25	Humboldt penguin	2/03/2018	9m7d	N/A
21-0664	0.5	0.25	0.5	0.25	Humboldt penguin	29/03/2018	19y17d	N/A
21-0665	2	0.5	1	0.25	Crested partridge	4/04/2018	5m15d	N/A
21-0666	0.5	0.25	0.5	0.125	Humboldt penguin	7/04/2018	13y11m23d	N/A
21-0667	1	0.5	0.5	0.25	Humboldt penguin	9/05/2018	14y1m2d	2017†
21-0668	1	0.5	0.5	0.25	Humboldt penguin	18/07/2018	11y1m17d	N/A
21-0669	0.5	0.25	0.5	0.064	Humboldt penguin	19/07/2018	11y1m24d	N/A
21-0670	1	0.55	1	0.25	Humboldt penguin	25/07/2018	11y2m	N/A
21-0671	0.5	0.25	0.5	0.25	Humboldt penguin	7/08/2018	2y1m6d	N/A
21-0672	0.5	0.5	0.5	0.25	Humboldt penguin	9/08/2018	2y1m8d	N/A
21-0673	0.5	0.5	0.5	0.125	Humboldt penguin	8/08/2018	12y1m24d	N/A
21-0674	0.5	0.5	2	0.25	Humboldt penguin	17/08/2018	7y4m7d	N/A
21-0675	1	0.5	2	0.25	Humboldt penguin	5/09/2018	17y4m15d	N/A
21-0676	1	1	1	0.5*	Humboldt penguin	5/09/2018	7y3m11d	N/A
21-0677	0.5	0.5	1	0.25	Humboldt penguin	4/09/2018	3y4m13d	N/A
21-0678*	0.5	0.5	1	0.5*	Humboldt penguin	13/09/2018	9y5m1d	N/A
21-0679	1	1	2	0.25	Humboldt penguin	22/09/2018	22y5m1d	16/2/2017
21-0680	1	1	1	0.5*	Humboldt penguin	28/09/2018	17y5m8d	2018†
21-0034	0.5	0.25	0.5	0.125	Red-billed blue magpie	17/11/2019	4m, 4d	N/A
21-0035	0.25	0.25	0.25	0.125	Chilean flamingo	22/02/2020	45y 7m 19d	N/A

ID *A. fumigatus* strain	VOR	ITC	ISA	POSA	Host species	Date of death	Age	Treatment since**
21-0036	0.5	0.25	0.5	0.125	Chestnut-backed thrush	8/10/2020	6y1m5d	N/A
21-0353	0.5	0.25	0.5	0.125	Crested oropendola	5/03/2021	6 y 11 m 21 d	N/A
21-0428/21-0488	0.25	0.25	0.25	0.125	Humboldt penguin	26/05/2021	12y1m17d	2020†
21-0494	2	1	2	0.5*	Humboldt penguin	1/08/2021	4y3m3d	N/A
21-0523	0.5	0.25	0.5	0.125	Humboldt penguin	6/09/2021	20y7m23d	N/A
21-0524	4*	2	4*	0.5*	Humboldt penguin	1/08/2021	5y3m12d	N/A
21-0525	0.5	0.5	0.5	0.125	African penguin	12/08/2021	11y3m2d	N/A
22-0592	0.25	0.25	0.5	0.032	Humboldt penguin	29/08/2022	22y4m12d	N/A

N/A = Not applicable, VOR = voriconazole, ITC = itraconazole, ISA = isavuconazole, POSA = posaconazole, *indicates resistance following the EUCAST clinical breakpoints for fungi, v10.0 (Arendrup, Friberg, et al., 2020), **starting date of treatment with 20mg/kg itraconazole daily until death, † exact starting date of treatment is unknown.

3 Broth microdilution antifungal susceptibility testing and *cyp51A* sequencing

All clinical (n = 35) and environmental (n = 68) *A. fumigatus* isolates were subjected to broth microdilution antifungal susceptibility testing. A total of six clinical (17.14%) and eight environmental (11.76%) *A. fumigatus* isolates displayed resistance against at least one medical azole (Table 19).

Table 19 - Antifungal susceptibility testing results of the isolates displaying antifungal resistance against at least one medical azole, and associated *cyp51A* mutation.

Source	ID Strain	Date of isolation	VOR	ITC	ISA	POSA	*cyp51A* mutation
Environmental	21-0468	29/06/2021	2*	2*	4*	0.5*	TR34/L98H
	21-0503	13/10/2021	4*	>16*	4*	0.5*	TR34/L98H, G54R
	21-0506	13/10/2021	4*	>16*	4*	0.5*	TR34/L98H
	21-0510	13/10/2021	4*	>16*	16*	2*	TR34/L98H
	21-0515	13/10/2021	2*	2*	4*	0.5*	TR34/L98H
	21-0516	13/10/2021	2*	2*	4*	0.5*	TR34/L98H
	21-0517	13/10/2021	2*	2*	4*	0.5*	TR34/L98H
	21-0518	13/10/2021	2*	2*	4*	0.5*	TR34/L98H
Veterinary	21-0662	26/022018	4*	>16*	4*	0.5*	TR34/L98H
	21-0676	5/09/2018	1	1	1	0.5*	no known mutations found
	21-0678	13/09/2018	0,5	0,5	1	0.5*	no known mutations found
	21-0680	28/09/2018	1	1	1	0.5*	F46Y, M172V, E427K
	21-0494	1/08/2021	2*	1	2*	0.5*	no known mutations found
	21-0524	1/08/2021	4*	2*	4*	0.5*	TR34/L98H

* = phenotypical resistance according to the EUCAST clinical breakpoints (v10.0) (Arendrup, Friberg, et al., 2020).

All *A. fumigatus* isolates showing resistance against at least one medical azole were further characterized by *cyp51A* sequencing. Two clinical isolates showed the presence of the TR34/L98H mutation (Table 19) and one displayed several nucleotide mutations, resulting in three amino acid substitutions F46Y, M172V, E427K. Three other clinical isolates did not show any mutations known to cause resistance in the *cyp51A* gene. All environmental isolates showed the presence of the TR34/L98H mutation and one (21-0503) showed an additional G54R nucleotide mutation.

4 Microsatellite genotyping of the *Aspergillus fumigatus* isolates

Genotyping was performed on a selection of 45 *A. fumigatus* isolates (Figure 44, Table A 1). The discriminatory power of the combined markers reached 98.8. A total of 21 veterinary isolates were included: 18 from Humboldt penguins and 3 from other avian species (Psarocolius decumanus, Spheniscus demersus and Rollulus rouloul) to investigate if transmission between bird species was possible. No identical genotype was shared between the veterinary isolates. Twenty-four environmental isolates, representing both susceptible and resistant strains, were also analyzed and resulted in 20 different genotypes. The same genotype was found in four environmental strains (21-0515, 21-0516, 21-0517 and 21-0518), and were closely related to two other genotypes: a first one shared by two environmental strains (21-0503 and 21-0510), and a second one corresponding to an isolate (21-0524) originating from a Humboldt penguin (Figure 44, Table A 1). Genotypes within this cluster differed by only 1 marker (STRAf2A). All seven isolates in this cluster were resistant against at least one medical azole (Table 19). The six environmental strains originated from the study time point in autumn (October 2021). The Humboldt penguin (21-0524) died during the same period, in August 2021 (Table 18).

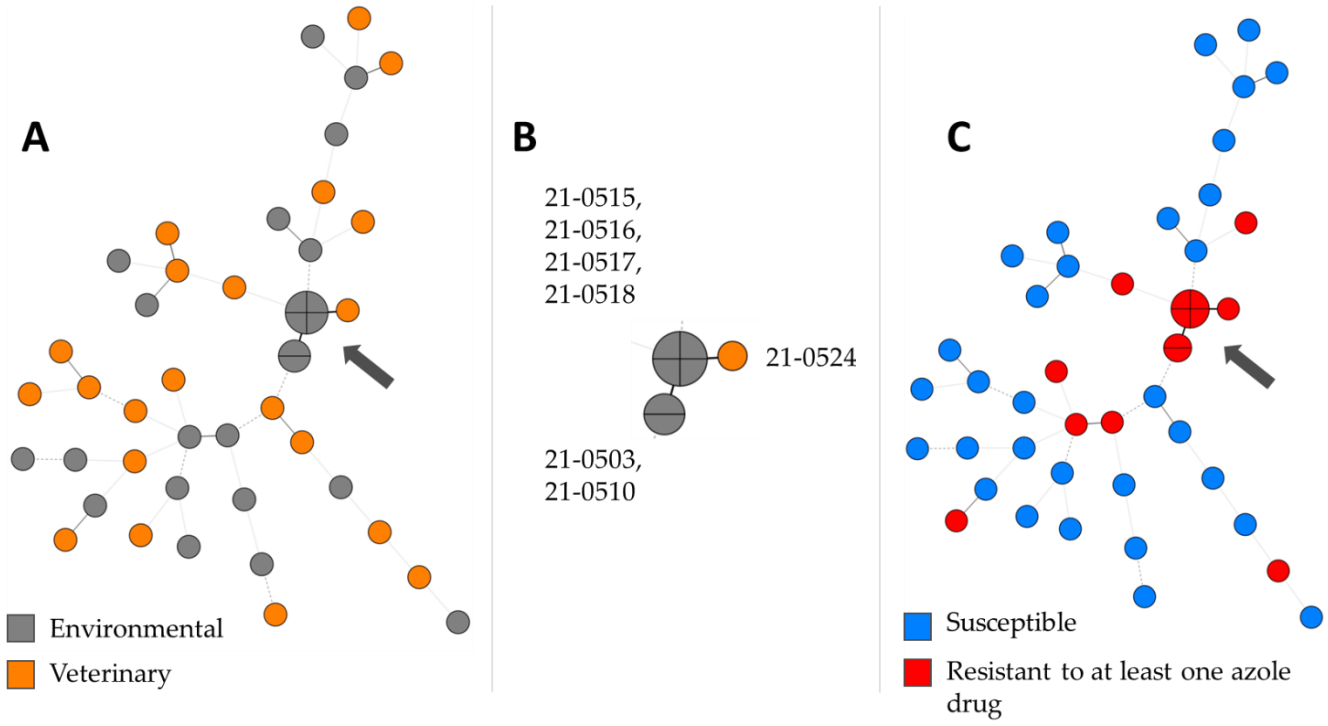

Figure 44 - Minimum spanning tree displaying the 41 different genotypes obtained from 45 *Aspergillus fumigatus* isolates, based on 9 variable-number tandem-repeat loci. The genotypes are represented by circles. The length and thickness of the connecting lines between the circles show the similarity between the profiles. (A) Mapping of the source of the isolate (i.e., isolated from the environment or from an infected animal). (B) Detailed map of the cluster indicating the strains ID. (C) Mapping of the resistance profile towards azole drugs (i.e., susceptible to all tested drugs, or resistant to at least one tested drug). The arrow shows the seven clustered isolates.

Discussion

Aspergillosis in captive birds plays an important role which can pose problems in their management. All avian aspergillosis cases in our study arose by *A. fumigatus*, confirming its role as the main causative fungal agent of avian aspergillosis in penguins, as previously described (A. M. Melo et al., 2021; Sabino et al., 2019). The naturally elevated body temperature of the birds, ranging from 39 °C to 41 °C, combined with the thermotolerance of *A. fumigatus*, favor its incidence. The second most common agent of bronchopulmonary aspergillosis in birds is *A. flavus*, accounting for about 5% of avian aspergillosis cases (Tell, 2005), but this species was not detected in this study.

In the present study, the mortality due to aspergillosis reached 86.7% in 2017 and 76.5% in 2018. In 2018, we see a clear rise in aspergillosis incidence compared to the other years (Table 1). This might be explained by the extreme weather conditions in 2018, especially due to the high temperatures in summer: 65% of the deaths in 2018 occurred during the summer months. Additionally, the population size at that time was large and a breeding program was operative in 2018. The effect of overcrowding was demonstrated in a study performed over 6 years in Magellanic penguins (*Spheniscus magellanicus*), where 65% of aspergillosis cases took place during the year with the highest population density (Silva Filho et al., 2015b). Measures were taken in the Belgian zoo in 2019 to lower the mortality of aspergillosis: ventilation holes were installed in the nests, monitoring of the water temperature below 20 °C to mimic their natural habitat, shade was provided in summer, breeding program was interrupted and direct contacts between visitors and penguins were discontinued. The breeding program started again in 2020 with 4 breeding couples in 2020 and 8 in 2021. The mortality in penguins increased again when breeding restarted. Over the period 2017-2021, it appeared that the aspergillosis incidence was 3 times higher in animals rearing young (n = 22, 12 female and 10 male) than those that did not (n = 7, 5 female and 2 male). The highest mortality was observed in adults, which is in contrast with previous findings where most of the cases of aspergillosis occur in juveniles (Cateau et al., 2022; Flach et al., 1990; Silva Filho et al., 2015b). Avian aspergillosis in adult penguins is mostly due to the chronical form and is linked to immune suppression (Beernaert et al., 2010). Noteworthy, juveniles younger than 2 months (n = 20) were not tested in this study for the presence of aspergillosis.

