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Étude *in vitro* et *in vivo* de la virulence et du réassortiment génétique des sérotypes 4 et 9 du virus de la peste équine

In vitro and *in vivo* study of the virulence and genetic reassortment of serotypes 4 and 9 of African horse sickness virus

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TABLE DES MATIERES

Table des matières…….....iii

Liste des abréviations……..v

Chapitre I : Introduction……1

1.1 Historique……..……..2

1.2 Le virus de la peste équine au sein de la famille des *Reoviridae*……2

1.3 Virologie moléculaire du virus de la peste équine……..…4

1.4 Variabilité du virus de la peste équine……8

1.4.1 Diversité génétique des populations virales de virus à ARN……..8

1.4.2 Diversité génétique du virus de la peste équine……..9

1.5 Epidémiologie de la peste équine……..10

1.5.1 Cycle de transmission……...10

1.5.2 Peste équine en Afrique……12

1.5.3 Peste équine en Europe…….13

1.6 Risque d'introduction de la peste équine en Belgique……..13

1.7 Pathogénie et aspects cliniques de la peste équine……15

1.7.1 Pathogénie……..……15

1.7.2 Réponse immunitaire……16

1.7.3 Signes cliniques…….17

1.7.4 Lésions nécropsiques……18

1.8 Diagnostic de l'infection par le virus de la peste équine……..19

1.8.1 Diagnostic moléculaire…….19

1.8.2 Diagnostic sérologique……..19

1.8.3 Isolement en culture cellulaire……..20

1.9 Vaccination……21

1.9.1 Vaccins commercialisés……21

1.9.2 Vaccins expérimentaux…….21

1.10 Modèle murin d'infection par le virus de la peste équine……..…….22

Chapitre II : Objectifs……...24

Chapitre III : Section expérimentale……...27

Étude 1 : Modèle murin expérimental d'infection par le virus de la peste équine……...28

Study of the virulence of serotypes 4 and 9 of African horse sickness virus in IFNAR -/-, Balb/C and 129 Sv/Ev mice

Étude 2 : Étude du réassortiment génétique du virus de la peste équine……..43

In vitro and *in vivo* characterization of *in vitro* generated African horse sickness reassortant viruses

Partie 1 : Étude *in vitro* du réassortiment génétique entre les sérotypes 4 et 9……48
du virus de la peste équine

Partie 2 *:* Étude de la virulence *in vivo* des réassortants obtenus *in vitro*……61
à partir des sérotypes 4 et 9 du virus de la peste équine

Chapitre IV : Discussion……...70

Chapitre V : Conclusions et perspectives……82

Chapitre VI : Résumé – Summary……...85

Chapitre VII : Références bibliographiques……...90

Annexes……106

***Préambule*……...107**

Risk profiling of introduction and dissemination of Eastern, Western and Venezuelan equine encephalitis virus in Belgium……108

Diversity and ecology survey of mosquitoes potential vectors in Belgian equestrian farms: a threat prevention of mosquito-borne equine arboviruses……140

LISTE DES ABREVIATIONS

AAE : acides aminés essentiels

ADN : acide désoxyribonucléique

ADNc : ADN complémentaire

AHSV : African horse sickness virus, *virus de la peste équine*

ARN : acide ribonucléique

BHK : Baby Hamster Kidney, *rein de jeunes hamsters*

CERVA : Centre d'Etude et de Recherche Vétérinaires et Agrochimiques

Ct : cycle seuil de détection

CPE : cytopathic effect, *effet cytopathogène*

$DICC_{50}$: dose infectieuse en culture de cellules à 50 %

DMEM : Dulbecco's Modified Eagle Medium, Gibco®Invitrogen

dNTPs : désoxyribonucléotides triphosphates

EDTA : acide éthylène diamine tétraacétique

ELISA : enzyme-linked immunosorbent assay, *dosage immuno-enzymatique sur support solide*

ENSO : El Niño Southern Oscillation, *oscillation australe associée à El Niño*

FCO : fièvre catarrhale ovine

FCS : Fetal Calf Serum, *sérum de veau fœtal*

IFNAR : Interferon-α Receptor knock-out, *déficient en récepteur à l'interféron α*

MDA5 : melanoma differenciation-associated gene 5

MOI : Multiplicity Of Infection, *multiplicité d'infection*

MS : Monkey Kidney, *rein de singe*

NGS : next-generation sequecing, *séquençage haut débit*

NS : non structural protein, *protéine non structurale*

OIE : Office *international des épizooties*

***ORF** : open reading frame, cadre ouvert de lecture*

PAMP : pathogen-associated molecular patterns, *motif moléculaire associés aux pathogènes*

PRR : pattern recognition receptors, *récepteurs de reconnaissance de motifs moléculaires*

Pb : paires de bases nucléotidiques

PBS : Phosphate Buffered Saline, *tampon phosphate salin*

PCR : Polymerase Chain Reaction, *réaction de polymérisation en chaîne*

PS : Pénicilline-Streptomycine

qRT-PCR : réaction de polymérisation en chaîne quantitative après transcription inverse

RIG : retinoid acid-inducible gene-I

rpm : Rotation per minute, *tour par minute*

Spf : Service public fédéral

$TCID_{50}$: Tissue Culture Infectious Dose, *dose infectieuse en culture de tissu à 50 %*

TLR : toll-like recepteur, *récepteur de type toll*

VP : (structural) viral protein, *protéine virale (de structure)*

Introduction

1.1 Historique

Le premier épisode clinique d'un syndrome ressemblant à la peste équine a été rapporté au Yémen en 1327. Cependant, le virus de la peste équine ou AHSV (African horse sickness virus) est avant tout un virus africain et y a été observé les premières fois à la suite de l'introduction de chevaux en provenance d'Inde pendant l'exploration de l'Afrique de l'Est et centrale par les colons portugais en 1569. En Afrique du Sud, le virus était déjà probablement présent mais des signes cliniques concordant avec la peste équine n'ont pas été décrits avant 1657, après la première introduction de chevaux par les colons néerlandais. Une épidémie majeure y eut d'ailleurs lieu en 1719 pendant laquelle quelques 1700 chevaux moururent. Ensuite, différents épisodes de peste équine furent rapportés et des dizaines de milliers de chevaux périrent de la maladie (Maclachlan et Guthrie, 2010). Ces faits historiques ne se basent que sur la présence de signes cliniques similaires à ceux observés lors d'une infection de peste équine confirmée (Mellor et Hamblin, 2004).

En effet, le premier isolement du virus de la peste équine date du début des années 1900 et la distinction antigénique en 9 sérotypes connus date de 1962. Depuis lors, tous les sérotypes de la peste équine ont été retrouvés en Afrique sub-saharienne et le sérotype 9 a provoqué des épidémies en dehors des zones endémiques et notamment en Egypte, Turquie, Liban, Jordanie, Iran, Afghanistan, Inde, Maroc, Tunisie, Algérie mais également en 1965 en Espagne. L'exception s'est avérée en 1987 lors d'une épidémie de peste équine en Espagne et au Portugal (Portas *et al.*, 1999) qui, cette fois, a été attribuée au sérotype 4 de l'AHSV. Le virus s'y est maintenu pendant les quatre étés suivants, provoquant des épidémies à répétition et montrant la capacité de l'infection virale à se maintenir en dehors des zones endémiques africaines (Wilson *et al.*, 2009; Johnson *et al.*, 2012).

