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Equine ulcerative keratitis in Belgium: Associated bacterial isolates and in vitro antimicrobial resistance in 200 eyes

Eline M. Vercruysse[1] | **Florine P. Narinx**[1] | **Albane C. M. Rives**[2] | **Aurélie C. Sauvage**[1] | **Magda F. Grauwels**[1] | **Sébastien J. Monclin**[1]

[1]Ophthalmology Service, Veterinary Teaching Hospital, University of Liège, Liège, Belgium

[2]Department of Veterinary Management of Animal Resources, Veterinary Teaching Hospital, University of Liège, Liège, Belgium

Correspondence
Eline M. Vercruysse, Steenbeekpad 2 8880 Sint-Eloois-Winkel, Belgium.
Email: eline.vercruysse@hotmail.be

Abstract

Purpose: To describe bacterial isolates and associated antibiotic resistance from horses with ulcerative keratitis in Belgium.

Methods: Medical records from horses with ulcerative keratitis presented to the ophthalmology service of the Veterinary teaching hospital of Liege, Belgium, between 2014 and 2021 were evaluated. Bacterial isolates were identified and VITEK® 2 (Biomérieux) provided antimicrobial susceptibility testing and resistance detection.

Results: Two hundred eyes of 196 horses were sampled. Ninety-seven eyes had a positive bacterial culture (48.5%) and 139 bacterial isolates were identified. *Staphylococcus* (63/139: 45.3%) and *Streptococcus* (33/139: 23.7%) were the most frequent genus isolated. *Staphylococcus aureus* (21/139: 15.1%) was the most frequent species isolated of which half were methicillin-resistant (MRS). *Streptococcus equi* subsp. *zooepidemicus* (19/139: 13.7%) was the second most identified bacterial isolate. Only two *Pseudomonas* species were isolated (2/139: 1.4%). The overall resistance of all bacterial isolates against chloramphenicol (12.4%) and fluoroquinolones (14.3%) was low. Resistance against tobramycin, polymyxin B, gentamicin, fusidic acid, tetracycline, and neomycin ranged from 40.8% to 58.6%. When separating the MRS from the other staphylococci, a significant difference was noted in percentage of resistance to gentamicin (p = .00026) and tetracycline (p = .00015). MRS were highly resistant to gentamicin (75%) and tetracycline (100%), whereas the remaining staphylococci were significantly less resistant to gentamicin (17%) and tetracycline (40.4%).

Conclusion: Although *Pseudomonas* species has been rarely cultured, our results are roughly consistent with previous studies. Multiple drug resistance was high and resistance to first-choice antibiotics in ulcerative keratitis was noted. These results warrant continued monitoring of susceptibility profile.

KEYWORDS

antimicrobial resistance, bacterial isolates, Belgium, horses, ulcerative keratitis

1 | INTRODUCTION

The prominent eye position, physical activity, and the environment of the horse are such that the ocular surface is constantly exposed to potential injury and potential pathogens. Ocular surface defense mechanisms, including antimicrobial components of the tear film and mechanical action of the eyelids and tear film, are effective against invasion of potential pathogenic bacteria. When bacteria break through this defense, they have the opportunity to invade the cornea.[1] Bacterial infection and inflammation of the cornea can cause an imbalance between proteolytic enzymes and proteinase inhibitors and result in keratomalacia.[2] When ulcers do not heal, have stromal involvement, enlarge, increase in depth, increase in cellularity or start melting, they become complicated ulcers.[1,3]

Primary therapy for ulcerative bacterial keratitis includes first and foremost a topical antibiotic.[4] The appropriate antimicrobial selection, specifically prophylactic vs. therapeutic antimicrobials, depends on the clinical presentation and whether or not an infection is suspected.[5] Many traumatic ulcers heal uneventfully in horses treated early with a prophylactic topical antibiotic, including tetracyclines, macrolides, and aminoglycosides.[5] In case of a complicated ulcer with signs of infection, specimens for cytology, bacteriology, and fungal culture should be obtained. The main objective of an antimicrobial treatment is a rapid elimination of the pathogen, preventing the release of proteases and toxins, and obtaining control of the innate PMN driven inflammation responsible for the progressive destructive process of the cornea.[6] Increasing antimicrobial resistance has been reported in several common bacterial pathogens.[4,7–9] In addition, overuse or inappropriate use of topical antimicrobial drugs is shown to alter the ocular surface microflora and may be responsible for the development of antimicrobial resistance.[10]

A variation in bacterial isolate and antimicrobial susceptibility has been demonstrated to depend on geographical location, season, and previous antimicrobial therapy.[3,9–11] Currently, there are no publications on equine ulcerative keratitis in Belgium. The purpose of this study was to describe bacterial isolates and associated antibiotic resistance against topical antibiotics from horses with ulcerative keratitis in Belgium.

2 | MATERIALS AND METHODS

2.1 | Case selection

Medical records from client owned horses with ulcerative keratitis presented to the ophthalmology service of the equine Veterinary teaching hospital of Liege from January, 2014, to May, 2021, were reviewed. All eyes with a corneal defect, including epithelial-, stromal-, or melting-ulcers, descemetocele, abscess, (non)perforating laceration, corneal foreign body, or any primary ocular disease with secondary corneal ulceration, that underwent sampling for bacterial culture, were included. Cases were excluded in the absence of corneal fluorescein uptake or when bacterial culture results were not available. Following information was collected for each individual: age, sex, breed, affected eye, date of admission, topical medication prior to presentation, clinical diagnosis, and results of bacterial and fungal culture.