Environmental sampling in the penguin housing did not show significant differences between the four study time points. However, mean fungal loads in the environment were slightly more elevated in spring and summer. This is in line with the findings of Cateau *et* al. reporting higher fungal burden in September compared to April and December (Cateau et al., 2022). Similarly, a study performed on African Penguins in the Maryland Zoo in Baltimore, described higher environmental fungal load during the warmer period from the end of spring to the beginning of autumn (Rivas et al., 2018).

Artificial nests made up of plastic in the synthetic rock formations did not reveal the presence of *A. fumigatus* in this study. Here, only 2 nests were consistently sampled on 3 out of the 4 sampling days, which might explain the absence of *A. fumigatus*. However, unpublished data from the zoo obtained on a larger sampling campaign of all the nests (n=25), confirmed that in the period of 2017-2018, *A. fumigatus* colonies were found in all nests, with higher average counts from May to September, as compared to October to March. This is in agreement to the study of Cateau *et* al. which found a high fungal load in the nests (Cateau et al., 2022).

Azole resistance in *A. fumigatus* represents an emerging problem in human and veterinary medicine and was detected in 14 isolates, both from the environment and from penguins. Most of the mutations conferring azole resistance in *A. fumigatus* are found in the *cyp51A* gene encoding for lanosterol-C14-α-demethylase, the target protein of azole drugs (Resendiz Sharpe et al., 2018). *Cyp51A* mutations can be tandem repeats (TR) in the promotor region of the gene, single-nucleotide polymorphisms (SNPs), or both (Resendiz Sharpe et al., 2018). Many articles describe the TR34/L98H mutation conferring resistance to azole drugs, which has been linked to the intense use of agricultural azoles for crop protection (Snelders et al., 2009). The zoo is located near a larger city, however, it is also surrounded by many agriculturally cultivated plots. In this study, the TR34/L98H mutation was found in both clinical and environmental samples. This mutation usually leads to pan-azole resistance phenotype in human clinical samples, which can be seen in several samples in this study (Table 19). In contrast, the most common mutations in *cyp51A* leading to azole-resistance that develop during antifungal treatment, occur in amino acid sites G54, G138, M220, and G448 (Fan et al., 2021). Prophylaxis with azoles in distressed penguins, as well as the treatment of aspergillosis in captive penguins, are very common (Bunting et al., 2009). Several penguins included in our study received treatment for extended periods due to an increased chance of disease development.

However, only the TR34/L98H mutation was detected, in two strains isolated from Humboldt penguins, suggesting that the resistance of these strains was acquired from the environment. The isolate 21-680 harbored several other amino acid substitutions in the *cyp51A* gene: F46Y, M172V, E427K. The combination of these amino acid substitutions was reported in approximatively 10% of all *A. fumigatus* isolates tested worldwide (Garcia-Rubio et al., 2018), including in patients receiving azole treatment (Alanio et al., 2012). Generally, they display elevated MIC values for the medical azoles compared to the wild-type (WT) *cyp51A* (Alanio et al., 2012). However, their susceptibility profiles are inconsistent and were described as both azole-susceptible or resistant by different authors (Abdolrasouli et al., 2015; Alanio et al., 2012; Escribano et al., 2011; Garcia-Rubio et al., 2018; Howard et al., 2009; Hsu et al., 2022; Snelders et al., 2009; Won et al., 2020; Y. Zhao et al., 2013). No known resistance conferring mutations in the *cyp51A* gene were found in the remaining veterinary isolates. Other mechanisms, such as mutations in other genes [24,28,38,39] or efflux pumps [26] could be involved in the decreased

susceptibility of these isolates to POSA. All eight environmental azole-resistant strains harbored the TR34/L98H mutation. Three of them displayed the typical pan-azole phenotype, whereas the remainder were resistant against ISA and POSA, but had MIC values in the area of technical uncertainty (ATU) for VOR and ITC.

Genotyping of *A. fumigatus* isolates in this study revealed a broad diversity in both environmental and veterinary strains, suggesting independent events of contamination. Also, the azole resistant strains were not all closely related to each other, indicating that the resistance was acquired multiple times and has different origins. No relation was observed between the veterinary isolates included in this study. In contrast, Cateau *et* al. found identical genotypes among veterinary isolates, but their sampling was performed within a short timespan (Cateau et al., 2022). A veterinary strain from a Humboldt penguin (which died in August 2021) however clustered with 6 environmental isolates from the study time point in autumn. Moreover, all seven isolates of this cluster were resistant against at least one medical azole and harbored the TR34/L98H mutation. This suggests that the Humboldt penguin acquired the strain from the environment. Interestingly, isolates 21-0503 and 21-0510 had identical genotypes, but differed in MIC values and *cyp51A* sequencing. Isolate 21-0503 indeed had an additional G54R amino acid mutation, alongside the TR34/L98H mutation. The observed MIC values were higher for 21-0510 than for 21-0503 for ISA and POSA. Additionally, two strains (21-0428 and 21-0488) originating from the same animal had different unrelated genotypes, indicating that Humboldt penguins can be infected by multiple *A. fumigatus* strains. Altogether, within this study we were able to capture a small proportion of the large diversity present in the environment and veterinary *A. fumigatus* strains present in the Belgian zoo.

This research has several limitations. The first is the timeframe of the environmental sampling which was performed on 4 non-consecutive days. This gives a limited view on the seasonality since single time point measurements depend on many different aspects such as temperature, wind or humidity. Future studies should therefore consider continuous long-term sampling. Secondly, the environmental sampling was only performed in 2021 and 2022, while the majority of the veterinary strains were isolated during the previous years.

There is limited evidence in humans that the infection can be spread from patient to patient (T. G. P. Engel et al., 2019). There is however no evidence reported yet of such events in animals. But considering the anatomy of the respiratory system of birds, and the high fungal burden in their air sacks/lungs, it could also be true for birds. This would need more research with multiple time point sampling of penguins living in the same habitat. However, this might be a challenge considering the invasive nature of sampling living penguins and the poor reliability of diagnostic tools ante mortem.

The One Health concept envisages a tripartite health system based on the environment, animals and humans and their mutual interactions. This concept directs us towards a more holistic approach in the surveillance of infectious diseases on a global scale. The interactions between the environment, the ubiquitous mold *A. fumigatus*, and the birds, illustrate such complex ecosystem. The latter is changing in an accelerated manner due to global warming, which should be considered when addressing research on infectious diseases. In this paper, we were able to show a probable interaction between the environment and animals, but transmission between animals was not evidenced. However, we observed the same resistance profile and gene mutations in the environment and in the animals, which are also observed in human isolates (Snelders et al., 2009). The effect of extreme weather conditions was demonstrated. We could predict these phenomena to occur more often with the effects of global warming.

In conclusion, this report contributes to a better understanding of the molecular epidemiology of avian aspergillosis in penguins, dominated by *A. fumigatus*. It is also the first report of a TR34/L98H mutation in *A. fumigatus* isolates obtained from penguins, showing the relevance of monitoring azole resistance of *A. fumigatus* in veterinary science. Genotyping revealed infection by multiple *A. fumigatus* strains in the same penguin individual, as well as a clustering between environmental and veterinary isolates. More frequent sampling could provide more insight on the diversity and possible transmission of *A. fumigatus* between Humboldt penguins and their environment. The high mortality rates due to aspergillosis observed in this study also questions the best combination of practices in captive penguins management.

Materials and Methods

1 Environmental *Aspergillus fumigatus* sampling within the penguin enclosure

The environmental sampling was conducted between April 2021 and January 2022 at a Belgian zoo. The maximal population size of the group was 52 in 2021 and 28 in 2022. Since the start of the Humboldt penguin program in 2013, the zoo welcomed a total of 214 animals. The penguin habitat, which is exclusively outdoors, includes a temperature-monitored swimming pool (< 20 °C) and artificial rock formations with 25 built-in nests (Figure 45). The entire habitat is roofed with a steel wired net to prevent the entry of other birds.

Four study time points of environmental sampling were performed on April 1st 2021, 29 June 2021, 13 October 2021 and 12 January 2022, covering spring, summer, autumn and winter, respectively. At each sampling day, four types of samples were taken: air, water, sand and nest surface (except for nest swabs on April 1st since breeding couples were present and could not be disturbed). Sand and air were taken at four predefined locations (Figure 42). Two nest samples and one water sample of the pool were taken (Figure 42).

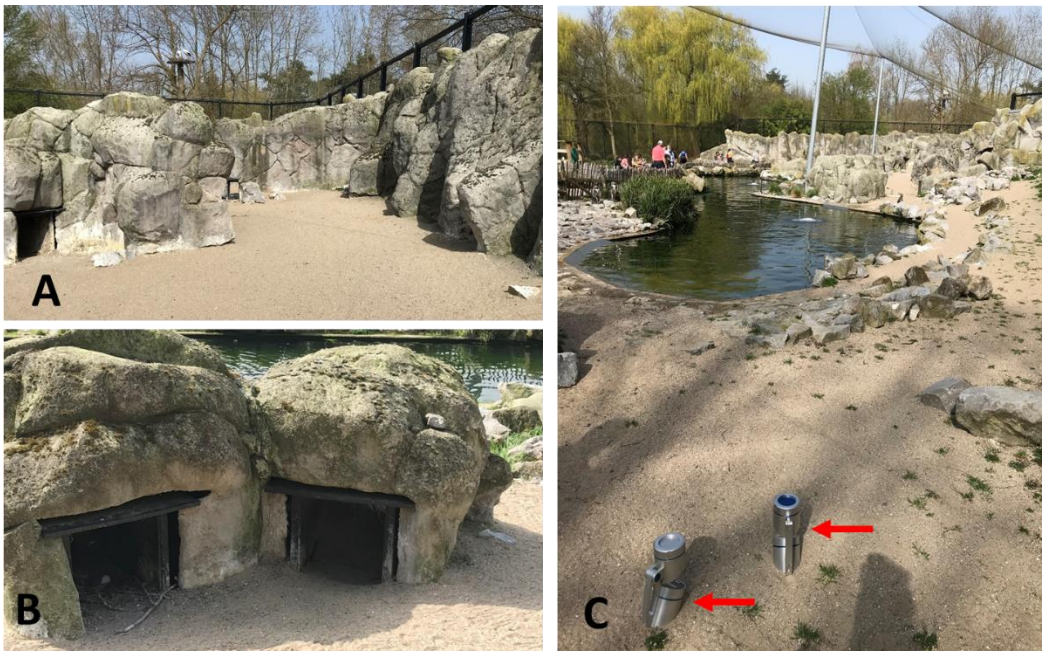

Figure 45 -Presentation of the Humboldt penguin enclosure. (A) Artificial rocks with built-in nests; **(B)** two nests made up of plastic; **(C)** temperature-controlled pool surrounded by sand and artificial rock formations. Air sampling was performed using MAS-100 NT air samplers (red arrows).

2 Isolation of *Aspergillus fumigatus* from environmental samples

All samples were plated on two media: malt extract agar + chloramphenicol (MC) and MC supplemented with 4mg/l of tebuconazole (MC+T). The plates were incubated at 48°C ± 1°C for 48h ± 2h to prevent the growth of most environmental fungi. For the air samples, a total of 1000L of air was impacted on each medium using the MAS-100 NT™ impactor (Merck®, Darmstadt, Germany) with a rate of 100 L/min. Quantitative results were expressed as log10 CFU/1000L air. From each sand sample, 1g was dissolved in 9 mL of 0.85% NaCl + 0.01% Tween 20 solution. After thorough vortexing, 100 microliters were plated onto the two media. A total of 100 mL of water from the swimming pool was filtered using a nitrocellulose membrane filter (0.45µm, Sartorius, Göttingen, Duitsland) in two times, after which each filter was placed on a different medium. For the surface sampling of the nests, swabs were taken on the inside of the nests, (floor and walls) and inserted in 1 mL of Amies liquid (eSwab, Copan, Menen, Belgium). Following a 1 min vortexing step, 100µl of each suspension was seeded onto the two media.

The MC medium was used to determine the total number of *A. fumigatus* isolates. Fungal colonies on MC+T were isolated and identified for further analysis to detect potential azole resistant *A. fumigatus* (ARAF) isolates. The microscopic and macroscopic features of each colony were used to identify every *A. fumigatus* isolate. Matrix-assisted laser desorption ionization-time of flight mass spectrophotometry (MALDI-TOF MS) was used to confirm the identity of the suspected ARAF isolates that grew on MC+T (A.-C. Normand et al., 2021b).