1.2 Le virus de la peste équine au sein de la famille des *Reoviridae*

Le virus de la peste équine appartient à la famille des *Reoviridae*, genre *Orbivirus*.
La famille des *Reoviridae* comprend deux sous-familles : *Sedoreovirinae* et *Spinareovirinae* avec 14 genres répartis entre ces deux sous-familles (Figure 1) (http://www.ictvonline.org/virusTaxonomy.asp). Le genre *Orbivirus* est un des 6 genres que comporte la sous-famille des *Sedoreovirinae*. La famille des *Reoviridae* inclut des pathogènes des vertébrés, des arthropodes et des plantes.

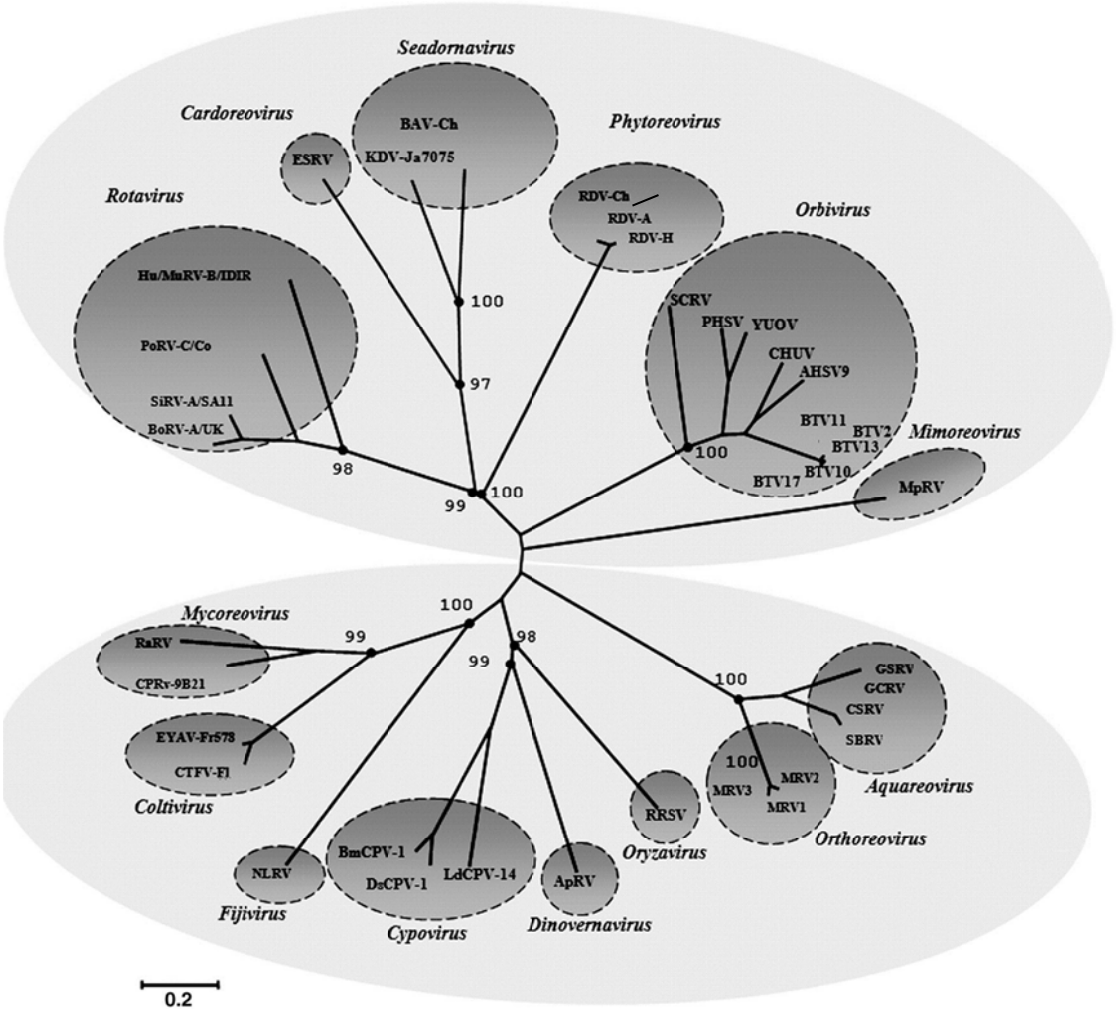

Figure 1 : Arbre phylogénétique de la famille des *Reoviridae* sur base des séquences de la polymérase (segment 1). La figure représente 14 des 15 genres de la famille des *Reoviridae*. *Mimoreovirus* : MpRV (Micromonas pusilla reovirus) ; ***Orbivirus* :** BTV (Bluetongue virus), AHSV (African horse sickness virus), CHUV (Palyam virus), YUOV (Yunnan orbivirus), PHSV (Pervuvian horse sickness virus), SCRV (St Croix river virus) ; ***Phytoreovirus*** : RDV (Rice dwarf virus) ; ***Seadornavirus*** : BAV (Banna virus), KDV (Kadipiro virus) ; ***Cardoreovirus*** : ESRV (Eriocheir sinensis reovirus) ; ***Rotavirus*** : RV (Rotavirus) ; ***Mycoreovirus*** : RaRV (Rosellinia anti-rot virus), CPRV (Cryphonectria parasitica reovirus) ; ***Coltivirus*** : EYAV (Eyach virus), CTFV (Colorado tick fever virus) ; ***Fijivirus*** : NLRV (Nilaparvata lugens reovirus) ; ***Cypovirus*** : BmCPV (Bombyx mori cytoplasmic polyhedrosis virus), DsCPV (Dendrolimus punctatus cytoplasmic polyhedrosis), LdCPV (Lymantria dispar cytoplasmic polyhedrosis) ; ***Dinovernavirus*** : ApRV (Aedes pseudoscutellaris reovirus) ; ***Oryzavirus*** : RRSV (Rice ragged stunt virus) ; ***Orthoreovirus*** : MRV (Mammalian orthoreovirus) ; ***Aquareovirus*** : SBRV (Striped bass reovirus), CSRV (Chum salmon reovirus), GCRV (Grass Carp reovirus), GSRV (Golden shiner reovirus). (D'après Attoui et al., 2009)