2.2 | Sampling and microbiology

Each horse underwent a full ophthalmic examination, often under sedation due to ocular discomfort. Samples for bacterial culture were collected before fluorescein staining and after the topical application of tetracaine (MINIMS® tetracaine hydrochloride 10 mg/mL). A sterile culture swab (eSwab™, Copan diagnostics Inc., California) was rolled on the surface and borders of the ulcers, taking care to avoid touching the eyelid margins and eyelashes. The samples were placed immediately in tubes containing sterile transport medium and submitted to Synlab veterinary diagnostics laboratory (Heppignies, Belgium) for further analyses. Bacterial swabs were subjected for aerobic culture and inoculated onto various culture media: Columbia colistin and aztreonam (CAP) agar with sheep blood (CNA; Becton Dickinson, Erembodegem, Belgium), Mac Conkey agar (MCK; bioMérieux), and Trypticase soy agar with sheep blood (TSS; bioMérieux). The agar plates were incubated for 24 h at 37°C. Individual colonies were further identified using matrix-assisted laser desorption—/ionization time-of-flight (MALDI-TOF) mass spectrometry. VITEK® 2 provided antimicrobial susceptibility testing and resistance detection according to Biomérieux CLSE recommendations. Minimum inhibitory concentrations (MICs) were assessed and isolates were classified as susceptible, intermediate, or resistant to a standard list of antimicrobials. Susceptibility to methicillin was evaluated in case *Staphylococcus* spp. was isolated. For purposes of data analyses, results characterized as susceptible and intermediate were considered sensitive. Antimicrobial susceptibility was classified according to percentages of resistance: lower than 30%, between 30 and 70% or higher than 70%, were considered of "low resistance," "moderate resistance," or "high resistance," respectively. Only antimicrobials that were available in Belgium as a topical drug were comprised in this study and included gentamicin, tobramycin, neomycin, chloramphenicol, tetracycline, polymyxin B, fluoroquinolones, and fusidic acid. Fluoroquinolones included enrofloxacin and marbofloxacin. Antibiotic susceptibility testing was not standardized but depended on the isolate identified. As such, gram-positive bacteria were not always subjected to gram-negative spectrum antimicrobials susceptibility testing.

2.3 | Statistical analyses

Limited statistical analysis were performed due to the retrospective design of this study. The influence of sex, age, and season on the culture outcome was determined using a Fisher's exact test. The horses were divided into three age groups as the authors saw fit: young horses (<5 years), adult horses (between 5 and 15 years), and older horses (>15 years). The effect of age on isolating more gram-negative or gram-positive bacteria was investigated. In addition, the Fisher's exact test was used to compare isolation rates between ulcers treated with a topical antimicrobial and non-treated ulcers prior to referral. The horses that had received a combination of a topical antimicrobial with a (non) steroidal anti-inflammatory drug, were not taken into account. The significance level for all Fisher's exact tests were set at 5% ($p < 0.05$).

3 | RESULTS

Two hundred eyes of 196 horses with ulcerative keratitis between January, 2014, and May, 2021, matched the inclusion criteria. The age of the horses varied from 14 days to 30 years, with a mean age of 12 years (median of 12 years, 4 months and 27 days). Ninety-six of the horses were mares, 90 were geldings, and 14 were stallions. There were 103 right eyes and 97 left eyes affected. Four horses suffered a bilateral ulcerative keratitis. The majority of horses were warmbloods (127/200: 63.5%), followed by Crossbred ponies ($n = 11$) and Thoroughbreds ($n = 10$). Other breeds represented were Friesian ($n = 5$), Standardbred ($n = 4$), Shetland pony ($n = 4$), American Quarter horse ($n = 4$), Welsh pony ($n = 3$), American Miniature horse ($n = 3$), Haflinger ($n = 2$), Drafthorse ($n = 2$), Fjord ($n = 2$), Irish cob ($n = 2$) and Lusitano horse ($n = 2$), Paint ($n = 1$), Mérens ($n = 1$), and Appaloosa ($n = 1$). In 16 horses, the breed was not specified. Cases are distributed according to season as follows: 40 cases (20%) during winter, 42 (21%) during spring, 63 (31%) during summer, and 55 cases (28%) during autumn.

One hundred twenty-six cases of the 200 cases had a superficial to deep stromal ulcer or descemetocele without a known cause or underlying disease. Five cases had a perforating corneal lesion due to a deteriorating corneal ulcer. Eleven eyes were diagnosed with an ulcerated corneal abscess, eight with a (non) perforating laceration, and two eyes had a corneal foreign body. Nineteen eyes with immune-mediated keratitis, 17 eyes with eosinophilic keratitis, and 12 eyes with uveitis, were ulcerated.