3 Clinical incidence of avian aspergillosis in Humboldt penguins

Aspergillosis incidence in the Humboldt penguins in the zoo was assessed for the period 2017-2022. Necropsy was performed on all dead animals older than 2 months with suspected aspergillosis (Figure 46). Confirmed cases of pulmonary aspergillosis were verified by light microscopy using a lactophenol cotton blue stain and culture of *A. fumigatus* of the lungs and/or the surface of the air sacs.

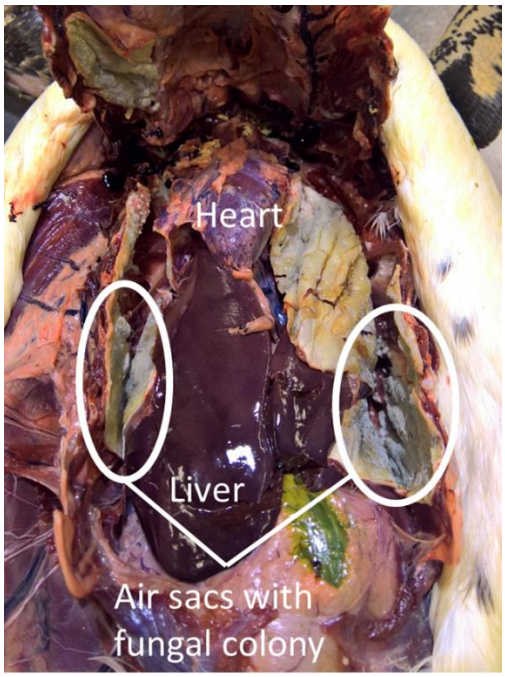

Figure 46 - Gross lesions of aspergillosis found in a captive Humboldt penguin. View of the opened coelomic cavity with dissected air sacs that show confluent green to gray velvety fungal growth in the air sac.

4 Broth microdilution antifungal susceptibility testing and *cyp51A* sequencing

All strains able to grow on MC+T were tested by broth microdilution method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (Arendrup, Meletiadis, et al., 2020). Briefly, a cell suspension of 1-5 x 10^6 CFUs (colony-forming unit) per ml was prepared in 10 ml of saline water (8.5g/L NaCl) from a 5 day old subculture on Sabouraud chloramphenicol agar tube. Subsequently, 1 mL of the cell suspension was added and mixed with 10 mL of RPMI-1640 medium (Sigma-Aldrich, Saint-Louis, MO, USA). A total of 100 µL of this cell suspension was added to each well of a 96-well plate containing 100 µl of serial dilutions of the antifungals and a control. The plates were incubated at 35°C ± 1°C for 48 h. The minimal inhibitory concentration (MIC) of four medical azoles (itraconazole (ITC), voriconazole (VRC), posaconazole (POSA) and isavuconazole (ISA)) was determined on suspected ARAF strains. The MIC was determined visually as the lowest concentration of antifungal drugs causing complete inhibition of fungal growth. Candida krusei (IHEM 9560 = ATCC 6258), Candida parapsilosis (IHEM 3270 = ATCC 22019) and *A. fumigatus* (IHEM 28944 = ATCC 204305) were used as quality control strains. Azole resistance was defined according to the EUCAST clinical breakpoints (v10.0)(EUCAST, 2020), for ITC and VOR with MIC >1 mg/L, POSA MIC >0.25 mg/L and ISA MIC >2 mg/L) .

5 Genotyping

A selection of environmental and clinical strains were analyzed by microsatellite polymorphism genotyping using three multiplex PCRs. A total of nine microsatellite markers consisting of di-, tri-, or tetranucleotide short tandem repeats (STR) were used (de Valk et al., 2005). Fungal DNA extraction was performed by freeze-drying the cultures and mechanically breaking the cells by bead-beating. DNA was then extracted using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research) following the manufacturer's instructions. Genotyping was performed by Genoscreen (Lille, France) using the PCR conditions described by De Valk et al (de Valk et al., 2005) with the fluorophores 6FAM/HEX/NED. The size of the amplicons was determined with a ABI 3730XL genetic analyzer using the GeneScan 500 ROX size standard (ABI) and the GeneMapper v5.0 software. The size of each microsatellite fragment was measured to determine the number of repetitions for each marker according to de Valk et al (de Valk et al., 2005). All results are reported as repeat numbers. The relatedness of the strains was estimated by a minimum spanning tree analysis in Bionumerics 8.0 (Applied Maths, St-Martens-Latem, Belgium). The discriminatory power of the microsatellite markers was calculated using the Simpson index of diversity (Hunter 1990).

6 Statistics

Statistical analysis was performed to achieve global comparison between each study time point, using the non-parametric Friedman test. The statistical and graphics software R (version 4.2.0) was used. The significance level was set at p-value < .05.

Author Contributions: Conceptualization, H.D., A.P. and F.V.; methodology, H.D. and A.P.; formal analysis, H.D. and P.B.; investigation, H.D..; resources, F.V. and R.H.; data curation, H.D. and P.B.; writing—original draft preparation, H.D.; writing—review and editing, H.D., P.B., A.P., K.L., C.S., F.V., R.H.; supervision, A.P. and K.L.; project administration, H.D.; funding acquisition, A.P. and C.S. All authors have read and agreed to the published version of the manuscript.

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Supplemental material

Table A 1. Genotyping results displayed by STR numbers for 9 microsatellite markers according to de Valk et al . (de Valk et al., 2005)

source	ID	2A	2B	2C	3A	3B	3C	4A	4B	4C
Env	21-0174	17	10	8	37	10	26	9	8	10
Env	21-0179	NA	8	12	24	9	23	8	14	10
Env	21-0466	17	10	8	14	9	20	9	8	5
Env	21-0470	23	17	16	26	12	18	7	15	8
Env	21-0477	19	10	11	31	12	22	10	8	8
Env	21-0479	17	10	8	27	14	21	8	7	7
Env	21-0498	19	17	19	19	11	18	16	15	10
Env	21-0501	21	21	18	25	10	16	9	12	8
Env	21-0504	19	10	11	25	9	22	17	7	5
Env	21-0509	10	14	10	22	12	13	7	4	5
Env	21-0512	22	21	15	28	9	29	10	8	5
Env	21-0514	24	20	8	29	9	8	8	9	19
Env	21-0515	22	20	16	17	9	8	5	8	10
Env	21-0516	22	20	16	17	9	8	5	8	10
Env	21-0517	22	20	16	17	9	8	5	8	10
Env	21-0518	22	20	16	17	9	8	5	8	10
Env	21-0649	13	18	11	NA	9	8	8	9	27
Env	21-0652	10	12	10	17	13	9	7	4	6
Env	21-0658	11	10	11	17	9	20	8	7	7
Env	21-0177	NA	20	9	37	9	8	8	8	19
Env	21-0468	13	8	11	19	10	8	8	8	20
Env	21-0503	23	20	16	17	9	8	5	8	10
Env	21-0506	NA	8	11	19	11	8	8	8	21
Env	21-0510	23	20	16	17	9	8	5	8	10
Vet	21-0029	12	18	10	9	10	11	8	8	20
Vet	21-0030	13	19	10	23	13	22	7	4	6
Vet	21-0353	11	13	9	28	9	21	8	7	5
Vet	21-0428	22	17	15	10	11	8	13	8	5
Vet	21-0488	23	17	8	15	12	20	7	8	8

source	ID	2A	2B	2C	3A	3B	3C	4A	4B	4C
Vet	21-0525	19	10	8	19	10	21	8	8	5
Vet	21-0528	20	10	9	9	8	11	8	8	10
Vet	21-0659	23	19	16	NA	11	8	13	8	5
Vet	21-0662	NA	8	8	51	10	12	8	6	11
Vet	21-0664	NA	19	9	9	10	11	8	9	10
Vet	21-0665	13	10	17	NA	11	19	17	13	5
Vet	21-0670	22	10	11	26	9	22	17	7	5
Vet	21-0673	13	17	8	35	16	8	8	26	5
Vet	21-0674	17	19	15	NA	NA	29	27	26	8
Vet	21-0676	13	23	9	39	13	15	8	8	15
Vet	21-0678	NA	21	21	27	10	18	16	8	8
Vet	21-0680	22	20	16	16	13	9	7	5	6
Vet	21-0494	18	10	8	27	10	21	9	7	7
Vet	21-0523	24	18	8	9	10	23	9	9	5
Vet	21-0524	10	20	16	17	9	8	5	8	10
Vet	22-0592	NA	15	10	23	13	21	7	4	6

CHAPTER 4

GENERAL DISCUSSION - FUTURE PERSPECTIVES

This doctoral thesis aimed at addressing the most important knowledge gaps for the implementation of a 'One Health' surveillance approach. This was tackled by investigating the prevalence of *K. pneumoniae* and *A. fumigatus* in Belgium, the occurrence and mechanisms of antimicrobial resistance, and their transmission dynamics (Figure 47).

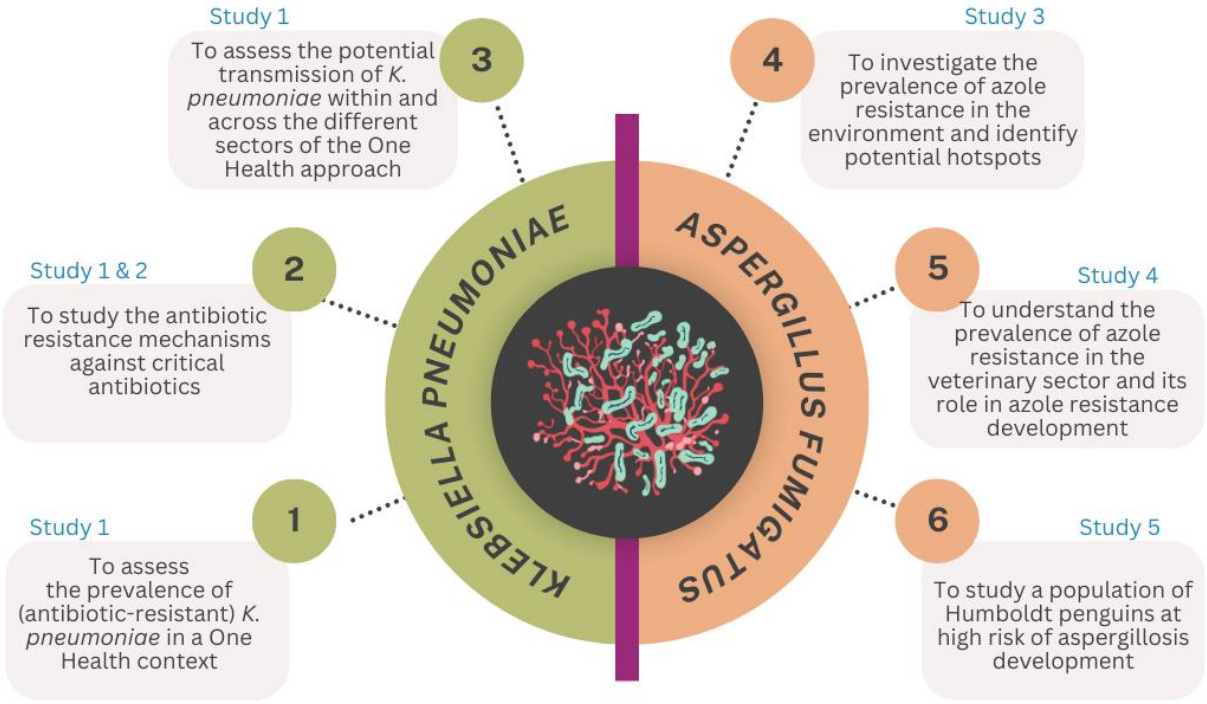

Figure 47 - Overview of the specific objectives and corresponding studies in this doctoral research

First, the prevalence of *K. pneumoniae* in the food chain and in wastewater was evaluated (**Study 1**). Subsequently, the occurrence of antibiotic resistance in *K. pneumoniae* in the food chain, diseased animals, wastewater and the human clinical isolates was studied (**Study 1**). A rare resistance mechanism conferring ertapenem resistance was elucidated in a *K. pneumoniae* strain isolated from a companion animal (**Study 2**). Furthermore, whole genome sequencing was performed on a subset of *K. pneumoniae* isolates to study the potential transmission of strains between the different sectors of the One Health approach (**Study 1**). The prevalence of azole resistant *A. fumigatus* in the environment (agriculture, horticulture and composting facilities) was studied and identification of potential hotspots was performed (**Study 3**). Additionally, the occurrence of azole resistance in *A. fumigatus* isolated from veterinary samples was evaluated (**Study 4**). Finally, a study linking the environment and a high risk veterinary population in the zoo environment was performed (**Study 5**).

1 Klebsiella pneumoniae

1.1 The prevalence of a critical pathogen in the human sector

Klebsiella pneumoniae is a major cause of hospital-acquired infections, including pneumonia, bloodstream infections, and urinary tract infections, especially in immunocompromised individuals. On top of this, *K. pneumoniae* is called a key-trafficker due to its remarkable ability to acquire and spread ARGs, particularly those conferring resistance to carbapenems, making treatment increasingly challenging. The emergence of hvKp has expanded the threat to healthy individuals, causing severe conditions such as liver abscesses, meningitis, and endophthalmitis. Combating *K. pneumoniae* requires an integrated approach across various health sectors, highlighting the need for coordinated strategies.