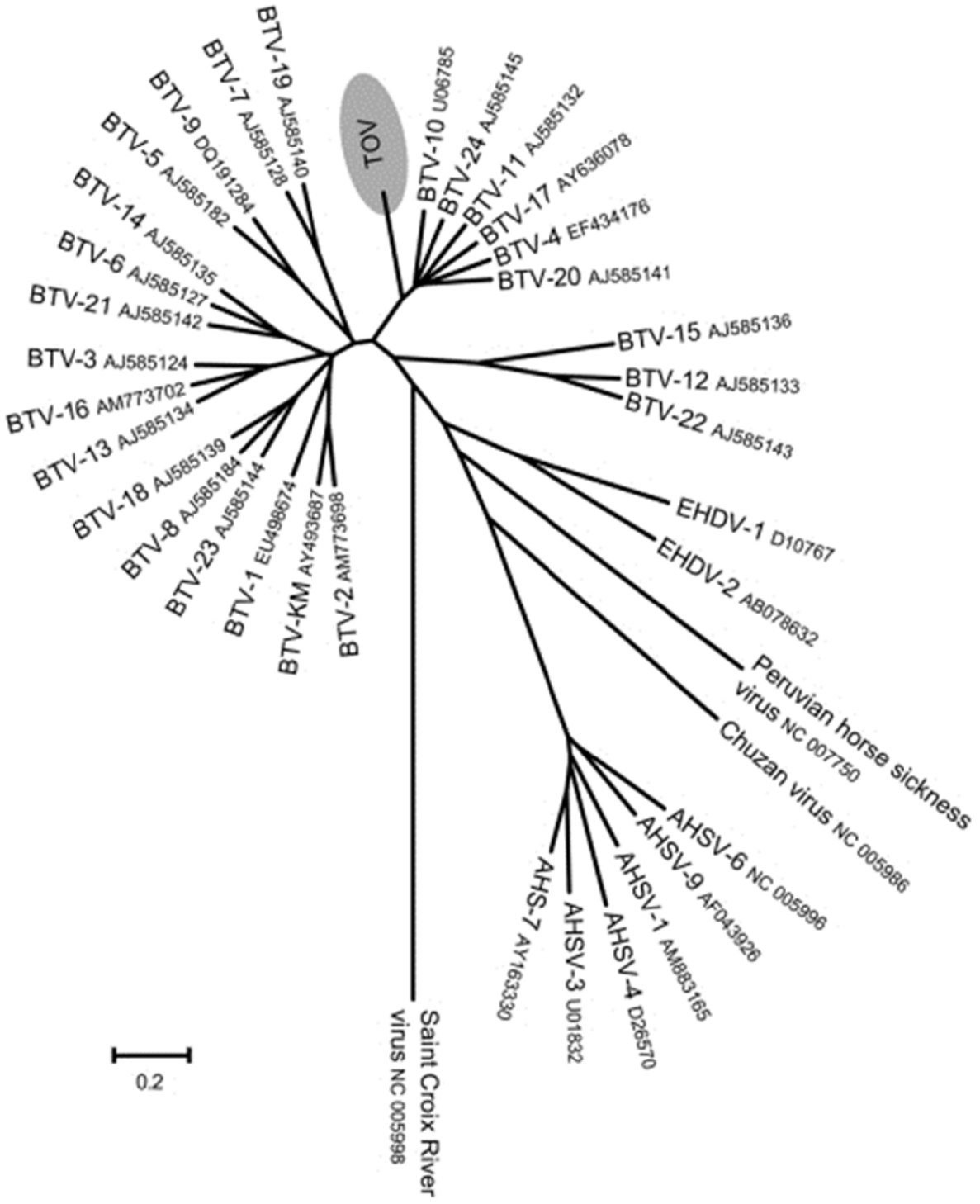

Figure 2 : Analyse phylogénétique du segment 2 du genre *Orbivirus*. Six espèces du genre *Orbivirus* sont représentées ici. BTV, Bluetongue virus comprend actuellement 27 sérotypes décrits; EHDV, Epizootic hemorrhagic disease virus compte entre 8 et 10 sérotypes; AHSV, African horse sickness virus a 9 sérotypes décrits. (D'après Hofmann et al, 2008).

Même si certaines similarités avec les autres membres de la famille des *Reoviridae* peuvent être trouvées, les *Orbivirus* sont assez différents du point de vue de leur structure, de leurs propriétés physico-chimiques, de leur cycle de réplication, de leur pathogénie et de leur épidémiologie. La première différence majeure est que les *Orbivirus* sont des arbovirus donc transmis par des vecteurs, que les *Reovirus* ou encore les *Rotavirus* n'en sont pas et sont transmis par voie féco-orale.

Le sérotype est la catégorie dans laquelle on classe les virus selon leur réaction en présence de sérums contenant des anticorps spécifiques ; pour les Orbivirus, les anticorps neutralisants induits par VP2 (protéine de capside externe) définissent le sérotype. Le sérogroupe est l'ensemble de plusieurs sérotypes possédant en commun un facteur caractéristique qui est la protéine VP7 (protéine de capside interne) pour le genre *Orbivirus*. L'isolat est un virus produit en culture pure autant que cela est connu. L'isolat peut ultérieurement être un mélange de virus et nécessite un isolement du virus en culture de cellules. La souche virale se définit comme un isolat viral qui ressemble au virus prototype de l'espèce dans les propriétés majeures qui définissent l'espèce, mais qui diffère dans des propriétés mineures comme la spécificité de l'espèce de vecteur, les signes cliniques induits, les propriétés sérologiques et génétiques (Thiry *et al.*, 2008). Sur base de leur profil sérologique, 22 sérogroupes, différents ont été identifiés pour les *Orbivirus* et certains isolats sont encore non classés (Figure 2). A l'intérieur de ces sérogroupes, différents sérotypes peuvent être différenciés, notamment 9 pour l'AHSV et 27, pour le virus de la fièvre catarrhale ovine (FCO) (Jenckel *et al.*, 2015) qui est le virus le plus apparenté à l'AHSV et est le virus de référence pour le genre *Orbivirus*.

1.3. Virologie moléculaire du virus de la peste équine

Le virion de l'AHSV est non enveloppé, de forme icosaédrique avec une taille d'environ 70 nm et contient 10 segments d'ARN double-brin dans son génome (Figure 3). Chaque segment d'ARN est numéroté en fonction de sa vitesse de migration en gel d'électrophorèse (agarose 1 %) et code une protéine majeure. Les 10 segments sont classés en segments longs (L1 à L3), moyens (M4 à M6) et courts (S7 à S10).

Au total, le génome viral de l'AHSV code 7 protéines de structure (VP1-7) organisées en une double couche de capside et 4 protéines non structurales (NS1-NS4). Les protéines VP2 et VP5 forment la partie externe de la capside du virion, et les protéines VP3 et VP7 sont

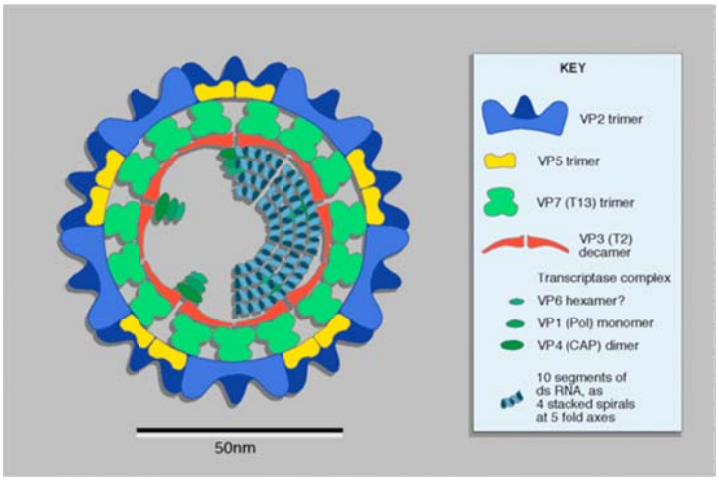

Figure 3 : Structure des *Orbivirus*. Les protéines VP2 et VP5 constituent la capside externe du virus alors que VP7 et VP3 constituent la capside interne. Le génome de l'AHSV est organisé en 10 segments d'ARN double-brin à l'intérieur du core viral (Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses, Mertens P.P.C., Attoui H., Duncan R., Dermody T.S. (Eds.), Reoviridae).