Information about the previous treatment was not always available and was only included if the duration of the treatment was more than 1 day. About 137 eyes received a previous topical antimicrobial treatment: 89 eyes had at least one antimicrobial on the affected eye before sampling, 46 eyes received an antimicrobial in combination with a steroidal and/or non-steroidal anti-inflammatory, and two eyes received a non-steroidal anti-inflammatory only. Thirty-four eyes did not receive any treatment prior to referral. The information was not available for the remaining cases. Most often used antibiotics included oxytetracycline in combination with polymyxin B sulfate (43/89: 48%), tobramycin (40/89: 45%), and gentamicin (29/89: 33%). Other antibiotics less often used included chloramphenicol (17/89: 19%), ofloxacin (17/89: 19%), chlortetracycline (4/89: 4%), ciprofloxacin (1/89: 1%), and fusidic acid (1/89: 1%). Dexamethasone, neomycin, and polymyxin B sulfate (18/48: 28%) was the most used combination of a steroidal anti-inflammatory drug and an antibiotic, followed by the combination of hydrocortisone acetate, oxytetracycline, and polymyxin B (11/48: 23%).

3.1 | Bacterial isolates

Ninety-seven eyes had a positive bacterial culture (48.5%), and 139 bacterial isolates were identified. Sex ($P = .18$), age ($P = .85$), and season ($P = .77$) had no significant effect on the microbial isolation rate. Of the four horses with bilateral disease, two horses had identical bacterial isolates and cultures in both eyes and two horses had a negative culture in the left and a positive culture in the right eye. Polymicrobial infections were noted in 30 eyes (30.9%): 2 bacteria were isolated from 18 eyes, 3 bacteria from 8 eyes, 4 bacteria from 3 eyes, and 5 bacteria from one eye.

Forty-nine different species were isolated and listed in Table 1. All isolates were aerobes or facultative anaerobes and the vast majority were gram-positive organisms (112/139: 80.6%). Staphylococcus was the most frequent genus isolated (63/139: 45.3%), followed by Streptococcus (33/139: 23.7%). *Staphylococcus aureus* was the most frequent species isolated (21/139: 15.1%) followed by *Streptococcus equi* subsp. *zooepidemicus* (20/139: 14.4%). Half of *S. aureus* (11/21) and one *Staphylococcus epidermidis* (1/4) were methicillin-resistant (methicillin-resistant staphylococci MRS). Other frequently identified bacteria included *Staphylococcus xylosus* (7/139: 5%), *Pantoea agglomerans* (7/139: 5%), *Bacillus* species (7/139: 5%), *Staphylococcus warneri* (6/139: 4.3%), *Enterococcus*

TABLE 1 Bacterial isolates from horses with ulcerative keratitis in Belgium

	Isolate species	N° isolates: 139
Staphylococcus spp.	*S. aureus*	10
	Methicillin-resistant *S. aureus*	11
	S. xylosus	7
	S. warneri	6
	S. epidermidis	3
	Methicillin-resistant *S. epidermidis*	1
	S. hyicus	3
	S. equorum	3
	S. pseudointermedius	3
	S. haemolyticus	2
	S. intermedius	2
	S. pasteuri	2
	S. succinus	2
	S. vitulinus	2
	S. capitis	1
	S. chromogenes	1
	S. coagulase negative	1
	S. delphini	1
	S. schleiferi	1
	S. sciuri	1
Total		63 (45.3%)
Streptococcus spp.	*S. equi* ssp. *zooepidemicus*	20
	S. canis	2
	S. dysgalactiae equisimilis	2
	S. equi	2
	S. pluranimalium	2
	S. constellatus	1
	S. dysgalactiae	1
	S. schleiferi	1
	S. viridans	1
	β-hemolytic group C *S. equisimilis*	1
Total		33 (23.7%)
Gram-negative bacteria	*Pantoea (enterob) agglomerans*	7
	Escherichia coli	4
	Acinetobacter baumannii	3
	Acinetobacter haemolyticus	1
	Actinobacillus sp.	1
	Actinobacillus equuli	1
	Enterobacter cloaca	1
	Klebsiella oxytoca	1
	Klebsiella pneumoniae	1
	Kluyvera intermedia	1
	Moraxella	1
	Pasteurella multocida	1

(Continues)

TABLE 1 (Continued)

	Isolate species	N° isolates: 139
	Proteus vulgaris	1
	Providencia rettgeri	1
	Pseudomonas aeruginosa	1
	Pseudomonas fluorescens	1
Total		27 (19.4%)
Miscellaneous gram-positive bacteria	*Bacillus* spp.	7
	Enterococcus faecalis	5
	Rothia mucilaginosa	2
	Listeria monocytogenes	1
	Arthrobacter gandavensis	1
Total		16 (11.5%)

faecalis (5/139: 3.6%), and *Escherichia coli* (4/139: 2.9%). Only two *Pseudomonas* species were isolated (1.4%). *Clostridium perfringens* was isolated in one eye, together with five aerobic bacteria. Nine fungi were identified (9/193: 4.7%) of which two in combination with a positive bacterial culture. *Aspergillus* species was the most frequent fungi identified (5/9: 56%).

Bacterial isolates were identified in 45 of the 89 eyes that were treated with a topical antimicrobial treatment and in 11 of the 34 eyes that had not receive any treatment prior to sampling. There was no significant effect of an antimicrobial treatment prior to sampling on the microbial isolation rate ($p = .104$).