In 2022, Belgium saw a significant 18.8% increase in community antibiotic consumption compared to 2021, reversing a decade-long decline. This rise, following a marked reduction in antibiotic use during the COVID-19 pandemic, is mainly due to the easing of pandemic restrictions and the return to normal healthcare and social activities. However, antibiotic consumption remained slightly below pre-pandemic levels of 2019 but still above the EU average. Most antibiotic subgroups saw mild to moderate increases, except for tetracyclines, which slightly decreased. Fluoroquinolone use, which had declined after stricter reimbursement criteria in 2018, increased by 12.6% in non-reimbursed consumption in 2022.

The 2023 WHO European Region AMR surveillance report, based on 2021 data, showed significant variability in AMR depending on bacterial species, antimicrobial groups, and geography. *K. pneumoniae* represented 12.3% of reported invasive isolates in EU/EEA countries, with resistance to third-generation cephalosporins and carbapenems being more prevalent in *K. pneumoniae* than in *E. coli*. While carbapenem resistance in *E. coli* remained low in most countries, 15 countries (33%) reported 25% or higher resistance in *K. pneumoniae*, particularly in southern and eastern Europe. Eight countries reported a resistance rate exceeding 50%. The north-to-south and west-to-east gradient of resistance was especially evident for third-generation cephalosporins and carbapenems in *K. pneumoniae.*

Although Belgium's rates of resistance to third-generation cephalosporins (18.3%) and carbapenems (1.4%) in *K. pneumoniae* in 2022 were below the EU/EEA average (32.7% and 10.9%, respectively) in the EARS-Net surveillance, prevention remains crucial (European Centre for Disease Prevention and Control, 2022). Understanding potential sources of infection is key, and the One Health concept, which links human, animal, and environmental health, provides a comprehensive framework for addressing AMR.

In this doctoral thesis, we performed a study looking at the prevalence rate of (antibiotic resistant) *K. pneumoniae* using the One Health approach, identified their resistance mechanisms and found evidence of transmission across the sectors from the One Health approach **(Study 1)**.

A large proportion (n = 51, 53.68%) of human clinical *K. pneumoniae* isolates in our analysis (n = 95) were grouped into seven high-risk clonal lineages, with ST307 being the most prevalent (16.84%). Other major lineages included ST15 (15.79%), ST147 (7.37%), ST11 (6.32%), ST14 (3.16%), ST101 (3.16%), and ST512 (1.05%). Comparing this to large European studies like EURECA, which examined carbapenem-resistant Enterobacterales across southern Europe, there are differences in clonal lineage prevalence. The carbapenem-resistant isolates were predominantly grouped into 11 common clonal lineages, with most high-risk clones producing carbapenemase genes (Budia-Silva et al., 2024). In our collection of human clinical isolates, 59.7% (n = 56) of the *K. pneumoniae* isolates were carbapenemase producing, with the majority (n = 38, 67.9%) belonging to those seven high-risk clonal lineages. Results should however be interpreted with caution as we focussed on presumptive ESBL-producing and carbapenem resistant human clinical *K. pneumoniae* isolates, likely not representing the resistance prevalence observed in the general population.

Budia-Silva et al. highlighted regional differences in the dominant *K. pneumoniae* clonal lineages across Europe. In Italy, ST258/512 was the most prevalent, associated with bla_{KPC}-like genes, while Greece displayed greater clonal diversity, with both ST11-bla_{NDM} and ST258/512 being prominent. In Serbia and Romania, ST101 and ST11 were the dominant clones, whereas Spain and Türkiye showed a mixture of lineages, with ST258 now prevailing in Spain and ST14 in Türkiye. The bla_{OXA-48} gene, carried on highly transmissible plasmids, was widely observed in Serbia, Spain, and Türkiye. This is consistent with our findings, where bla_{OXA-48} was detected in 69.6% (39/56) of our carbapenemase-producing isolates. These regional variations underscore the importance of genomic surveillance in Europe, providing essential insights for local risk mapping and informing adjustments to control strategies (Budia-Silva et al., 2024).

A study investigating the epidemiology of CRKP on a global level describes that bla_{KPC} is endemic in some states of the USA and some countries in South-America. Several countries in Asia describe nationwide distribution of bla_{KPC}, bla_{NDM} and bla_{IMP}. Data on Africa is mainly lacking, apart from South Africa and some countries in the north of Africa (Logan & Weinstein, 2017). Sporadic outbreaks are described worldwide (Logan & Weinstein, 2017).

In 2017, Belgium participated in the EuSCAPE study, which included 455 sentinel hospitals across 36 countries, examining a total of 2,703 clinical isolates (85% *K. pneumoniae* and 15% *E. coli*). Among these, 37% of the *K. pneumoniae* and 19% of the *E. coli* isolates were carbapenemase producers, with KPC being the most detected enzyme (31.5%), followed by OXA-48-like (25.8%), NDM (7.7%), and VIM (5.7%). In Belgium specifically, 48 carbapenem non-susceptible isolates were submitted, and 68.8% (n = 33) were confirmed as carbapenemase-producing *K. pneumoniae*. OXA-48-like was the most common enzyme (37.5%), followed by KPC (27.1%) and NDM (4.2%), with no VIM detected (Grundmann et al., 2017). Similarly, in our collection, $bla_{OXA-48-like}$ was the most frequently observed carbapenemase (83.9%, n = 47). Unlinke in the EuSCAPE study, we detected more bla_{NDM} (17.9%, n = 10) than KPC (10.7%, n = 6). The bla_{VIM} gene was observed the least VIM (1.8%, n = 1). Budia-Silva

et al. also noted some isolates lacking carbapenemase, suggesting other resistance mechanisms or plasmid loss (Budia-Silva et al., 2024). Likewise, 31.3% of the carbapenem-resistant isolates from Belgium in the EuSCAPE study did not carry a carbapenemase gene (Grundmann et al., 2017), which aligns with our findings, where some cases were likely related to ESBL or AmpC genes, along with outer membrane protein deficiencies.

In our collection, the majority of isolates with a virulence score of 1 (n = 81) were primarily from human clinical samples (n = 47, 58.02%). Additionally, we observed isolates with a virulence score of 3 (n = 1) and 4 (n = 3) among the human clinical isolates. One notable clinical isolate exhibited both a virulence score of 4 and a resistance score of 3, suggesting the possible emergence of a hypervirulent and MDR *K. pneumoniae* strain. However, the specific phenotypic or genotypic markers (such as mucoviscosity or siderophore concentration) that best predict the hypervirulent phenotype remain unclear (Russo et al., 2024). Since we did not conduct phenotypic tests, such as a murine sepsis model, we cannot confirm whether this strain exhibits the hypervirulent phenotype (Russo et al., 2018, 2024). These convergent strains, where a hypervirulent strain acquires ARGs or an MDR strain gains virulence factors, could significantly heighten the risk posed by *K. pneumoniae* (Wyres, Lam, et al., 2020). Hybrid vectors may arise through several mechanisms. Hypervirulent lineages (e.g., ST23/ST86/ST65) acquire carbapenem resistance through MGEs or chromosomal mutations. This resistance is often linked to specific mechanisms that vary by geography, such as NDM and OXA carbapenemases in India. These strains typically carry a congenital virulence plasmid and acquire an additional plasmid with a carbapenemase gene plasmid (S. Harada et al., 2019). Hypervirulent lineages, especially CG23, have fewer replicon markers and less plasmid diversity compared to MDR lineages (Wyres, Wick, et al., 2019). Conversely, CRKP lineages like ST11 can acquire virulence plasmids. CRKP strains more readily acquire virulence genes than hypervirulent strains do resistance genes (Wyres, Wick, et al., 2019), and pLVPK-like plasmids, once thought non-conjugative, can spread and enhance CRKP virulence (Kochan et al., 2020; X. Yang et al., 2019). CRKP strains maintain multiple plasmids with minimal fitness cost (Buckner et al., 2018; Conlan et al., 2016), facilitating plasmid exchange in hospital settings and increasing outbreak risks (J. Martin et al., 2017). A third, more concerning pattern is the convergence of virulence and carbapenem resistance on a single plasmid, seen in hybrid plasmids like the pLVPK-like plasmid carrying blaKPC-2 in China in 2013 (Dong et al., 2018) and three hybrid plasmids found in London (2018) carrying virulence fragments and blaNDM-5 (Lan et al., 2021). These high-risk plasmids likely form through transposon-mediated integration of resistance genes into virulence plasmids, raising significant concerns in hospital environments (Lan et al., 2021). This phenomenon has not been observed in our collection of human clinical isolates from Belgium.

Recognizing these associations is a key step toward focusing efforts on reconstructing the mobile elements that carry critical virulence genes, with long-read sequencing approaches proving effective in unraveling MDR plasmids (Holt et al., 2015). This approach could help target known epidemic clones and increase the chances of identifying emerging strains. Active surveillance, both

phenotypically and genotypically, of key virulence and AMR genes and clonal backgrounds will be essential to track the emergence of MDR and extremely drug-resistant (XDR) hypervirulent clones.

1.2 The environmental sector as a reservoir of ARGs and a valuable tool for surveillance

Current AMR surveillance programs primarily focus on healthcare-associated infections and aim to detect the potential threats these bacteria could create to the population. Consequently, this could result in poorly monitored and understood AMR prevalence in the healthy population (Chau et al., 2022). To address this gap, alternative options for AMR surveillance at the population level, such as wastewater based epidemiology (WBE), have been explored (Chau et al., 2022; Conte et al., 2017; Rocha et al., 2022). WBE offers the potential for population-level assessment and survey of AMR while avoiding the ethical issues associated with sampling of individuals (Heljanko et al., 2024). Furthermore, wastewater can act as a reservoir and transmission pathway for *K. pneumoniae* and it can effectively monitor AMR strains such as ESBL producers and CRKP (Conte et al., 2017; Galarde-López et al., 2022; Rocha et al., 2022; Surleac et al., 2020; Wyres, Lam, et al., 2020).

We conducted an analysis of wastewater from 10 different WWTPs covering four non-consecutive months (**Study 1**). Our results showed a prevalence rate of *K. pneumoniae* of 69.4% on McC agar and of 54.4% of presumptive ESBL- and/or AmpC-producing isolates identified on McC+CTX agar. Cefotaxime resistance was found in 45.2% of *K. pneumoniae* isolates, closely matching the 42.5% ESBL-producing *K. pneumoniae* from wastewater reported by other researchers (Ludden et al., 2020). Resistance rates to other antibiotics included 41.71% for ceftazidime, 39.70% for sulfamethoxazole, 37.19% for ciprofloxacin, and 36.18% for trimethoprim. Notably, 55.32% (52/94) of the *K. pneumoniae* isolates from wastewater were resistant to ertapenem, with 25% (13/52) and 21.15% (11/52) also showing resistance to meropenem and imipenem, respectively. A German study (Savin, Bierbaum, Schmithausen, et al., 2022) reported higher meropenem resistance rates than observed in our study, though the use of a selective isolation of ertapenem-resistant isolates in their study may have influenced the observed rates of meropenem and imipenem resistance (Savin, Bierbaum, Mutters, et al., 2022).

The high prevalence of *K. pneumoniae* resistant to 3GCs and carbapenems in municipal WWTPs poses a significant public health risk. In this study, phylogenomic analysis using cgMLST revealed clusters between human clinical isolates and those from WWTPs, suggesting potential transmission from hospital effluents into municipal treatment plants. Other studies have also reported high concentrations of CRKp in clinical wastewater that ultimately ends up in municipal sewers (Kehl et al., 2022; Kizny Gordon et al., 2017; Müller et al., 2018; Surleac et al., 2020). While WWTPs reduce the concentration of ARB and ARGs by around 2 log, they still release these contaminants into the environment (J. Wang et al., 2020). The presence of these bacteria in surface waters indicates that conventional biological treatment methods are not enough to fully eliminate microbial loads (Cimen et

al., 2023; Savin, Bierbaum, Schmithausen, et al., 2022). Rodríguez et al. (2021) conducted a metagenomic analysis of the urban wastewater resistome and mobilome, demonstrating that WWTPs are hotspots for AMR (Rodríguez et al., 2021). Over six months, they studied the microbial composition and the pool of ARGs and MGEs at a traditional activated sludge WWTP in Colombia using both shotgun metagenomics and culture-based methods. While some ARG categories decreased in the aeration tanks and activated sludge, an increase was noted in the effluent, following a drastic reduction in the aeration tanks. This suggests that while activated sludge remediation effectively eliminates certain ARGs, others may persist or even increase post-treatment (Rodríguez et al., 2021).