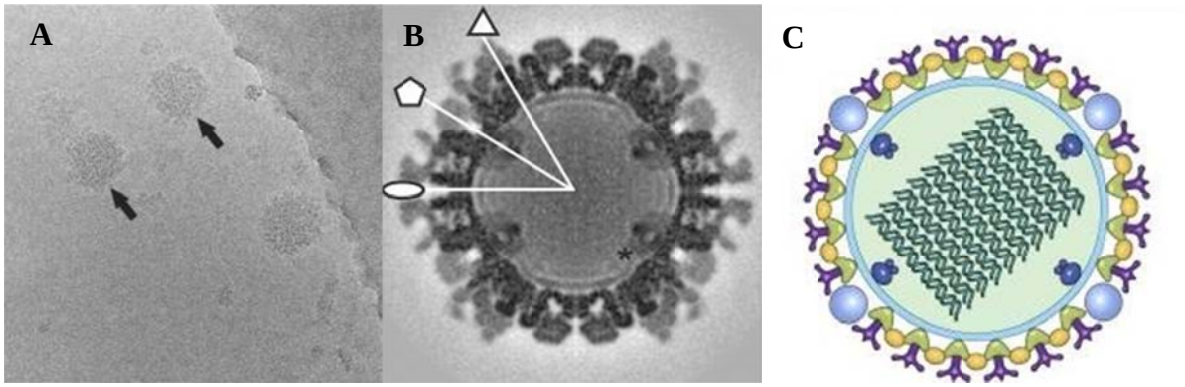

Figure 4 : Organisation générale de l'AHSV. A : microscopie électronique des particules virales intactes d'AHSV-4 de 87 nm de diamètre ; **B** : Coupe transversale à travers une reconstruction de l'AHSV-4 ; les axes de symétrie double (ellipse), triple (triangle) et quintuple (pentagone) sont indiqués ; **C** : Organisation schématique de l'AHSV-4 ; les segments d'ARN sont représentés en vert foncé, les complexes enzymatiques (VP1, 4 et 6) en bleu foncé sont entourés par VP3 en bleu ciel ; les trimères de VP7 en vert clair sont attachés à VP3 et surplombés par les trimères de VP2 (mauve) ; VP5 en jaune vient compléter la capside externe ; enfin le virion de l'AHSV-4 présente une densité supplémentaire représentée par les petites sphères bleu pâle correspondant à des protéines de nature indéterminée (adapté de Manole *et al.*, 2012).

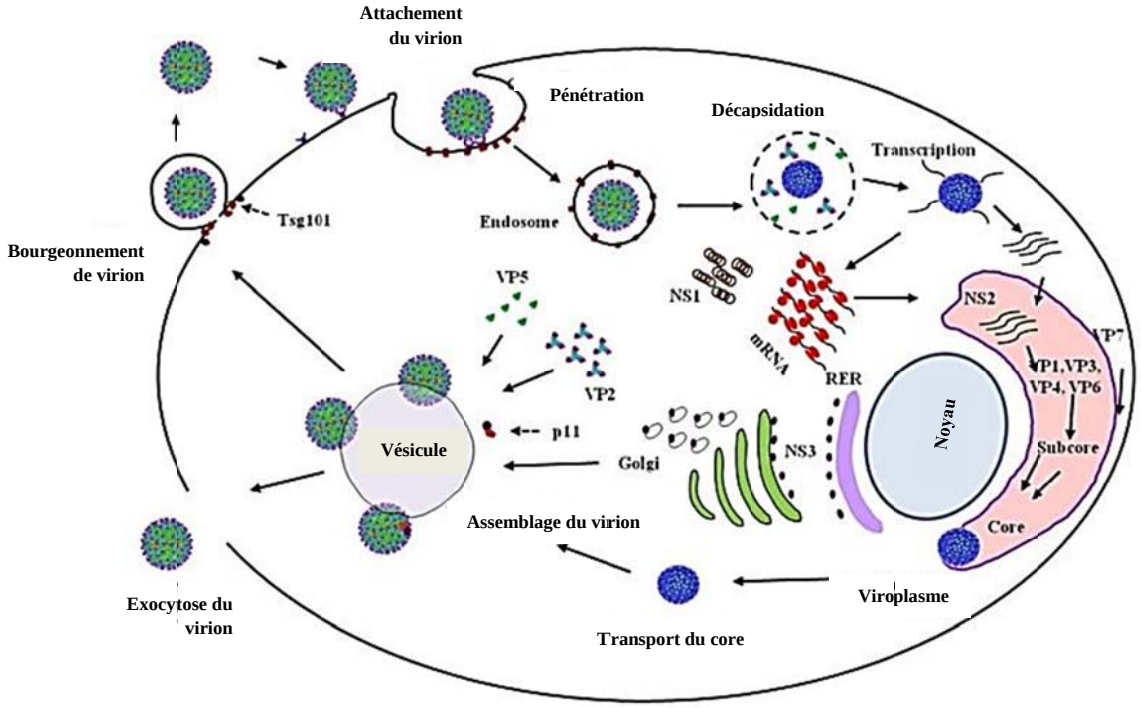

Figure 5 : Représentation schématique du cycle de multiplication des *Orbivirus*. Le site de fixation du virus sur son récepteur cellulaire est situé sur VP2. La décapsidation du virus est induite par VP5 qui possède des propriétés déstabilisantes de la membrane. Cette décapsidation est nécessaire à l'activation de la transcription virale dans le core viral. NS1 soutient la traduction des ARN simples brins et forme les tubules dans le cytoplasme. NS2 assemble les corps d'inclusion cytoplasmiques au cœur desquels sont rassemblées les protéines virales et les nouveaux brins d'ARN synthétisés. Après l'assemblage du core et sa sortie hors des corps d'inclusion cytoplasmiques, le transport du core dans le cytoplasme se fait sur des vésicules d'exocytose grâce à l'interaction de NS3 avec des calpectines. La maturation du virion est achevée par l'acquisition des protéines VP2 et VP5, le virion étant désormais une particule infectieuse complète. Enfin NS3 favorise la libération des virions par bourgeonnement membranaire (adapté de Mohl et Roy, 2014).

les constituants majeurs de la couche interne de la capside (Roy, 1996) (Figure 3 et 4) alors que VP1, VP4 et VP6 sont des constituants mineurs de la partie interne de la capside.

La protéine VP2, codée par le segment 2, est la protéine la plus variable et est spécifique aux différents sérotypes viraux rencontrés. VP2 est la protéine la plus variable entre les différents sérotypes avec environ 60 % d'homologie nucléotidique entre les différents sérotypes et environ 90 % d'homologie nucléotidique entre souches au sein du même sérotype. Avec VP5 (segment 6), VP2 est également responsable de la neutralisation virale. VP2 et VP5 ensemble sont les médiateurs de l'attachement viral à la cellule et de sa pénétration dans la cellule. L'attachement de VP2 sur des récepteurs cellulaires contenant de l'acide sialique est suivi par une endocytose contrôlée par des molécules de clathrine.