3.2 | Antimicrobial susceptibility

Due to the high number of bacterial isolates, susceptibility results were divided into four different groups: *Staphylococcus* spp., *Streptococcus* spp., gram-negative bacteria and miscellaneous gram-positive bacteria (Table 1). The staphylococci were divided into two groups depending on their resistance against methicillin. The results of antimicrobial susceptibility of the different groups are listed in Table 2.

Susceptibility testing was performed on each isolate except for *Bacillus* spp. and *Listeria monocytogenes*. Because of the low number of isolates in comparison with the other groups and the few susceptibility testing available, susceptibility results of the miscellaneous gram-positive bacteria were not further interpreted.

Resistance of the 139 bacterial isolates was mostly seen against tobramycin (58.6%), polymyxin B (54.5%), and gentamicin (48.6%). Resistance to tetracycline, fusidic acid, and neomycin was observed in 43.1%, 42.7%, and 40.8%, respectively, whereas only 15.5% were resistant to fluoroquinolones and 11.4% to chloramphenicol (Table 2).

All staphylococci presented a high resistance to polymyxin B (14/15: 93.3%), a moderate resistance to tetracycline (31/59: 52.5%) and tobramycin (9/20: 45%), and a low resistance against neomycin (6/37: 16.2%), fusidic acid (6/56: 10.7%), chloramphenicol (5/49: 10.2%), and fluoroquinolones (5/57: 8.8%) (Table 2, Figure 1A). When separating the methicillin-resistant staphylococci (MRS) from the remaining staphylocci, a difference was noted in the percentage of resistance to tetracycline, tobramycin, and gentamicin: MRS were highly resistant to tetracycline (12/12: 100%), tobramycin (4/5: 80%), and gentamicin (9/12: 75%) (Table 2, Figure 1C), whereas the remaining staphylococci showed a moderate resistance to tetracycline (19/47: 40.4%) and tobramycin (5/15: 33.3%) and a low resistance to gentamicin (8/47: 17%) (Table 2, Figure 1B). The difference in antimicrobial susceptibility between the MRS and remaining staphylococci was significative for gentamicin ($p = .00026$) and tetracycline ($p = .00013$).

Almost all *Streptococcus* spp. were resistant to tobramycin (10/10: 100%), neomycin (18/19: 94.7%), gentamicin (24/27: 85.7%), fusidic acid (18/22: 81.8%), and polymyxin B (6/8: 75%). They presented a moderate resistance against tetracycline (11/30: 36.7%) and a low resistance against fluoroquinolones (3/30: 10%) and chloramphenicol (1/26: 3.8%) (Table 2, Figure 2).

All gram-negative bacteria were resistant to fusidic acid (14/14: 100%). They showed a moderate degree of resistance to gentamicin (10/24: 41.7%), tetracycline (8/24: 33.3%), chloramphenicol (6/19: 31.6%), tobramycin (5/16: 31.3%), fluoroquinolones (7/25: 28%), and neomycin (5/18: 27.8%), and only 11.1% (2/18) of gram-negative bacteria were resistant to polymyxin B (Table 2, Figure 3).

TABLE 2 Antimicrobial susceptibility of microbial organisms isolated from horses diagnosed with ulcerative keratitis in Belgium from January 2014 to May 2021

		GENT	TOBR	NEO	CHLO	TETRA	PB	FLUO	FUS
All isolates	Resistant	51	27	31	10	47	24	17	38
	Sensitive	54	19	45	78	62	20	93	51
	Total	105	46	76	88	109	44	110	89
	% resistance	48.6	58.6	40.8	11.4	43.1	54.5	15.5	42.7
Staphylococcus spp.	All staphylocci								
	Resistant	17	9	6	5	31	14	5	6
	Sensitive	42	11	31	44	28	1	52	50
	Total	59	20	37	49	59	15	57	56
	% resistance	28.8	45.0	16.2	10.2	52.5	93.3	8.8	10.7
	Methicillin sensitive								
	Resistant	8	5	4	5	19	9	3	5
	Sensitive	39	10	26	35	28	1	43	39
	Total	47	15	30	40	47	10	46	44
	% resistance	17.0	33.3	13.3	12.5	40.4	90.0	6.5	11.4
	Methicillin resistant								
	Resistant	9	4	2	0	12	5	2	1
	Sensitive	3	1	5	9	0	0	9	11
	Total	12	5	7	9	12	5	11	12
	% resistance	75.0	80.0	28.6	0.0	100	100	18.2	8.3
Streptococcus spp.	Resistant	24	9	18	1	11	6	3	18
	Sensitive	4	0	1	25	19	2	27	4
	Total	28	9	19	26	30	8	30	22
	% resistance	85.7	100	94.7	3.8	36.7	75.0	10.0	81.8
Gram-negative bacteria	Resistant	10	5	5	6	8	2	7	14
	Sensitive	14	11	13	13	16	16	18	0
	Total	24	16	18	19	24	18	25	14
	% resistance	41.7	31.3	27.8	31.6	33.3	11.1	28.0	100