Blaak et al. performed a nationwide surveillance study in Dutch municipal WWTPs and analyses of untreated wastewater demonstrated that CPE are widely distributed among the population throughout the Netherlands. Prevalence of CPE in wastewater was related to WWTP size and inhabitant equivalents but not to the presence of hospitals in the WWTP service areas, suggesting circulation of CPE in the general population (Blaak et al., 2021). All WWTPs in our study received hospital effluents, however the influence of prevalence of *K. pneumoniae* in the hospital effluents on the content in the WWTPs was not studied in this research project.

Additionally, a study in Austria identified two MDR *K. pneumoniae* ST985 isolates with identical cgMLST profiles, sampled from the same river at locations 200 km apart, indicating that *K. pneumoniae* can survive over long distances in river water (Lepuschitz et al., 2019). Similarly, in our study, a cluster of environmental *K. pneumoniae* ST307 isolates, separated by only one SNP, was found in the WWTPs from Ghent and Bruges (about 50 km apart), sampled one week apart. This could indicate the presence of a common source or by human carriage of individual events. *Klebsiella* species in wastewater can retain clinically relevant traits, including those acquired through HGT, even after treatment. This suggests the possibility of further dissemination of 3GC-resistant and CRKp isolates to animals and humans, along with potential colonization and infection (Rocha et al., 2022).

Our study provided valuable insights into WBE and proves it could be a valuable tool for the monitoring of *K. pneumoniae* on a population-based level. However, research is still needed before it could be routinely implemented. For example, standardization of sampling, processing, and analyzing wastewater is necessary to ensure reliable and comparable results across different studies and locations. As such, WBE can serve as a public health early warning in a non-invasive way and decision support tool (Blaak et al., 2021).

1.3 Antimicrobial resistance in diseased animals and the food chain

Carriage and infection of ESBL producing and CRKp is usually healthcare-associated in Europe. However, public health authorities and clinicians were alarmed when cases of CRE were reported in German swine farms in 2012 (Kluytmans, 2013), as well as in companion animals (Guerra et al., 2014). In veterinary medicine, carbapenems are not approved for livestock (Madec et al., 2017).

However, studies on ESBL-producing *E. coli* and *K. pneumoniae* have demonstrated that these multidrug-resistant bacteria are often transmitted between pets and their owners, as well as livestock and occupationally exposed humans (Hamza et al., 2016; He et al., 2017; Lazarus et al., 2015; Madec et al., 2017; Manges & Johnson, 2012; Marques, Belas, et al., 2019; Silva et al., 2022; Valentin et al., 2014).

In this doctoral thesis, we conducted a large-scale sampling of *K. pneumoniae* from the food chain (**Study 1**). We detected *K. pneumoniae* in 18.6% of fresh meat samples at the distribution level, which is lower than the prevalence reported in other studies: 50% in chicken meat in a European study (Rodrigues et al., 2022), 47% in retail turkey, chicken, and pork products from the United States (Davis et al., 2015), and 35% in calf and chicken meat samples from Turkey (Gundogan et al., 2011). The highest rates of *K. pneumoniae* were observed in chicken meat, consistent with other literature. (Hamza et al., 2016; Lazarus et al., 2015). Several factors may explain the difference in prevalence rates. First, the isolation method we used (McConkey agar) may have made it more challenging to differentiate *K. pneumoniae* from other bacteria, as McConkey agar is known to have limitations in distinguishing *Klebsiella* species (Rodrigues et al., 2022). Some studies have suggested that Simmons citrate agar with 1% inositol (SCAI) may be a more effective and specific medium for isolating *Klebsiella* species (Rodrigues et al., 2022; Van Kregten et al., 1984). Second, species identification using MALDI-TOF has been shown to be challenging for *K. pneumoniae* sensu stricto, potentially leading to misidentification (Rodrigues et al., 2018; Thorpe et al., 2022). This was confirmed in our study, where 15 isolates initially identified as *K. pneumoniae* by MALDI-TOF MS were later reclassified as other species within the KpSC through WGS. Lastly, large variations in prevalence of *K. pneumoniae* per country can be expected.

Despite the relatively high prevalence of *K. pneumoniae* in fresh meat samples, ESBL-producing *K. pneumoniae* carrying the CTX-M-15 gene was detected in only 1.2% (2 out of 168) of the samples, similar to the 1.3% observed in a European study (Rodrigues et al., 2022). In Germany, 60% of chicken meat samples were positive for ESBL producing Enterobacterales. However, the majority was *E. coli*, with only 1.67% of samples positive for the presence of ESBL producing *K. pneumoniae*, which is similar to our findings (Belmar Campos et al., 2014).

At the primary production level, we observed a low prevalence (1.7%) of *K. pneumoniae* in the feces of food-producing animals. Sparse information is available regarding the prevalence of *K. pneumoniae* fecal carriage in food-producing animals, but a prevalence of 29% was detected in fecal and hind limb swabs of dairy herds in Brazil (Nobrega et al., 2021). There are, however, multiple studies on the presence of ESBL/Ampc producing and/or carbapenem resistant isolates found in livestock, where selective isolation techniques were used. For example, Atterby et al. describe a prevalence of 2% of ESBL-producing *K. pneumoniae* in poultry, but detected none in cattle or pigs (Atterby et al., 2019). No ESBL/Ampc producing or carbapenem resistant isolates were detected.

Unlike in human medicine, reports of carbapenemase-producing organisms in animals are rare (Madec et al., 2017). While surveillance programs in Belgium monitor *E. coli* in the food chain, *K. pneumoniae* is not currently included in these efforts. In our monitoring study, one isolate (0.60%) from the food chain showed resistance to ertapenem, although no resistance to meropenem or imipenem was found. CPE contamination in food products remains rare in Europe. For instance, VIM-1-producing *E. coli* has been detected in retail seafood and pigs in Germany, as well as in retail meat in Belgium (Garcia-Graells et al., 2020; Köck et al., 2018; Roschanski et al., 2017). In contrast, CPE contamination is more common in Africa and Asia, where carbapenemase-producing *E. coli*, *K. pneumoniae*, and *Enterobacter cloacae* have been found in poultry, chicken meat, cows, milk, and pigs (Köck et al., 2018). In India and China, NDM producers are the most frequently identified CPEs in animals and food (Köck et al., 2018).

In contrast to food producing animals, carbapenems are authorized in exceptional clinical cases in companion animals, under the cascade according to Article 112 of the veterinary medicinal products Regulation 2019 of the European Union Legislation. As such, reports on CRKp are available and frequently investigate the association with clinical isolates (Garcia-Fierro et al., 2022; Marques, Belas, et al., 2019; R. Zhao, 2022). Here, we observed a CRKp from a companion animal, lacking carbapenemase genes. The observed resistance mechanisms was probably due to the presence of $bla_{\text{DHA-1}}$ and $bla_{\text{SCO-1}}$ in the combination with permeability defects of outer membrane proteins (*OmpK37*) (Debergh et al., 2022) (**Study 2**). Later we observed that this isolate from a companion animal was closely related to two human clinical isolates, confirming the possible transmission between humans and companion animals.

We identified a cluster involving two *K. pneumoniae* isolates from meat and one isolate from a wastewater sample in Leuven, with no SNP differences detected among the three isolates. Unfortunately, due to a lack of information on the food processing locations, further investigation into this intriguing cluster was not possible. However, Projahn et al. described contamination of chicken meat with ESBL-producing *K. pneumoniae* during the defeathering process (Projahn et al., 2019), with the water from defeathering machines ultimately being discharged into a WWTP. A similar contamination pathway could potentially explain the origin of this cluster.

2 Aspergillus fumigatus

2.1 The increase of antifungal resistance in the human sector

Reports of azole resistance in clinical settings have been steadily increasing since its first identification, posing significant public health challenges. The rise in azole-resistant *A. fumigatus* complicates the treatment of aspergillosis, leading to increased morbidity and mortality, particularly among vulnerable populations such as transplant recipients and patients with chronic lung diseases. Although monitoring of clinical *A. fumigatus* was not within the scope of this doctoral thesis, the establishment of continuous local surveillance programs is crucial. These programs are essential for tracking the evolving epidemiology of triazole resistance in *A. fumigatus* and for assisting clinicians in selecting appropriate treatments for *Aspergillus*-related diseases. Furthermore, implementing effective mitigation strategies is critical. These strategies include reducing the use of azole fungicides in agriculture, which contributes to environmental resistance, and enhancing the monitoring of resistant strains in both clinical and environmental settings. By addressing these factors, we can better manage and reduce the impact of azole resistance on public health.

Since 2016, all *A. fumigatus* complex isolates are screened for triazole-resistance at the national reference center (NRC). A fluctuating but stable trend of triazole resistance was observed over the years. From 2016 to 2020, surveillance data from the tertiary care center University Hospitals Leuven in Belgium indicated an average azole resistance prevalence of 7.1% [95% CI 6.6-7.7%], with 73% of isolates resistant to all three tested antifungals (Resendiz-Sharpe et al., 2021). This prevalence contrasts with data from the Netherlands, where a surveillance network reported an increase in the prevalence of culture-positive patients from 7.6% (58/760) in 2014 to 15% (114/774) in 2017. This rise prompted recommendations to combine a triazole antifungal with an echinocandin or liposomal amphotericin B for the initial treatment of IA (Lestrade et al., 2020). Similarly, an epidemiological study from the Danish Mycology Reference Center reported an increase in azole resistance from 1.8% (2/111) in 2010 to 3.8% (7/185) in 2014 among culture-positive patients (Jensen et al., 2016). As expected, *cyp51A* gene mutations were found in the majority of the Belgian isolates (81%), with the TR34/L98H mutation being the most prevalent (83%), followed by the TR46/Y121F/T289A mutation (13.8%). Interestingly, other or no *cyp51A* gene mutations were reported in 21.5% of the analyzed triazole-resistant cases (Resendiz-Sharpe et al., 2021).

As a tertiary care facility, the center treats patients from various regions in Belgium, making the reported triazole-resistance prevalence likely representative of the broader area. However, some regions or centers might have higher resistance rates due to their proximity to environmental hotspots or their specific patient populations, such as those with a high number of patients treated with triazoles for chronic *Aspergillus* diseases. The last multicenter cohort study on *Aspergillus* resistance among approximately 300 patients in Belgium was conducted in 2011-2012. Given the need for updated data, a new prospective nationwide surveillance study was carried out in 2022-2023. This study collected

isolates from patients with invasive *Aspergillus* infections across hospitals nationwide, with susceptibility testing performed at the NRC. Preliminary data indicate that the triazole resistance rate for *Aspergillus fumigatus* has nearly doubled in 2022-2023 compared to the 2011 study (9.7% vs. 4.6%), with over 85% of isolates resistant to all four tested azoles.

2.2 The environmental sector as the basis of azole resistance development

As detailed throughout this doctoral thesis, the widespread use of azole fungicides in the environment is considered one of the cornerstones in the development of azole resistance in *A. fumigatus*. The use of fungicides is critical for securing the food supply, as plant pathogenic fungi can cause up to 30% crop loss. However, the application of fungicides in agriculture and horticulture has been associated with the development of medical azole resistance due to their chemical similarity to medical azoles. The azole fungicides tebuconazole, epoxiconazole, propiconazole, and bromuconazole, are or have been intensively used for crop protection and in 2019, these were, respectively, the second, fourth, seventh, and tenth most sold triazoles in agriculture in Belgium. From a One Health perspective, the essential role of azole fungicides to secure the food supply should be balanced with the need to preserve the activity of structurally related azoles in the clinical practice.

Numerous studies have identified specific environments in Europe as hotspots for azole resistance in *A. fumigatus*, particularly in settings such as flower bulb waste, green waste, and woodchips (Burks et al., 2021; Doughty et al., 2021; Fraaije et al., 2020; Schoustra et al., 2019). Hotspots are defined as environments that not only support the growth, reproduction, and dispersal of *A. fumigatus* populations but also promote the selection of resistant phenotypes within a mixed population of susceptible and resistant strains. This selection occurs when substrates contain residues of DMIs at sufficient concentrations (Doughty et al., 2021). To date, there is limited data on the prevalence of azole-resistant *A. fumigatus* in the environment in Belgium. Therefore, we conducted this pilot study, incorporating air and soil/compost sampling in agriculture, horticulture, and composting facilities (**Study 3**). The study aimed to assess the prevalence of resistance and identify potential hotspots for the development of azole-resistant *A. fumigatus* (ARAf).