La capside externe est enlevée après pénétration grâce aux propriétés de déstabilisation de la membrane de VP5 (pH=5), ce qui expose le core viral constitué par la capside interne et l'acide nucléique (VP1, 3, 4, 6 et 7) (Figure 5 et Tableau 1).

VP7 codée par le segment 7, est la protéine la plus conservée entre les différents sérotypes et elle est spécifique du sérogroupe. VP7 est le constituant majeur de la capside interne de l'AHSV. Une infectiosité similaire du virus de la FCO est décrite pour des particules virales composées uniquement du core et des particules virales complètes, lors de l'infection de cellules d'insectes, suggérant que VP7 participe à l'entrée du virus dans la cellule d'insecte et joue un rôle dans l'infectiosité de la particule de core pour des vecteurs adultes et les cellules d'insectes (Mertens *et al.*, 1996; Xu *et al.*, 1997).

VP3 (segment 3) présente un rôle de protection des enzymes actives du core viral. VP1 (segment 1), VP4 (segment 4) et VP6 (segment 9) sont des protéines associées aux enzymes du core du virion et sont responsables de la transcription avec VP1 de l'ARN Polymérase ARN dépendante, de l'acquisition de la coiffe des nouveaux brins d'ARN avec VP4 et de l'activité hélicase avec VP6 qui déroule les brins d'ARN en présence d'ATP. La transcription précoce a lieu dans le core viral et produit des ARNm coiffés qui permettront la synthèse des protéines.

La protéine non structurale NS1 codée par le segment 5 est très conservée entre les différents sérotypes et forme les tubules caractéristiques dans le cytoplasme de la cellule infectée. Le rôle de ces tubules n'est pas connu précisément mais leur association aux protéines du cytosquelette suggère leur participation dans les mécanismes de transfert intracellulaire. Récemment des études sur le virus de la FCO suggère également que la

Segment ARN	Taille segment (pb)	Protéine codée	Fonction
1	3965	VP1	ARN Polymérase ARN dépendante
2	3203	VP2	Constituant externe de la capside externe ; séquence très variable ; détermine le sérotype viral ; induit anticorps neutralisants ; médiateur de l'attachement viral et de la pénétration dans la cellule
3	2792	VP3	Constituant mineur de la capside interne ; protection des protéines avec activités enzymatiques actives (VP1, VP4 et VP6) ;
4	1978	VP4	Guanyl-transférase ; coiffe et polyadényle les ARNm précoces
5	1748	NS1	Forme des tubules dans le cytoplasme de la cellule infectée ; implication dans la morphogenèse de la particule virale ; participe aux mécanismes de transport intracellulaire
6	1566	VP5	Composant mineure de la capside externe ; propriétés de déstabilisation de la capside virale ce qui permet au core d'être relargué dans le cytoplasme
7	1050	VP7	Composante majeure de la capside interne ; séquence très conservée entre les différents sérotypes, antigène spécifique du groupe ;
8	1165	NS2	Constituant majeur des corps d'inclusion cytoplasmique ; favorise la traduction des ARNm au sein de ces corps d'inclusion
9	1169	VP6 (NS4)	ARN hélicase ; déroule les brins d'ARN en présence d'ATP ; active dans le core viral ; un ORF alternatif code NS4, pour le virus de la FCO, cette protéine participerait à la modulation de réponse aux voies de l'IFN de type I
10	758	NS3-NS3a (NS5-virus de la FCO)	Protéines associées à la membrane des vésicules intracellulaires et dans la membrane cellulaire ; favorise la libération des virions par bourgeonnement ; pour le virus de la FCO, participe à la modulation de la production des IFN suite à l'infection ; des études avec le virus de la FCO ont mis en évidence un ORF alternatif codant NS5 dont la fonction doit encore être explorée

Tableau 1 : Segments génomiques et protéines du virus de la peste équine. VP : viral protein ; NS : non structural protein ; ORF : open reading frame (cadre ouvert de lecture) ; sérotype : catégorie dans laquelle on classe les virus selon leur réaction en présence de sérums contenant des anticorps spécifiques. Subdivision de l'espèce ; pour l'AHSV, le sérotype est défini par VP2 ; sérogroupe : ensemble de plusieurs sérotypes possédant en commun un facteur caractéristique ; pour l'AHSV, le sérogroupe est définit par VP7 **;** core ou nucléocapside : ensemble formé par la capside interne et l'acide nucléique viral.

protéine NS1 joue un rôle crucial dans la régulation de l'expression des gènes viraux (Matsuo et Roy, 2013 ; Boyce *et al.*, 2012) . NS2 (segment 8) est le constituant majeur des corps d'inclusion cytoplasmiques observés dans les cellules infectées. NS2 serait responsable du recrutement des différents segments d'ARN de manière à n'en avoir qu'une copie par génome grâce à un mécanisme encore inconnu. L'assemblage du core viral a lieu dans ces corps d'inclusion cytoplasmiques. Les particules de core viral une fois assemblées, quittent ensuite ces corps d'inclusion et sont transportés sur des vésicules d'exocytose grâce à l'interaction de NS3 avec des calpectines. D'autre part, pendant ce processus, les protéines VP2 et VP5 sont acquises afin de former des particules virales complètes. NS3-NS3a (segment 10) est une protéine associée à la membrane qui est impliquée dans le relâchement du virion par altération de la perméabilité de la membrane cellulaire (Meiring *et al.*, 2009). D'autre part, par analogie avec le virus de la FCO, on reconnaît à NS3, un rôle dans la virulence. En effet, des propriétés de modulation de NS3 dans la production d'interférons suite à l'infection par le virus de la FCO ont été reconnues (Chauveau *et al.*, 2013).

Récemment, un cadre de lecture ouvert (open reading frame ou ORF) alternatif au niveau du segment 9 (VP6) a été mis en évidence et code une protéine non structurale supplémentaire NS4 mais son rôle dans le cycle de réplication de l'AHSV est encore inconnu (Zwart *et al.*, 2015). Pour le virus de la FCO, NS4 module la réponse IFN de type I de l'hôte car cette protéine favorise la réplication virale in *vitro* en cellules pré-traitées à l'IFN de type I (Ratinier *et al.*, 2011).

De même, un ORF alternatif au niveau du segment 10 du virus de la FCO a été mis en évidence et code une cinquième protéine non-structurale, NS5. Cependant, le rôle précis de cette dernière n'est pas encore connu (Stewart *et al.*, 2015) et des études spécifiques pour l'AHSV sont nécessaires pour confirmer la présence de cet ORF alternatif pour ce virus également.

Quelques réactions sérologiques croisées entre les sérotypes ont été observées : entre les sérotypes 1 et 2, entre les sérotypes 3 et 7, entre les sérotypes 6 et 9 et entre les sérotypes 5 et 8 mais aucune réaction croisée avec d'autres *Orbivirus* connus n'a encore été mise en évidence (Von Teichman *et al.*, 2010). Les caractéristiques physico-chimiques de l'AHSV sont sa sensibilité aux acides, son inactivation à un pH inférieur à 6,0, sa résistance aux solvants lipidiques et sa résistance relative à la chaleur. Son infectiosité reste relativement stable à 4°C (Mellor et Hamblin, 2004).