4 | DISCUSSION

Bacterial isolates were identified in almost 50% of all the horses with an ulcerative keratitis that were presented to the ophthalmology service of the Veterinary teaching hospital of Liège. Bacteria were not always isolated when infection was clinically suspected (mucopurulent discharge, keratomalacia, stromal cellular infiltration, and/or anterior chamber inflammation) while bacteria were isolated in some cases when clinical signs of infection were not present. In accordance with previous published data based on culture-dependent methods, gram-positive bacteria were most commonly identified (80.6%) in horses with an infectious ulcerative keratitis in Belgium.[3,9,11–14] *Staphylococcus* spp. (45.3%) was the most common aerobic bacteria isolated, followed by *Streptococcus* spp. (23.7%). *Staphylococcus aureus* and *Streptococcus equi* subsp. *zooepidemicus* were the far most common gram-positive bacteria isolated. Other frequently isolated gram-positive bacteria were *Staphylococcus xylosus*, *Bacillus* species, *Staphylococcus warneri*, and *Enterococcus faecalis*. The most common gram-negative bacteria identified was *Pantoea agglomerans*, followed by *Escherichia coli*. Our results are compared with previously published reports of horses suffering an ulcerative keratitis.[3,9,11–14] However, prominent differences are discussed below.

Staphylococcus aureus was identified in 15.1% of our bacterial isolates. Other studies reported a prevalence of *S. aureus* in less than 7% of the isolates.[3,7,9,11,13,14] *S. aureus* is a gram-positive bacteria and its virulence is related to its ability to adhere to injured epithelium, to resist phagocytosis during corneal invasion, and to produce cytodestructive toxins.[1] All *Staphylococcus* spp. isolated in this study were tested for methicillin susceptibility. Nearly 20% of all staphylococci were methicillin-resistant and half of the *Staphylococcus aureus* were methicillin-resistant (MRSA),

FIGURE 1 Antimicrobial resistance of Staphylococcus species to commonly used antibiotics from horses with an ulcerative keratitis. (A) All *Staphylococcus* spp., (B) *Staphylococcus* spp. methicillin sensitive and (C) methicillin-resistant *Staphylococcus* spp. (MRS). Abbreviations: CHLO, chloramphenicol; FLUO, fluoroquinolone; FUS, fusidic acid; GENT, gentamicin; NEO, neomycin; PB, polymyxin B; TETRA, tetracycline; TOBR, tobramycin

whereas MRSA were not identified in previous reports of healthy or diseased horses eyes.[3,9,11,13–15] The high prevalence of MRSA in our ocular samples reflects the high frequency of horses carrying MRSA presented to Belgium equine hospitals. Van den Eede et al.[16] reported that 10.9% of horses that were hospitalized in a Belgium equine hospital for a short period carry MRSA on the skin and in the nose. In case of a long-term hospitalization, up to 40% of the horses carried MRSA.[17] Another explanation for the high amount of MRSA in our study is that these virulent organisms do not respond to typical first-line therapy and thus are most commonly seen at referral institutions. In

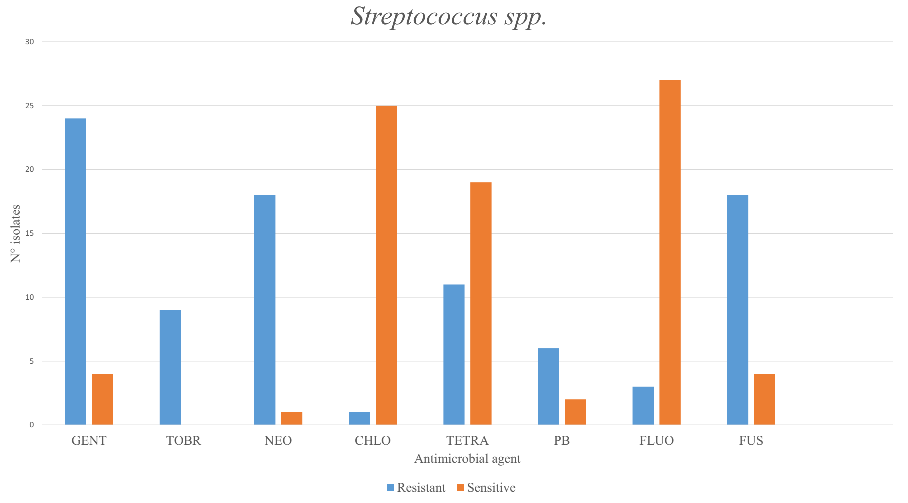

FIGURE 2 Antimicrobial resistance of *Streptococcus* spp. to commonly used antibiotics from horses with an ulcerative keratitis. Abbreviations: CHLO, chloramphenicol; FLUO, fluoroquinolone; FUS, fusidic acid; GENT, gentamicin; NEO, neomycin; PB, polymyxin B; TETRA, tetracycline; TOBR, tobramycin