Between June 2020 and July 2022, a comprehensive sampling effort in Belgium collected 232 environmental samples to investigate the prevalence of azole-resistant *Aspergillus fumigatus* (ARAf). Of these samples, 136 were from agricultural settings (58.6%), 67 from horticultural settings (28.9%), and 29 from composting facilities (12.5%). In the first composting facility, a significant number of airborne spores were captured on RBCA medium. Due to contamination by other fungi, only a subset of these spores was analyzed on MC + T medium, resulting in the growth of 24 *A. fumigatus* isolates, with 16 colonies isolated from indoor air and 8 from outdoor air. Additionally, eight compost samples were collected, but no *A. fumigatus* colonies were detected in these samples. In the second composting facility, 170 colonies were isolated from air samples on RBCA medium. From these, 30 non-

contaminated *A. fumigatus* colonies were subcultured onto MC + T medium, with 29 colonies isolated from indoor air samples. Similar to the first facility, no *A. fumigatus* colonies were detected in the compost samples from this facility. Overall, 54 *A. fumigatus* colonies from both facilities were subjected to antifungal susceptibility testing, revealing that two isolates displayed phenotypic resistance to at least one medical azole. In horticulture, a total of 29 *A. fumigatus* colonies were isolated from the MC + T medium in air samples from greenhouses.

In the experimental cropland trials, 128 soil samples and 8 air samples were collected, yielding 925 *A. fumigatus* colonies. A high number of airborne spores (197) were captured on the MC medium, but no *A. fumigatus* was isolated from the MC + T medium. Similarly, soil samples showed a high spore count of *A. fumigatus* (728) on the MC medium, yet no *A. fumigatus* was isolated from the MC + T medium. This finding aligns with low prevalence rates of ARAf reported in Germany, the United Kingdom, France, and Italy, suggesting that cropland may not provide ideal conditions for *A. fumigatus* proliferation and dispersal. International data indicate that cropland is considered a 'coldspot' for ARAf (Doughty et al., 2021; Godeau et al., 2023).

In this investigation, a total of six isolates from horticulture and composting facilities exhibited phenotypic resistance to at least one medical azole, with four being pan-azole resistant. The prevalence of ARAf was found to be 2.62% (6/229). Resistant isolates were detected in air samples adjacent to the composting facility and in the residue heap of a horticultural research site. In five isolates, genetic mutations were identified in the *cyp51A* gene. Specifically, 66.7% of these isolates exhibited the TR34/L98H mutation, and 16.7% had the TR46/Y121F/T289A mutation, which are comparable to those reported in clinical cases in Belgium (Resendiz-Sharpe et al., 2021). One isolate did not display known resistance mutations in the *cyp51A* gene, suggesting the presence of other resistance mechanisms. The study faced challenges in isolating *A. fumigatus* from composting facilities due to the presence of other fast-growing species like *Mucorales* spp. This could indicate that the reported prevalence rates might be underestimated. Improved isolation techniques, such as the use of selective flamingo medium, are recommended to enhance detection accuracy (J. Zhang, Debets, et al., 2021).

Reports on resistance prevalence in the environment vary widely between studies. In Germany, the prevalence of ARAf in the environment and clinics was estimated at 1.3% and 3.2%, respectively (Barber, Riedel, et al., 2020). Environmental resistance rates across Europe range from negligible to nearly 14%, influenced by urban versus rural settings and the type of plant material involved (Doughty et al., 2021). Furthermore, literature indicates that ARAf has been recovered from various agronomic settings, with only sufficiently detailed investigations from flower bulb waste and cereal settings. Other crop-related findings are mainly from soil sampling, often showing low recovery rates of resistant isolates and inconsistent results across different sites and studies. This suggests that soil sampling alone is likely insufficient for distinguishing potential hotspots from background levels of ARAf distributed and deposited from airborne sources (Doughty et al., 2021). Comparing prevalence rates between countries is challenging due to the lack of standardized sampling procedures, underscoring the need for

European guidelines. Furthermore, there is a need for antifungal resistance stewardship in all areas of DMI (including comprehensive fungicide residue quantification of azoles, SDHIs, and QoIs) use as a critical component of the One Health concept to preserve the availability and efficacy of both the available medical antifungals and agricultural fungicides (Chowdhary et al., 2013; Chowdhary & Meis, 2018; Fisher et al., 2018). The primary aim of mitigation should be avoiding the mass release of *A. fumigatus* spores, and particularly of ARAf, to limit human exposure (Anon., 2017).

Studies indicate that ARAf hotspots typically involve plant waste materials containing DMI residues rather than directly resulting from fungicidal treatment of specific crops. This suggests that controlled waste management can mitigate the selective pressure of DMIs in these environments. For crops where residual organic material is neither processed in the field after harvest nor directed towards uses like straw, hay, or silage, the drive for a circular economy encourages the return of organic "waste" material to the original setting. However, this necessitates appropriate waste management and composting practices to prevent the creation of ARAf hotspots (Verweij et al., 2020). To avoid the long-term accumulation of plant waste in unmanaged piles, it is crucial to prioritize static, undisturbed heaps that are protected from the outdoor environment and where temperatures are allowed to reach 55–60°C for a sufficient duration (Anon., 2017; Doughty et al., 2021; Schoustra et al., 2019). Activities such as screening, shredding, and turning of compost, which can release significant numbers of conidia, need to be optimized (Millner et al., 1980; Mullins et al., 1976; O'Gorman, 2011; Pearson et al., 2015). It will be important to identify the stages and aspects of the cropping cycle that most effectively facilitate the amplification of ARAf, with particular reference to the generation and management of plant waste, both in- and off-field (Doughty et al., 2021). Additionally, hydrolysis of waste at centralized collection sites has been shown to reduce *A. fumigatus* populations (Anon., 2017; Schoustra et al., 2019). Creating unfavorable growth conditions in compost for *A. fumigatus* is likely to reduce the probability of resistant genotypes emerging via spontaneous mutations. Current research is exploring this approach for managing flower bulb waste hotspots in the Netherlands (Verweij et al., 2020). Further waste management options that avoid the creation of hotspots include using residual organic matter for animal feed, as a feedstock for biogas production, for alcohol fermentation, or for incineration to generate power (Doughty et al., 2021).

At the European level, the joint mandate of European Centre for Disease Prevention and Control (ECDC), European Chemicals Agency (ECHA), European Environment Agency (EEA), European Food Safety Authority (EFSA), European Medicines Agency (EMA), and the Joint Research Centre (JRC), is currently investigating the impact of azole fungicides, other than as human medicines on the development of ARAf. In this mandate, on which I participated for TOR 7 ("studies by applicants"), the goal is to generate a complete report on the current knowledge about ARAf epidemiology, the data gaps and future research needed. Coordinated national and international actions, along with European standardized guidelines and protocols, are essential to harmonize environmental surveillance and improve understanding of *A. fumigatus* resistance epidemiology. Effective management and

surveillance require mapping of fungicide use and waste streams within agriculture and horticulture, especially those involving fungicide-treated crops. Additionally, studies should extend beyond agricultural and horticultural settings to include residential gardens and hospital environments. Research on ARAf in retail products remains scarce and warrants further investigation.

These findings indicate a varied presence of *A. fumigatus* across different environmental settings in Belgium. Notably, azole-resistant *A. fumigatus* was identified in composting facilities, while no resistant isolates were found in agricultural samples. Here we observed the predominance of TR34/L98H strains associated with pan-azole resistance, which is in line with the observations in the clinics. This highlights the need for further research to better understand the distribution and drivers of azole resistance in *A. fumigatus*, particularly in different environmental contexts, to prevent the spread of resistant strains to clinical settings.

In conclusion, this study reveals the presence of azole-resistant *A. fumigatus* with an environmental background in Belgium, underscoring the importance of the One Health perspective to track resistance development and prevent its impact on both animal and human health. With a prevalence of 2.62% of ARAf and the first occurrence of the TR34/L98H and TR46/Y121F/T289A mutations in horticulture and compost isolates in Belgium, future work should focus on extensive sampling, azole residue measurements, and standardized environmental surveillance. Collaboration with public health authorities is crucial for effective management and mitigation of azole resistance.

2.3 The role of the veterinary sector

Aspergillus fumigatus is a significant opportunistic pathogen affecting a wide range of animals, including birds and mammals, leading to illness and often death (Tell, 2005). This fungus is particularly problematic in captive birds, such as penguins, where aspergillosis is prevalent (Cateau et al., 2022; Ewbank et al., 2021; Flach et al., 1990; Graczyk & Cockrem, 1995; Reed et al., 2020). In contrast, aspergillosis is less common in free-ranging birds and relatively rare in mammals, despite the increasing number of immunocompromised animals (Ewbank et al., 2021; A. M. Melo, Silva-Filho, et al., 2020; A. M. Melo, Stevens, et al., 2020; Tell, 2005). Exceptions include canine sinonasal aspergillosis, equine guttural pouch mycosis and bovine mycotic abortion caused by *Aspergillus* spp (Dobesova et al., 2012; Elad & Segal, 2018; Tell, 2005). Predisposing factors for the development of aspergillosis in birds and mammals consists of immunosuppression, previous debilitating illnesses, stress factors and environmental factors (Higgins & Pusterla, 2006; U. P. Melo et al., 2024; Tell, 2005).

The study aimed to fill gaps in knowledge about *A. fumigatus* and azole resistance in animals by examining 152 cases of aspergillosis in birds and mammals (**Study 4**). The overall resistance rate in veterinary isolates was found to be 3.3%, with 2.8% in birds and 3.7% in mammals. The resistance rate in birds was higher than in some other studies but lower than what has been reported in the Netherlands (van Dijk et al., 2024). The findings align with the general trend observed in Europe, where

environmental practices and hotspots contribute to variations in resistance prevalence in the veterinary sector (Barber, Riedel, et al., 2020; Barber, Scheufen, et al., 2020; Nawrot et al., 2019; van Dijk et al., 2024).

In **Study 5** we described the mortality rates and the associated resistance prevalence rates in Humboldt penguins, a population with high risk of developing aspergillosis. We observed higher resistance rates in Humboldt penguins (17.14%) compared to the overall resistance rate in birds and mammals from study 4. This can be explained on the one hand by the anatomy of the Humboldt penguins (the high body temperature of birds - 39°C to 41°C - and the thermotolerance of *A. fumigatus*, a scarcity of immune surveillance cells in the air sacs and the absence of structures like an epiglottis or diaphragm to prevent spore inhalation) and on the other hand by the external factors (thermal discomfort, overcrowding, stress, high spore load in the environment, contaminated feed, soil and bedding and poor husbandry).

Preventive measures implemented in 2019, such as improving ventilation, monitoring water temperature, providing shade, and restricting direct contact between visitors and penguins, helped to reduce the mortality rate. However, the incidence of aspergillosis increased again when the breeding program resumed in 2020, with a threefold higher incidence in breeding animals compared to non-breeding ones. Mortality rates due to aspergillosis were notably high, reaching 86.7% in 2017 and 76.5% in 2018, with a significant increase in incidence during the summer of 2018, likely due to extreme weather conditions. The effect of overcrowding was also evident, as higher population densities correlated with increased aspergillosis cases. Furthermore, environmental sampling revealed slightly higher fungal loads in spring and summer. We observed higher mortality rates during summer months in the Humboldt penguin population, likely due to the higher spore load and the heat which is a stress factor for the animals, affecting their immune system.

Diagnosis of aspergillosis is challenging due to the lack of reliable tools, and treatment often comes too late to be effective (Desoubeaux et al., 2022). Additionally, because of the concurrent severe underlying diseases in mammals, treatment or prophylaxis is challenging (U. P. Melo et al., 2024). Treatment outcomes are often poor due to late diagnoses and advanced disease stages at the time of treatment initiation. If suspected early, treatment with oral itraconazole (ITC) can be started, and ITC prophylaxis is typically used for susceptible individuals. In **Study 5**, we described that some Humboldt penguins received itraconazole prophylaxis for a prolonged period of time (sometimes even over 1 year). In **Study 4** we have limited data on azole treatment regimens, and only a few animals were treated. In our studies, we did not observe evidence of resistance development through this prolonged treatment, however, this was not the goal of the study nor was there enough data to make this a hard conclusion. On the contrary, we mostly observed the TR34/L98H mutation, linked to the environmental route of resistance development in both studies. Interestingly, in the study discussed in **Study 5**, we also observed the G54R mutation, usually linked to the patient route, in an environmental isolate. Although

uncommon, this mutation has been detected before in some environmental isolates: from commercial vineyard soil mix (Riat et al., 2018) and fruit (Tangwattanachuleeporn et al., 2017).

The study underscores the need for more comprehensive surveillance and research on azole resistance in veterinary medicine. The lack of clinical breakpoints for *A. fumigatus* in animals and limited data on azole pharmacokinetics and effectiveness complicates treatment. The main driver of azole resistance in the veterinary sector appears to be environmental exposure rather than treatment, raising concerns about increasing resistance trends and highlighting the importance of careful antifungal use and regular monitoring in veterinary medicine.

3 Future perspectives and recommendations

Despite significant progress in understanding antimicrobial resistance in *K. pneumoniae* and *A. fumigatus* through a One Health approach, many questions remain unanswered, warranting further investigation. Key unresolved issues regarding triazole resistance in *A. fumigatus* involve understanding exposure risks, identifying resistance hotspots, and standardizing surveillance methods.