1.4. Variabilité du virus de la peste équine

1.4.1 Diversité génétique des populations de virus à ARN

Les mutations ne sont pas des évènements occasionnels mais constituent des évènements répétés au cours des cycles de multiplication des virus, leur permettant de ce fait une diversité génétique (Domingo, 2010). Alors que certaines mutations ponctuelles passent inaperçues et sont dites neutres, d'autres mutations peuvent apporter un avantage sélectif par rapport au virus parental et déclencher l'émergence du virus au sein d'une nouvelle population ou d'un nouvel environnement (Weaver *et al.*, 2004). L'évolution de la population virale peut être conduite par des mutations, et lors de coinfections, par des recombinaisons et du réassortiment de segments qui est un cas particulier de la recombinaison et peut survenir lorsque le génome du virus est segmenté. L'accumulation de mutations, de même que la recombinaison et le réassortiment génétique, peuvent conduire par exemple à un échappement du virus aux anticorps neutralisants ou aux lymphocytes T cytotoxiques, à une résistance du virus aux agents antiviraux, à un changement de tropisme cellulaire, de vecteur ou d'hôte, à une modification de la virulence, à une réversion de l'atténuation de virulence, à une variation dans la capacité d'induction des IFN et à une persistance virale.

Lors d'une coinfection, des échanges de segments se produisent entre les génomes des deux virus apparentés pendant le cycle de multiplication virale et conduisent à la production de réassortants. Alors que le phénomène de recombinaison peut être observé chez tous les virus, le réassortiment génétique ne peut avoir lieu que chez les virus à ARN segmenté. Les mutations ponctuelles peuvent conduire à la dérive antigénique (antigenic drift) qui est une stratégie du virus pour échapper à la réponse immunitaire développée par l'hôte infecté. Le réassortiment quant à lui peut amener une cassure antigénique (antigenic shift) par l'échange d'un segment codant une protéine externe reconnue comme antigène majeur, tel qu'observé régulièrement pour le virus de l'influenza A (Zambon *et al.*, 1999). Les virus à ARN ont en général un taux de mutation significativement élevé, soit 10^{-4} à 10^{-6} mutations par nucléotide, ce qui équivaut à environ une mutation par génome par cycle de réplication (Lauring *et al.*, 2013). En comparaison, les virus à ADN, grâce à l'activité correctrice de leur ADN polymérase et d'une exonucléase 5' vers 3', subissent généralement des mutations tous les 10^8 à 10^{10} nucléotides seulement et montrent de ce fait une évolution génétique plus lente que les virus à ARN. Les virus ARN peuvent évoluer au sein d'un même hôte en formant des quasi-espèces virales c'est-à-dire une population de virus caractérisée par une séquence génomique

dominante et stable autour de laquelle gravite des mutants dont certains peuvent être sélectionnés en fonction des conditions changeantes du milieu (Domingo, 2010).

1.4.2 Diversité génétique du virus de la peste équine

Le génome segmenté de l'AHSV lui confère la possibilité d'évoluer par mutations ou réassortiment génétique. En tant que virus à transmission vectorielle, l'AHSV rencontre cependant plus de contraintes lors de son évolution génétique qu'un virus à ARN à transmission directe. En effet, l'AHSV doit passer par deux goulots d'étranglement (bottlenecks) que sont les infections du vecteur et de l'hôte pour conserver et faire évoluer ses populations virales. Cependant, ces contraintes pourraient jouer un rôle dans la sélection de certaines sous-populations à partir de quasi-espèces virales présentes. Ces sous-populations auraient au moins acquis la capacité à se multiplier de manière efficace à la fois dans l'insecte vecteur et le mammifère hôte.

Le réassortiment génétique entre différents sérotypes de l'AHSV a été décrit en culture de cellules, au niveau de l'hôte vertébré ainsi qu'au niveau du vecteur (O'hara *et al.*, 1998; Meiring *et al.*, 2009). Le réassortiment entre souches vaccinales vivantes atténuées a été décrit pour l'AHSV mais aucune réversion de virulence n'a été observée (Von Teichman et Smit, 2008). Récemment le séquençage complet du vaccin polyvalent contenant AHSV-1, 3 et 4 a montré que la souche de sérotype 3 était un virus réassortant ayant 9 segments du sérotype 3 et le segment 1 (VP1) qui provient de la souche virale de sérotype 1 (Guthrie *et al.*, 2015). Pour le virus de la FCO, le réassortiment génétique serait plus fréquent au niveau du vecteur qu'au niveau de l'hôte infecté (Roy *et al.*, 1990). Une étude récente sur le réassortiment génétique du virus de la FCO a mis en évidence, lors du séquençage de 150 isolats européens, que le réassortiment est un mécanisme fréquent pour ce virus suggérant que celui-ci joue un rôle important dans l'évolution des populations virales du virus de la FCO. De plus, au cours de cette étude, il a été démontré que les vaccins vivants atténués utilisés en Europe ont fréquemment échangé des segments avec les souches virales circulantes contribuant à la variabilité du virus de la FCO sur le terrain (Nomikou *et al.*, 2015).