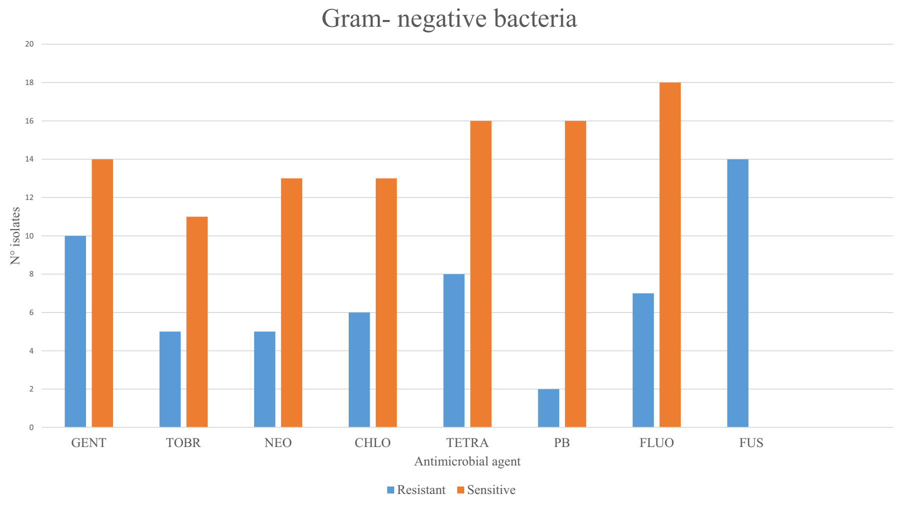

FIGURE 3 Antimicrobial resistance of gram-negative bacteria to commonly used antibiotics from horses with an ulcerative keratitis. Abbreviations: CHLO, chloramphenicol; FLUO, fluoroquinolone; FUS, fusidic acid; GENT, gentamicin; NEO, neomycin; PB, polymyxin B; TETRA, tetracycline; TOBR, tobramycin

this study, most of the MRSA cases were isolated from superficial non-healing ulcers without any signs of infection (6/11: 54.5%) of which two were infected after surgical superficial debridement. Four other cases had a superficial to stromal ulcer with no-to-mild signs of infection after several weeks of topical steroidal and non-steroidal anti-inflammatories for the treatment of uveitis (2), immune-mediated keratitis (1) and eosinophilic keratitis (1). Only one case had a large stromal ulcer with severe signs of keratomalacia.

Streptococcus equi subsp. *zooepidemicus* was identified in 15% of the isolates and is therefore one of the most frequent isolates identified. This was in line with previous studies from Tennessee (USA), Switzerland, and Finland.[3,11,13] *S. zooepidemicus* is a β-hemolytic coccus belonging to the Lancefield group C that is rarely found in conjunctival flora of healthy horses.[15,18–20] However, it is commonly found on mucus membranes of the upper respiratory tract and lower reproductive tract of healthy horses.[21] *S. zooepidemicus* is considered an opportunistic pathogen of horses that causes diseases ranging from mild-to-severe pneumonia, pleuropneumonia, strangles-like disease, or endometritis. It is also responsible for disease in humans and considered a zoonosis.[22] According to Brooks et al.,[23] ulcers infected with beta-hemolytic *Streptococcus equi* are characterized by excessive tear protease activity and severe uveitis induced by microbial death. A surgical intervention or an intensivemedical treatment with protease inhibitors and topical antibiotics are indicated.[23] Of the 20 *Streptococcus equi* subsp. *zooepidemicus* isolated in our study, 17 ulcers had signs of stromal melting and severe secondary uveitis of which 13 eyes were successfully managed with a surgical treatment, and three eyes were successfully managed with an intensive medical treatment. The remaining horse was euthanized.

Bacillus spp. have been isolated from normal and diseased equine eyes.[19] In our study, *Bacillus* spp. was frequently isolated but did not undergo susceptibility testing by the laboratory because they are considered to be rarely to non pathogenic. They are generally sensitive to beta-lactam antibiotics according to the laboratory. Nonetheless, *Bacillus* spp. has been reported to be an important ocular pathogen in humans, with the ability to kill corneal cells but with a very low percentage of antimicrobial resistance.[24] In our study, 71.4% (5/7) of the *Bacillus* spp. were isolated as the only bacteria of which 60% (3/5) had severe signs of infection. Despite the low percentage of resistance in humans, this study indicates the importance of susceptibility testing for all bacterial isolates.

We identified only two *Pseudomonas* spp.: *P. aeruginosa* and *P. fluorescens*. In contrast to studies from Florida[9] and Japan[14] where *Pseudomonas* spp. accounted for more than 22% of the bacterial isolates and was therefore the most common isolate. In the studies from Tennessee,[3] Switzerland,[13] and Finland,[11] *Pseudomonas* spp. represented 11.8%, 5.7%, and 0%, respectively, of all bacteria isolated in horses with infectious keratitis. Sauer et al.[9] suggested that their high percentage of *P. aeruginosa* isolates may be due to a selection bias and that only cases refractory to medical treatments were presented to their referral service. In our study, *Pseudomonas* spp. were rarely isolated despite the fact that the University of Liège serves as a referral-based ophthalmology service. Consequently, we suspect that it is climate and/or region dependent due to its low percentage of isolation in other European countries.

A single *Listeria monocytogenes* was isolated in our study. This gram-positive rod of low virulence is seldomly reported as a cause of keratitis in animals. The use of corticosteroids has been shown to increase the infectivity of *Listeria monocytogenes* in experimental animals.[25] Similar to a case report of Sanchez et al.,[26] *L. monocytogenes* was isolated in our study from a horse treated with corticosteroids prior to sampling.