One of the objectives of my PhD was to develop guidelines for monitoring azole resistance in *A. fumigatus* in the environment. However, it became evident that more research is needed before establishing a national surveillance system. To address the concern of the increasing prevalence of ARAf in the environment, further research focusing on hotspot identification is warranted, with the inclusion of azole concentration measurements. Comprehensive environmental sampling efforts should be conducted on a large scale, encompassing compost heaps from both ornamental and agricultural crops, fruit waste from conventional and organic fruit farming, and waste from the waste management industry. These efforts should be paired with assessments of fungicide concentrations, such as tebuconazole, prothioconazole, difenoconazole, and mefentrifluconazole. Additionally, mapping the quantities of plant protection products used across Belgium should be included in this initiative. Moreover, a pilot study on wastewater-based epidemiology in this field is in progress. Although research data is limited, our preliminary findings suggest that this could become a promising area for environmental surveillance on a broader scale. These ongoing research initiatives are included in the new project proposal I have written for the National Action Plan on Antimicrobial Resistance (NAP AMR) 2025-2029.

In addition, follow-up studies of this doctoral thesis include the project titled ‘Transmission of (azole resistant) *Aspergillus fumigatus* in the hospital environment in Belgium’ (S69061). This study, involving five university hospitals across Flanders, Brussels, and Wallonia, will conduct sampling during four non-consecutive periods throughout the year, focusing on wards with the highest-risk patients. Additionally, air sampling outside hospitals will be performed to investigate potential transmission routes within the hospital environment. Furthermore, a citizen science project will be initiated to assess the presence of ARAf isolates in the home environment of citizen scientists and examine the impact of home composting on indoor environments. In collaboration with Dr. Luc Cornet,

we are conducting an in-depth whole-genome sequencing (WGS) analysis on *A. fumigatus* isolates from various sources to perform ancestral reconstruction.

All these research efforts are essential for generating data specific to the Belgian context and for appropriately addressing the situation and setting up a surveillance system. However, since *A. fumigatus* is not confined by borders, European-wide surveillance is crucial. The lack of standardized surveillance systems for tracking azole-resistant *A. fumigatus* (ARAf) and an incomplete understanding of how antifungal resistance evolves or can be managed remain significant challenges. The GAP-AFR research project, funded by JPIAMR, aims to standardize research on azole resistance development. As discussed in my doctoral thesis, I am participating as an external expert in a joint European mandate by EMA/ECHA/EFSA/EEA on the impact of non-medical azoles on the development of azole resistance in *A. fumigatus*. My role involves providing scientific advice on the experimental data that applicants should submit for introducing new antifungals to the market. This document will prove to be a baseline document for further epidemiological studies on azole resistance selection in *A. fumigatus.*

Future research on *K. pneumoniae* is planned through a tailor-made update of the AMR-ARRAY (Timmermans et al., 2022), an assay featuring probes designed to identify ARGs conferring resistance for various antibiotic classes. This tool, specifically tailored for *K. pneumoniae*, promises cost-effective and efficient screening in large-scale sampling efforts, pinpointing isolates that warrant further WGS analysis. In this study, McC and McC+CTX media were employed to isolate *K. pneumoniae* and ESBL/AmpC *K. pneumoniae*, aligning the results with the food chain monitoring. However, several studies indicate that more effective media, such as the SCAI medium, exist for isolating this bacterium. Consequently, future research should explore testing diverse media types for isolating *K. pneumoniae* from sources like meat, feces, wastewater, and clinical human and veterinary samples.

Within this doctoral thesis, we successfully calculated the prevalence of *K. pneumoniae* in the food chain. However, due to selective sampling procedures and the inclusion of convenience samples, determining the prevalence of (antimicrobial-resistant) *K. pneumoniae* in each sector proved challenging. The current selection of clinical *K. pneumoniae* isolates focused on ESBL-producing and carbapenem-resistant strains, complicating prevalence rate calculations and cross-sector comparisons. Implementing a more systematic sampling approach could enhance comparisons of resistance prevalence and assist in identifying AMR hotspots in *K. pneumoniae*.

Our extensive research has uncovered several potential transmission events involving antibiotic-resistant *K. pneumoniae*. To delve deeper, future studies should incorporate long-read sequencing (using Oxford Nanopore Technologies) for clustered isolates to thoroughly analyze plasmid content. This could allow for tracking MGEs across pathogens and host compartments and could provide greater resolution of the sources of transmission of AMR and information on potential further spread. Furthermore, we observed great diversity in ARG content per sector and geographical location, indicating that local surveillance is necessary to take local actions. In certain instances in our collection, tracing the origin of samples was unsuccessful, hindering in-depth analysis of transmission routes and the identification of

common sources. For robust epidemiological studies, such data are indispensable; therefore, future efforts must prioritize obtaining all necessary information.

In conclusion, there is a pressing need for integrated surveillance systems that monitor *K. pneumoniae* across human, animal, and environmental sectors, enhancing the understanding of cross-sector transmission dynamics. Research to identify risk factors and transmission mechanisms, including direct contact and foodborne routes in animals, as well as the role of the environment as a reservoir for resistant strains is essential. Promoting interdisciplinary research collaborations that bring together experts from human medicine, veterinary science, environmental science, and public health is critical for addressing the challenges posed by *K. pneumoniae*. These research efforts should include phenotypic resistance testing (EUCAST) and WGS, preferably using hybrid sequencing to study plasmids and other MGEs in detail for potential transmission events. Policymaking should be informed by robust scientific evidence to develop effective control measures that span human, animal, and environmental health, as outlined by the NAP AMR. Addressing these research gaps through targeted studies and collaboration is essential for building a comprehensive understanding of *K. pneumoniae* and effectively managing its impact across sectors. All recommendations are summarized in Table 20 and Table 21.

Table 20 - Recommendations for improvements in *Klebsiella pneumoniae* surveillance and research per type of actor, ranked by importance. Recommendations are also categorised by short, middle and long term goals.

Type of actor	Priority	Recommendation	Short (S) / Middle (M) / Long (L) term
Competent authority	1	High prevalence rates of *K. pneumoniae* were observed in fresh meat compared to in the primary production. Therefore, sampling at **meat processing environments** in the national monitoring concerning the food chain should be included to investigate the potential contamination of the fresh meat in the meat processing plants. This should include regular sampling of the workers, the different working stations, the machinery, the floors, the drains and the effluent water, to elucidate the influence of these environments on the transmission of antimicrobial resistance genes in *K. pneumoniae.* Reporting of *K. pneumoniae* from diseased animals should be implemented in a national monitoring effort.	L
Research	1	Perform research regarding **wastewater-based epidemiology** for *K. pneumoniae*. It has the potential of being an early warning system regarding emerging resistant strains in humans. Research to delve deeper into the power of WBE regarding **antimicrobial resistance** adopting the **One Health approach** in *K. pneumoniae* should be conducted. Wastewater sampling represents a **non-invasive sampling effort** gathering information of a **large population size**. To broaden the knowledge about the influence of the **hospital environment** on the environment, hospital effluents should be investigated, alongside the influent and effluent of the wastewater treatment plant that receives the hospital wastewater. **Localized data** from Flanders, Brussels and Wallonia could inform local actions and allow comparison of the genomic determinants of ESBL/AmpC and/or carbapenemase gene prevalence at country level.	L
Laboratories / competent	1	Create a **Belgian One Health surveillance platform** regarding **AMR surveillance in critical pathogens** in the human sector, wastewater, the food chain and in diseased animals. This will allow faster response in case	L

Type of actor	Priority	Recommendation	Short (S) / Middle (M) / Long (L) term
authorities / Research		of **outbreaks** and a better understanding of the **epidemiology** and **transmission** of *K. pneumoniae*. This should involve **standardized MIC testing** per sector, **standardized and centralized WGS** and accompanying **standardized pipelines** (e.g., the *Klebsiella* pipeline developed during this PhD). Both short and long read sequencing should be included to allow **plasmid surveillance**. Tools that **predict phenotype based on genotype** should be included. These databases should be linked with **databases regarding treatment outcome**. **Bioinformaticians** should ensure good data analysis and interpretation at a central level.	
Competent authority	1	Include **metadata** collection regarding the **processing plants** in the food chain. Currently there is a gap in the traceability in the distribution sector.	M
Laboratories / competent authorities	1	**Design MIC panels specifically for *K. pneumoniae***, which will provide a robust framework for accurately **establishing ECOFF values**, enabling effective surveillance and detection of resistance trends in *Klebsiella* spp. These should include a wide gradient of antibiotic concentrations to accurately capture the full susceptibility range of *Klebsiella* isolates. The panel should contain antibiotics relevant to *Klebsiella* spp. The new MIC panels should be tested on a wide array of *Klebsiella* isolates from various sources and geographical regions to ensure the ECOFF values are representative. Protocols from EUCAST should be followed to ensure that results are reproducible and comparable. **MIC panels for broth microdilution tests** specific for **diseased animals**, the equivalent as available for diseased humans, should be made commercially available to guide treatment. Appropriate antibiotics and a wide range of concentrations for each antibiotic should be selected. The MIC panel should be tested on a collection of veterinary isolates from diseased animals across different species and geographic areas.	M

Type of actor	Priority	Recommendation	Short (S) / Middle (M) / Long (L) term
Research	2	Investigate the i**nfluence of water purification treatments** on the presence of antibiotic resistant bacteria and antibiotic resistance genes, accompanied by studying new methods that could limit the spread of those antibiotic resistant bacteria and antibiotic resistance genes. Implement a legal framework to establish contamination thresholds with AR. This should be precluded by risk assessment studies involving toxicology, epidemiology and exposure risks.	L
Research	2	Increase **fundamental research** into the epidemiology of *K. pneumoniae*. This research should focus on the transmission of antimicrobial resistance (AMR) genes across humans, animals, the food chain, and the environment, utilizing plasmid surveillance through hybrid sequencing and phylogenetic analysis. Comprehensive sampling should occur simultaneously across the entire chain, from farm and animal levels to production and processing sites, including workers at these locations and associated waste streams, such as wastewater. The roles of food-producing animals, companion animals, and environmental reservoirs (such as wastewater) in the transmission of MDR and hypervirulent strains of *K. pneumoniae* are poorly understood. The **zoonotic potential** of *K. pneumoniae* remains unclear, particularly regarding strains found in food-producing animals. More studies are needed to assess how frequently *K. pneumonia*e is transmitted from animals to humans and/or inversely, and under what conditions this occurs. There is limited information on the **virulence factors** and antibiotic resistance mechanisms of *K. pneumoniae* strains in animals and the environment.	L

Type of actor	Priority	Recommendation	Short (S) / Middle (M) / Long (L) term
Laboratories	3	To assess different **targeted protocols** to identify the optimal protocol for *K. pneumoniae* monitoring. Specific culturing methods should be investigated, alongside genomic protocols. This should be performed for all sectors implemented in the One Health approach.	S
Research	3	Implement the **AMR-ARRAY** as a screening tool for performing WGS in *K. pneumoniae*	S

WGS = whole genome sequencing; WBE: wastewater-based epidemiology; AMR: antimicrobial resistance; MIC: minimal inhibitory concentration; ARB: antibiotic resistant bacteria; ECOFF: epidemiological cut-off values; ESBL: extended-spectrum β-lactamase

Table 21 - Recommendations for improvements in *Aspergillus fumigatus* surveillance and research per type of actor, ranked by importance. Recommendations are also categorised by short, middle and long-term goals.