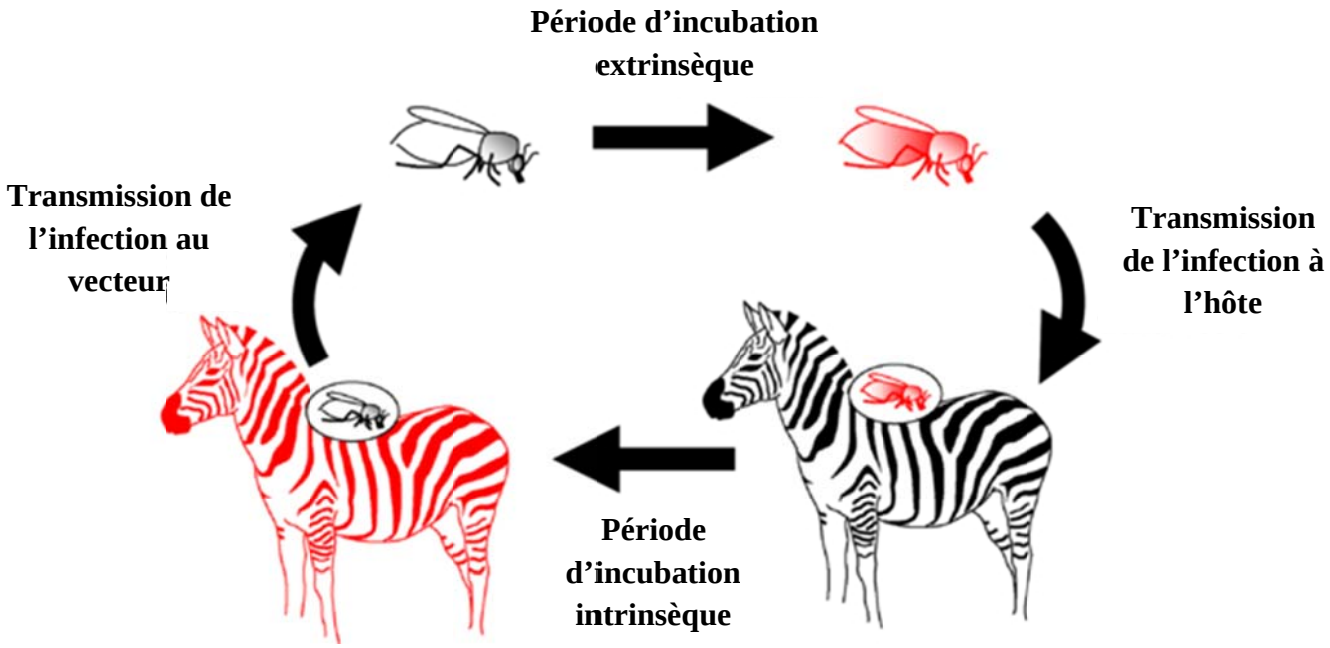

Figure 5 : Cycle naturel de transmission du virus de la peste équine (AHSV) entre le vecteur *Culicoides imicola*, vecteur principal et le zèbre, hôte réservoir. La période d'incubation extrinsèque (EIP) est fortement dépendante de la température extérieure ; à 25 °C, l'EIP dure de 7 à 10 jours. Adapté d'après Mellor *et al*., 2009.

1.5. Épidémiologie de la peste équine

1.5.1. Cycle de transmission

L'AHSV est maintenu naturellement en Afrique sub-saharienne entre un vecteur de genre *Culicoides,* principalement *C. imicola* et le zèbre (Figure 5). Les zèbres sont donc considérés comme les hôtes naturels du virus et constituent un réservoir important. Le rôle d'autres animaux dans la transmission du virus doit être investigué. En effet, le rôle des zèbres ne semble pas essentiel dans le cycle de transmission virale puisque, dans les zones géographiques où il n'y pas de zèbres comme en Afrique de l'Ouest (Oladosu *et al.*, 1993), l'AHSV circule toujours. Un nombre plus important de chevaux dans une région donnée peut jouer sur l'extension d'une épidémie d'AHSV en l'augmentant significativement (Backer et Nodelijk, 2011).

Toutes les espèces d'équidés sont sensibles à l'AHSV. Cependant, les zèbres et l'âne africain ne présentent que rarement des signes cliniques liés à l'AHSV, contrairement aux chevaux chez qui la peste équine est le résultat de l'infection des endothéliums vasculaires, avec en conséquence des fuites liquidiennes et des hémorragies. Les mulets et mules présentent une sensibilité intermédiaire au virus entre le zèbre et le cheval. De plus, une mortalité jusqu'à 95% est observée chez les chevaux car il n'existe pas de traitement spécifique contre la maladie. La vaccination et la lutte anti-vectorielle (rentrer les chevaux à l'écurie pendant la nuit ou éliminer les sites larvaires potentiels) sont donc primordiales dans les campagnes de prévention et de lutte contre la peste équine dans les régions endémiques d'Afrique (Mellor et Hamblin, 2004).

Les zèbres présentent une virémie qui dure jusqu'à plus de 40 jours contre 28 jours chez le cheval. La décision de se baser sur la durée de virémie chez le cheval pour contrôler l'entrée d'équidés en Europe a permis l'introduction de zèbres infectés de manière sub-clinique en Espagne en 1987. En plus des équidés, l'AHSV peut infecter certains carnivores via l'ingestion de viande ou de sang contaminés. Les chiens infectés montrent des signes cliniques graves (forme pulmonaire foudroyante de l'AHSV), ce qui suggère le développement d'une virémie même si le titre et la durée de celle-ci sont variables. Les chiens seraient des culs-de-sac épidémiologiques qui ne joueraient aucun rôle dans la transmission de l'AHSV (Alexander *et al.*, 1995 ; Braverman et Chizov-Ginzburg, 1996).

Chez les éléphants, des anticorps reconnus par la fixation du complément et des anticorps neutralisants ont été retrouvés à de faibles taux chez des individus sauvages. Cependant, des infections expérimentales ont été réalisées et n'ont pas permis de conclure clairement sur leur rôle dans l'épidémiologie du virus. En effet, aucune virémie n'a été détectée et leurs réponses immunitaires étaient limitées à des taux insignifiants d'anticorps (Barnard *et al.*, 1995; Barnard, 1997). L'infection des dromadaires est rare et inapparente. Aucun détail n'est disponible sur la durée de la virémie chez ces derniers (Mellor et Hamblin, 2004).

Une étude en Afrique du Sud, Namibie et Kenya, visant à connaître les maladies auxquelles les rhinocéros sont exposés, a mis en évidence des anticorps envers l'AHSV avec un taux variable selon la répartition géographique suggérant qu'ils y sont régulièrement exposés (Fischer-Tenhagen *et al.*, 2000). Cependant, une étude réalisée plus récemment dans la même région africaine, n'a pas détecté d'anticorps envers l'AHSV indiquant une prévalence variable au cours du temps (Miller *et al.*, 2011). Le rôle des rhinocéros dans l'épidémiologie de la peste équine n'a pas été investigué mais semble insignifiant.

Culicoides imicola est considéré comme étant le vecteur principal dans la transmission de l'AHSV en Afrique sub-saharienne, mais d'autres C*ulicoides* peuvent être des vecteurs potentiels aussi bien en Afrique qu'en Europe (Mellor *et al.*, 2000; Meiswinkel et Paweska, 2003). En effet, lors des épidémies espagnoles et portugaises, le virus de la peste équine a été isolé également de *C. obsoletus* et *C. pulicaris* (Portas *et al.*, 1999; Capela *et al.*, 2003), deux espèces de *Culicoides* qui, contrairement à *C. imicola*, sont retrouvées fréquemment et en grande proportion en Belgique. En outre, *C. imicola* est déjà présent dans le Bassin méditerranéen (Rawlings et Mellor, 1994; Capela *et al.*, 2003; Ramilo *et al.*, 2012) et au sud de la France et son nombre pourrait augmenter dans le nord de l'Europe avec le réchauffement climatique (Acevedo *et al.*, 2010; Cornell *et al.*, 2010; Ramilo *et al.*, 2012). En conditions de laboratoire, *C. sonorensis*, le vecteur nord-américain du virus de la FCO, a été trouvé capable de transmettre l'AHSV. Le deuxième vecteur compétent de l'AHSV dans le sud de l'Afrique est *C. bolitinos* (Meiswinkel et Paweska, 2003; Venter *et al.*, 2009; Venter *et al.*, 2010). L'AHSV a également été isolé de moustiques comme *Aedes spp*. dont on ignore l'espèce (Mellor *et al.*, 1990) pendant l'épisode espagnol-portugais et la compétence vectorielle a été testée en laboratoire pour *Anopheles stephensi*, *Culex pipiens*, et *Aedes aegypti* (Mellor, 1993) qui s'avèrent être capables de transmettre l'AHSV en conditions de laboratoire. Les tiques pourraient aussi servir de vecteurs pour l'AHSV.