The cause of varying dominant isolates in healthy horses and horses suffering ulcerative keratitis between studies is unclear. It has been associated with differences in season, geography, bedding, habitat, husbandry, and/or differences in analytic methods.[14,20] However, according to the study of Zak et al.,[15] identifying the commensal flora of the conjunctival sac, the maintenance conditions did not affect the number of microbial isolates. In another study by Andrew et al.,[18] no impact of the season and surroundings on the conjunctival sac bacterial flora were revealed. The age of the horse however had a significant effect on the culture outcome. Younger horses had an increased incidence of gram-negative isolates theorized to be the consequence of a variability in the ocular surface defense mechanisms.[18] In our study, however, the age did not significantly affect the culture outcome ($p = .85$) and in contrast to the commensal flora, we did not identify an increased incidence of gram-negative bacterial isolates in young horses ($p = .65$). Although earlier reports[15] have stated that commensal bacteria act as pathogens in case of damage of the ocular barrier, bacterial ulcerative keratitis should be diagnosed based on a swab obtained directly from the ulcer because conjunctival flora is not necessarily related to the corneal flora.[15]

The use of an initial empirical topical antibiotic while awaiting culture and sensitivity results is essential since a delay in effective therapy diminishes a successful outcome. When choosing a topical antimicrobial drug in the treatment of an ulcerative keratitis, understanding the underlying purpose of their use is essential: prevention vs. elimination of infection. Other characteristics that must be taken into consideration are their spectrum of activity, toxicity against the epithelium, bactericidal activity, and penetrance into the cornea. In case the epithelial barrier is absent, hydrophilic drugs such as aminoglycosides (gentamicin and tobramycin) easily penetrates the corneal stroma and rapidly reaches high concentrations. In case of a stromal abscessation, when the epithelial barrier is intact, only highly lipophilic antibiotics such as fluoroquinolones and chloramphenicol are likely to achieve therapeutic levels after topical application.[6]

In Belgium, gentamicin is registered for horses, is a first choice antimicrobial for the treatment of bacterial keratitis and is available as an ophthalmic gel (Clinagel®) or as viscous ocular drops (Soligental®). Due to its galenic properties, tobramycin (Tobrex®) drops are preferred when a sub palpebral eye lavage system is placed. Aminoglycosides used to be advocated as effective therapy for infectious keratitis.[5] In contrast to earlier studies[7,14] but in accordance with Sauer et al.,[9] our study demonstrated lower susceptibility profiles among ocular isolates to gentamicin and tobramycin. Although they both showed to be effective against the majority of *Staphylococcus* spp. (with the exception of MRS), gram-negative bacterial isolates were moderately resistant to both aminoglycosides. The high resistance of *Streptococcus* spp. is not surprising due to their gram-negative spectrum. Gentamicin, and to a lesser degree tobramycin, is known to inhibit canine corneal epithelial cell migration in tissue culture and thus are presumed to delay corneal healing.[27] The authors did not experience any obvious delayed healing in these clinical patients.

Prior to referral, horses with an ulcerative keratitis were mostly treated with a combination of oxytetracyclin and polymyxin B sulfate (Terramycine®), probably because it is available without prescription or medical advice. Susceptibility testing results of the present study indicated that colistin polymyxin B are non-effective against almost all bacterial isolates with exception of the gram-negative bacteria. Oxytetracycline had a moderate efficiency against *Streptococcus* spp., gram-negative bacteria and *Staphylococcus* spp. with the exception of MRSA, who showed to be 100% resistant.

Chloramphenicol is a broad-spectrum bacteriostatic antibiotic that is available as eyedrops (Chloramphenicol®) or topical ointment (Cavasan®, Ophtalon®, or Chlooramfenicol®) in Belgium. Compared with other studies,[3,9,14] a lower percentage of resistance of all bacterial isolates against chloramphenicol was noted in this study. However, these results are in line with those of Switzerland[13] and Finland[11] when chloramphenicol is tested against staphylococci and streptococci separately. In a study from Chalder et al.,[7] an increase in resistance was noted from 0% to 30% of ocular surface bacteria to chloramphenicol, isolated from diseased equine eyes in the UK. This could be a result of its empirical topical use in bacterial keratitis in the UK, which is not the case in Belgium yet. However, care should be taken when prescribing chloramphenicol as it is, according to Belgium's guidelines for the responsible use of antibiotics,[28] prohibited for the use in horses that are destined for the human food chain in Belgium.

Fluoroquinolones have a gram-negative spectrum and broaden in spectrum with increasing generation. Ofloxacin (Trafloxal®) is a second generation fluoroquinolone available in Belgium and is considered more effective against gram-negative isolates (particularly *Pseudomonas aeruginosa*).[5] Fluoroquinolones that were used in antimicrobial susceptibility testing in this study included enrofloxacin and marbofloxacin. Both are third generation fluoroquinolones that are effective against *Staphylococcus* spp., *Escherichia coli*, Proteus, *Klebsiella* spp., and *Pasteurella* spp. However, in our study, gram-negative bacteria together with MRS were generally more resistant to fluoroquinolones compared with gram-positive bacteria. The concern over the emerging resistance among ocular pathogens of fluoroquinolones[6] could not be proven in the UK[7] but a potentially high degree of variability among isolates in different geographic regions must be kept in mind. According to Belgium's guidelines for the responsible use of antibiotics,[28] fluoroquinolones should not be used for routine topical prophylaxis and be limited to cases of infectious keratitis confirmed by microbial isolation and based on antimicrobial susceptibility results.