Type of actor	Priority	Recommendation	Short (S) / Middle (M) / Long (L) term
Competent authorities	1	**Allocate sufficient budget** to research and governmental institutes for improving the knowledge about azole resistance in *A. fumigatus* and implement qualitative surveillance efforts.	L
Research	1	Identification of **hotspots for the selection of azole resistant *A. fumigatus* in Belgium** should be performed. This includes the measurement of azole residue concentration, mapping waste streams of treated green and wood waste, mapping the azole use in agriculture, horticulture and the wood industry. Research concerning limiting the spread of azole resistant *A. fumigatus* in those hotspots should be conducted.	M
Surveillance / laboratories / competent authorities	1	Implement **standardized** and linked **antifungal resistance surveillance networks** at national and international levels (**European and global surveillance**) together with **harmonized definitions and data types.** This initiative requires a structured, collaborative approach to ensure data consistency, accessibility, and the generation of actionable insights. Effective implementation should include establishing **standardized protocols** for sampling, testing, and interpreting antifungal resistance data. Developing secure **national repositories** to collect resistance data from hospitals, laboratories, and environmental sources will enable **real-time surveillance** and in-country trend analysis. Integrating data from human healthcare, veterinary services, and agriculture will help track resistance patterns across sectors in a **One Health approach**, essential for understanding antifungal resistance (AFR) origins and transmission.	L

Type of actor	Priority	Recommendation	Short (S) / Middle (M) / Long (L) term
		Building a **global AFR database** will support international data sharing, while implementing **whole genome sequencing** will help identify resistance mechanisms and track **transmission** routes across borders. Establishing **bioinformatics pipelines** and databases focused on resistance genes in fungal pathogens will allow for precise linkage between genotype and phenotype data, improving surveillance accuracy and risk assessment. To ensure uniformity, **laboratory staff** should **receive standardized training** in resistance testing and data entry. Technical support and funding should be directed toward **building surveillance infrastructure** in low- and middle-income countries, where resistance data may currently be limited. Creating **early warning systems** to flag emerging resistance trends will facilitate timely alerts to health authorities. Finally, **policy support** and **public awareness** are essential: findings should be communicated regularly to national and international health agencies to highlight trends and inform policy needs, while healthcare providers and the public should be educated on responsible antifungal use and resistance prevention strategies.	
Surveillance	1	Implement a **Belgian One health surveillance system.** Establishing a One Health antifungal resistance (AFR) surveillance system in Belgium requires a **cohesive framework** connecting human healthcare, veterinary services, agriculture, and environmental monitoring. A **national One Health working group**, composed of representatives from public health, animal health, agriculture, and environmental sectors, would lead this initiative, defining objectives, standardizing protocols, and overseeing implementation. Broad participation from hospitals, diagnostic laboratories, veterinary clinics, agricultural organizations, food industry representatives, and environmental agencies will ensure wide support and collaboration	M

Type of actor	Priority	Recommendation	Short (S) / Middle (M) / Long (L) term
		across sectors. To ensure consistency, the program would develop **standardized protocols for sampling**, **testing, and interpreting AFR data**. These protocols should define sample types (such as human clinical samples, animal and agricultural samples, and wastewater) and the frequency of sampling, and they would include uniform methods for antifungal susceptibility testing aligned with EUCAST standards. Clear criteria for interpreting resistance data would enable comparisons across sectors, improving consistency and reliability. A centralized, **secure national repository** would be crucial for collecting and storing AFR data from all sources, with real-time data entry capabilities and controlled access for authorized users. Privacy protocols, in line with Belgian and EU data protection regulations, would ensure data confidentiality. Integrating data from human healthcare, veterinary medicine, agriculture, and environmental monitoring in a centralized platform would facilitate comprehensive AFR tracking across sectors. By enabling **cross-sectoral analysis**, this One Health approach would reveal trends, transmission routes, and potential sources of AFR, providing a clearer understanding of its origins and pathways. **Whole genome sequencing** would be a valuable tool in tracking AFR by identifying resistance genes and tracing transmission routes. A **bioinformatics pipeline** and specialized databases would link genotype to the observed phenotype, allowing for precise surveillance and predictive analysis of resistance spread.	
Veterinarians / Medical doctors	1	**Raise awareness** about the occurrence of azole-resistant *A. fumigatus* in animals and human population at risk and its **consequences for the treatment**. Resistance testing should become a routine laboratory analysis to guide treatment, especially, if available, in those regions where environmental azole resistance	M

Type of actor	Priority	Recommendation	Short (S) / Middle (M) / Long (L) term
		rates above 10% have been detected. When possible, select antifungals based on sensitivity testing rather than empirical treatment, especially for immunocompromised patients or severe infections. Furthermore, reporting of cases of resistant *A. fumigatus* to regional or national health databases could help track resistance patterns and enable rapid response measures.	
Research	2	Conduct research concerning the **influence of the home environment** (at large) on azole resistant *A. fumigatus* exposure as they may present a risk for the introduction to the general population. This should involve citizen science to collect as much data as possible, by using air sampling in their home environment. Data collection should be performed at the same time by all citizen scientists and a geographical spread of sampling locations across Belgium is necessary. This will allow us to study the prevalence of azole resistance covering a broad geographic reach and investigate the risk for the population. This would raise awareness of antifungal resistance and environmental health in the general population	S
Research	2	Investigate the added value of **wastewater-based epidemiology** for environmental surveillance of azole resistant *A. fumigatus* in Belgium.	S
Research	2	Conduct research concerning the influence of the internal and external **hospital environment** on the exposure to azole resistant *A. fumigatus* to critical patients	S
Research	3	Increase **fundamental research** to identify molecular mechanisms of antifungal resistance and associated diagnostic markers	L

Type of actor	Priority	Recommendation	Short (S) / Middle (M) / Long (L) term
Laboratories	3	Bring **commercial EUCAST plates** on the market to perform antifungal susceptibility testing in *A. fumigatus* in a standardized and harmonized way	M
Research	3	Generate **globally accessible genomic antifungal resistance databases** for *A. fumigatus* that also link *in vitro* and *in vivo* resistance to clinical outcomes	L

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APPENDIX

Curriculum vitae

Personal information

PharmD. Hanne Debergh

PhD student

Sciensano, Service Mycology and Aerobiology

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Education

Academic

16-12-2019 - Present PhD research at the Scientific Service of Mycology and Aerobiology, in collaboration with food pathogen and veterinary bacteriology service. Sciensano, Belgium (Investigation of resistance mechanisms and their transmission in emerging pathogens applying the 'One Health' concept)

2017- 2019 Master in Biomedical science – Tropical and infectious diseases at Antwerp University (UA, Belgium)

2015-2017 Master in Pharmaceutical Sciences – Industry at Ghent University (UGent)

2013-2015 Bachelor in Pharmaceutical Sciences, Ghent University (UGent, Belgium)

Training

- Workshop data visualisation (Sciensano, 2023)
- Introduction to R course (Sciensano, 2022)
- Scientific writing (Liège, 2022)
- The floor is yours (Sciensano, 2022)
- Galaxy training (Sciensano, 2021)
- Systematic review (Cebam, Leuven, 2021)
- Next-generation sequencing in routine clinical microbiology and infectious diseases (ESGMD, 2021)
- Introduction to medical mycology (Sciensano, 2020)
- EURL-AR 2021

Skills

Wet lab experience, MALDI-TOF MS, Molecular Biology, Next-generation sequencing, Microbiology, FTIR, Antifungal susceptibility testing, wastewater analysis, antibiotic susceptibility testing

Project and management skills

Languages

Dutch native language

English fluent

French proficient

German basic

Scientific activities and projects

Member

- International Society for Human and Animal Mycology (ISHAM)
- ESCMID
- Belgian Society for Human and Animal Mycology (BSHAM)

Chair at conferences

One Health European Joint project – annual scientific meeting (OHEJP ASM), Orvieto, Italy, 2022

Publications

Debergh, H., Maex, M., Garcia-Graells, C., Boland, C., Saulmont, M., Van Hoorde, K., & Saegerman, C. (2022). First Belgian Report of Ertapenem Resistance in an ST11 *Klebsiella Pneumoniae* Strain Isolated from a Dog Carrying blaSCO-1 and blaDHA-1 Combined with Permeability Defects. Antibiotics (Basel, Switzerland), 11(9), 1253. https://doi.org/10.3390/antibiotics11091253

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Debergh H., Haesendonck R., Botteldoorn N., Martel A., Pasmans F., Saegerman C., Packeu A. (Accepted in *One Health*) Pan-azole resistance in clinical *Aspergillus fumigatus* isolates carrying TR34/L98H from birds and mammals in Belgium.

Reviewer for scientific papers

Molinero, R. L., Alava, K. S. H., Devoto, T. B., Sautua, F., Carmona, M., Cuestas, M. L., & Pena, G. A. (2024). Prevalence of azole-resistant *A. fumigatus* and other aspergilli in the environment from Argentina. *Medical Mycology*, myae098. https://doi.org/10.1093/mmy/myae098

Posters/Presentations/Reports

Posters

H. Debergh, C. Garcia-Graells, C. Boland, K. Van Hoorde; High rate of carbapenem resistant *Klebsiella pneumoniae* in wastewater from Belgium, **ECCMID, 15- 18 April 2023,** Copenhagen, Denmark

H. Debergh, K. Goens, F. Vercammen, C. Saegerman, A. Packeu; Aspergillosis in Humboldt penguins – susceptibility patterns of clinical and environmental isolates from a Belgian zoo, 2018-2022, The International Society for Human and Animal Mycology, **ISHAM, 20-24 September 2022**, New Delhi, India.

H. Debergh, P. Becker, C. Van den Eynde, F. Baert, E. D'hooge, R. De Pauw, A.-C. Normand, R. Piarroux, D. Stubbe; Screening of Belgian bats and hibernacula for the description of related fungal microbiomes and the detection of *Pseudogymnoascus destructans*; International Society for Human and Animal Mycology, **ISHAM, 20-24 September 2022**, New Delhi, India.

D. Stubbe, P. Becker, E. D'hooge, **H. Debergh**, A. Packeu; Validation panels for MALDI-TOF MS identifications of fungi; The International Society for Human and Animal Mycology, **ISHAM, 20-24 September 2022**, New Delhi, India.

H. Debergh, C. Garcia-Graells, C. Boland, K. Van Hoorde, C. Saegerman ; High impact of the meat processing environment on the prevalence of *Klebsiella* spp. In Belgium, One Health European Joint program Annual scientific meeting (**OHEJP ASM**) ; **11-13 April 2022**, Orvieto, Italy

H. Debergh, L. Delbrassine, C. Garcia-Graells, K. Van Hoorde, Kitchen sponges - home sweet home for *Klebsiella spp.*, Belgian society for microbiology, **BSFM**, **7 – 8 October 2021,** Brussels, Belgium

H. Debergh, C. Saegerman, R. Haesendonck, N. Botteldoorn, A. Packeu; High burden of azole-resistant *Aspergillus fumigatus* in veterinary medicine; **Trends in Medical Mycology** – **8 - 11 October 2021** – Aberdeen (Scotland), Online Hybrid Session.

Oral presentations

Debergh H.; The potential role of the environment in azole resistant veterinary *A. fumigatus* isolates – penguin in the coalmine?; ISHAM congress, 20-24 September 2022, New Delhi, India.

Debergh H, Environmental sampling of *A. fumigatus* in Belgium; Expert meeting on environmental *A. fumigatus*, Wageningen, 4 November 2022,The Netherlands.

Debergh H.; High impact of the meat processing environment on the prevalence of *Klebsiella* spp. In Belgium; KlebClub series; 10 May 2022, Online.

Debergh H.; High detection rate of *Klebsiella* spp. in used kitchen sponges from the domestic environment; BSM conference, 11 March 2022, Brussels, Belgium.

Debergh H.; Ertapenem resistance in an ST11 *Klebsiella pneumoniae* strain isolated from a dog; AMCRA scientific meeting, 27 June 2023, Brussels, Belgium

Debergh H.; Pulmonary Aspergillosis in Humboldt Penguins—Susceptibility patterns and molecular epidemiology of clinical and environmental *Aspergillus fumigatus* isolates from a Belgian zoo, 2017–2022; AMCRA scientific meeting, 25 June 2024, Brussels, Belgium

Debergh H.; Ertapenem resistance in an ST11 *Klebsiella pneumoniae* strain isolated from a dog; ARMB scientific meeting, 21 September 2024, Brussels, Belgium

Reports

- BELMAP report, OneHealth report on antibiotic use and resistance in Belgium, 2022
- National action plan on antimicrobial resistance in Belgium, NAP-AMR, 2019-2024

Projects

- Interagency mandate (EMA/ECHA/EFSA/ECDC/EEA): Request for a Scientific Report on the impact of the use of azole fungicides, other than as human medicines, on the development of azole-resistant *Aspergillus* spp. – TOR 7 (Studies for applicants) (external expert - finalising)
- National action plan AMR 2025- 2029 – Environnemental surveillance of *A. fumigatus* in Belgium (submitted)
- Transmission of (azole resistant) *Aspergillus fumigatus* in the hospital environment in Belgium, S69061 (ongoing)

Supervisor positions

- Bachelor internship of 3 months in the lab of food pathogens – Validation of wastewater filtration for the isolation of *Klebsiella* spp. Feb – April 2021
- Internship of 8 weeks learning routine diagnostics in the lab of mycology and aerobiology – MIC analysis and MALDI TOF analysis – March – May 2021
- Bachelor internship of 3 months in the lab of food pathogens – Biofilm formation of *Klebsiella* spp. isolated from wastewater. Feb – May 2022
- Internship of 8 weeks learning routine diagnostics in the lab of foodborne pathogens – DNA extraction for WGS and FTIR analysis on *K. pneumoniae*. Feb-March 2023
- Erasmus internship of 3 months in the lab of mycology and aerobiology - MIC analysis and MALDI TOF analysis of *A. fumigatus* and development of a new detection technique of fungi and yeasts in dust. April – June 2023
- Bachelor internship of 3 months in the lab of mycology and aerobiology - MIC analysis and MALDI TOF analysis of *A. fumigatus* isolated from wastewater treatment plants (WWTPs). January – May 2024
- Internship (Master student): validation of the LipidArt module of Bruker for the identification of fungi using lipidomics. February – June 2024

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