Fusidic acid is a narrow spectrum antibiotic that is useful for the treatment of ocular bacterial infections caused by *Staphylococcus*, but not *Streptococcus*.[29] As expected, more than 80% of the *Streptococcus* spp. in our study were resistant whereas only 6.1% of the *Staphylococcus* spp. were resistant against this antimicrobial. Therefore, fusidic acid should be used with caution for the treatment of equine ulcerative keratitis and be limited to ocular staphylocci and MRS infections.

In Belgium, recommendations on prudent and responsible use of antibiotics for farmers and veterinarians are listed in the guidelines from “Antimicrobial Consumption and Resistance in Animals” or AMCRA.[28] These guidelines are not specific for ocular use but systemic use of antimicrobials. Each antimicrobial is assigned into one out of three groups based on the importance of the molecule for public and animal health: (1) “yellow” antimicrobials are agents that may be used for curative treatment preferably supported by additional laboratory research, (2) “orange” antimicrobials are agents that may be used for curative treatment if additional laboratory research has been performed, and (3) “red” antimicrobials are agents that may be used for curative treatment only if additional laboratory research has been performed and antimicrobial susceptibility results are available. There are currently no “yellow” antimicrobials for topical use in Belgium. All antimicrobials described in this article are “orange” except for fluoroquinolones who belong to the “red” antimicrobials.

Due to the retrospective nature of this study and the presence of potential confounding factors, statistical analysis was limited. Furthermore, there was often a lack of power of the Fisher's exact tests, which could be the reason why our results failed to prove an effect although it may exist. No significant influence was found of sex, age, season, and a previous treatment on the microbial isolation rates. The authors therefore concluded that corneal ulcers should always be sampled for microbial isolation and antimicrobial susceptibility, even if the horse has received an antimicrobial treatment prior to sampling.

Gemensky-Metzler et al.[19] noted that a topical corticosteroid treatment did not influence a direct propagation of pathogenic bacterial or fungal species on the ocular commensal flora in healthy equine eyes. In case of injured or inflamed corneas, changes in the corneal environment (i.e., decreased oxygen, pH, and glucose concentration) may in conjunction with corticosteroids, alone or in combination with antimicrobials, potentiate or exacerbate a fungal infection.[30] To the best of our knowledge, there is no evidence that topical corticosteroids enhance the risk of a bacterial infection in case the cornea is injured or inflamed. However, they amplify the risk of a corneal infection by inhibiting epithelialization, vascularization, inflammation, and phagocytic abilities of inflammatory cells. Because of a lack of information, our study failed to prove the influence of corticosteroids prior to sampling on the culture outcome.

Limitations of this study are predominantly associated with its retrospective design. Information about a previous treatment was often missing, and a clinical description was not always available. Moreover, not all bacterial isolates were subjected to identical antibiotic susceptibility testing. This depended partially on the bacterial isolate and the spectrum of the antimicrobial tested. In addition, susceptibility testing was generally performed on antibiotics for systemic use and did not always included tobramycin and polymyxin B. The referral nature of this study could have influenced a higher resistance rate compared with the actual patient population. Furthermore, breakpoints for topical antimicrobial susceptibility tests were not available but based on systemic antibiotic concentrations. However, concentration of topical antibiotics may substantially exceed the levels used in standard antimicrobial susceptibility tests, and thus, therapeutic efficacy of topical treatments are underestimated.[6] Comparison of susceptibility testing with previous studies was not always accurate because susceptibility in this study was based on MIC values whereas most previous studies used a disk diffusion method. In contrast to MIC, the disc diffusion method cannot quantify the antibiotic concentration needed to achieve a therapeutic result. Finally, all samples for bacterial culture were collected after the topical application of tetracaine. Edwards et al.[31] has studied the effect of topical tetracaine on corneal culture in 19 horses with ulcerative keratitis and concluded that topical anesthetic did not significantly alter bacterial culture results in horses.

5 | CONCLUSION

Our study identified bacterial isolates in horses with ulcerative keratitis in Belgium and their antimicrobial resistance against topical antibiotics. The authors noted a moderate-to-high antimicrobial drug resistance among certain bacterial isolates and a large amount of methicillin-resistant staphylococci were identified. Because of its high resistance against first-choice antibiotics, care should be taken when using these antimicrobial drugs for routine topical prophylaxis. Therefore, culture and susceptibility testing should be standard clinical practice, and a continued monitoring of susceptibility profile is highly designated.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

ORCID

Eline M. Vercruysse https://orcid.org/0000-0003-0532-1703
Aurélie C. Sauvage https://orcid.org/0000-0002-3839-9569
Magda F. Grauwels https://orcid.org/0000-0003-3716-2989

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