L'isolement de l'AHSV d'*Hyalomma dromedarii* (Mellor, 1993; Wilson *et al.*, 2009) en conditions naturelles en Egypte ainsi que le test positif de sa capacité vectorielle en laboratoire, sont des facteurs à prendre en compte. En effet, en comparaison des culicoïdes et des moustiques, la durée de vie d'une tique est plus longue et en fait des candidates pour l' « overwintering » de l'AHSV. Cependant, l'overwintering du virus de la FCO-8 en Europe occidentale, suggèrent que les culicoïdes femelles seraient de meilleures candidates pour l'overwintering de l'AHSV dans nos contrées.

1.5.2. Peste équine en Afrique

L'AHSV est endémique dans les régions tropicales et subtropicales de l'Afrique au sud du Sahara. Le désert du Sahara semble être une barrière géographique qui protège le nord de l'Afrique du virus. A ce jour, la peste équine est endémique en Afrique sub-saharienne, avec des épisodes épidémiques annuels principalement en Ethiopie et en Afrique du Sud (Bitew *et al.*, 2011; Aklilu *et al.*, 2014). Tous les sérotypes de l'AHSV circulent en Afrique sub-saharienne et en 2015, des épidémies sont en cours au Mozambique et en Ethiopie (http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasedistributionmap, consulté le 5 mai 2015). En Afrique du Sud, les épidémies de grande ampleur d'AHSV ont lieu tous les 10-15 ans mais leur cause a été incertaine jusqu'à récemment où une corrélation étroite entre ces épidémies et le phénomène climatique nommé El Niño a été trouvée. El Niño est un courant côtier chaud, qui associé à des variations de pressions atmosphériques entre le Pacifique Est et Ouest (El Niño Southern Oscillation, ENSO), aurait des répercussions climatiques jusqu'en Afrique du Sud et amène des périodes de sécheresse suivies de fortes pluies. Dans le sol humide et dans les années à forte pluie, la population de *C. imicola* peut augmenter jusqu'à 200 fois (Baylis *et al.*, 1999). En plus de ces épidémies de peste équine, des cycles endémiques de transmission sont présents dans différentes régions de l'Afrique sub-saharienne comme l'Éthiopie et en Afrique du Sud où le virus peut être isolé de mars à septembre voire toute l'année selon les régions d'Afrique.

1.5.3. Peste équine en Europe

La première épidémie de peste équine en Europe a fait suite à l'introduction du sérotype 9 dans le nord de l'Afrique (Tunisie, Maroc et Algérie) en 1965. L'AHSV a remonté dans le nord du continent africain par l'intermédiaire d'ânes infectés et transportés à travers le Sahara. Le vent aurait été alors responsable de l'introduction du virus dans le sud de l'Espagne, en emmenant des vecteurs infectés à travers le détroit de Gibraltar (Wilson *et al.*, 2009).

L'incursion la plus récente de l'AHSV en Europe a eu lieu en Espagne en 1987. L'introduction du virus est due à l'importation de zèbres infectés par le sérotype 4 provenant de Namibie dans un parc animalier de la région de Madrid. Jusqu'au mois d'octobre 1987, des cas furent recensés en Espagne. Pendant l'hiver suivant, le cycle du virus semblait interrompu mais le virus a survécu grâce au climat tempéré de l'Espagne et a continué à sévir au cours des 3 années suivantes (Mellor, 1993; Rawlings et Mellor, 1994). Après la notification de la présence du virus à l'OIE par les autorités espagnoles, les services vétérinaires portugais ont mis sur pied un plan d'éradication du virus en cas d'introduction du virus sur leur territoire ainsi que des mesures préventives. Ce plan a dû être activé 2 ans plus tard, puisque l'AHSV a été isolé en Algarve, dans un village frontalier de l'Espagne. Grâce à ce plan comprenant une campagne de vaccination et des mesures de restriction des mouvements des équidés, la peste équine a pu être éradiquée du Portugal après 13 semaines. La campagne de vaccination a été poursuivie en 1990 et 1991. Ensuite, le Portugal a été déclaré indemne d'AHSV. Des enquêtes menées dans les écuries où les premiers cas de peste équine ont été déclarés ont conclu à l'absence de mouvement de chevaux depuis ou vers l'Espagne. L'hypothèse avancée pour expliquer l'émergence de l'AHSV au Portugal est l'introduction de vecteurs infectés depuis l'Espagne grâce au vent (Portas *et al.*, 1999).

1.6. Risque d'introduction de la peste équine en Belgique

Dans le contexte actuel de réchauffement climatique et d'intensification des échanges internationaux, le risque d'introduction de l'AHSV en Belgique doit être envisagé. L'AHSV peut être introduit par plusieurs voies, dont l'introduction d'un hôte ou d'un vecteur infecté depuis une région endémique. Les recommandations très strictes de l'OIE sur le transport d'équidés au départ de zones endémiques réduisent de façon importante le risque d'introduction par un hôte infecté. En effet, le code terrestre de l'OIE (Chapitre 12.1) prévoit

une période de quarantaine avec au moins une épreuve sérologique réalisée pendant cette période et des mesures de lutte contre le vecteur pendant la quarantaine et au moment du chargement vers le lieu d'exportation : http://www.oie.int/index.php?id=169&L=1&htmfile=chapitre_ahs.htm. Malgré tout, il convient de rester vigilant au moment de l'importation d'équidés, car si les chevaux développent une virémie de durée assez courte (entre 4 et 8 jours avec un maximum de 28jours), les ânes ou les zèbres développent une virémie durant une période plus longue. L'AHSV pourrait également être introduit par l'importation de vecteurs infectés exotiques ou indigènes par transport de bateau ou d'avion. Cette voie d'introduction est beaucoup plus difficile à contrôler que l'importation d'un hôte infecté. De plus, lors de la première épidémie européenne d'AHSV, l'introduction de *Culicoides* infectés via le détroit de Gibraltar a été incriminée. Cette hypothèse a également été avancée pour expliquer l'incursion du virus de la FCO de sérotype 8 en Europe (Maclachlan et Guthrie, 2010). *C. imicola*, le vecteur principal de l'AHSV, est déjà présent dans le bassin méditerranéen, dans le sud de la France, en Espagne, en Grèce, en Italie, au Portugal, en Turquie et à Chypre.

En plus des éléments présents dans le cycle de transmission de l'AHSV, il faut prendre en compte des facteurs d'émergence comme le climat, le commerce international, les transports internationaux d'équidés dans le cadre d'épreuves sportives ou d'expositions, les activités humaines et le virus en lui-même. Pour le climat, un temps chaud et humide est favorable à la création d'un environnement propice au développement des populations de vecteurs. Le virus en lui-même, de par son génome à ARN segmenté, a une capacité d'évolution importante par un taux de mutation élevé et un potentiel de réassortiment de ces différents segments. Des publications récentes ont mis par ailleurs en avant le fait que le vaccin atténué avait la capacité d'infecter le culicoïde vecteur et de s'y répliquer (Venter et Paweska, 2007; Venter *et al.*, 2009).

En conclusion, le risque d'introduction de l'AHSV en Belgique peut être qualifié de très faible grâce notamment aux mesures strictes imposées par l'OIE. Cependant, au vu de la gravité des conséquences économiques générées par une épidémie d'AHSV, il convient de rester vigilant et d'inclure l'AHSV dans le diagnostic différentiel avec d'autres maladies telles que l'encéphalose virale équine (equine encephalosis virus, *Orbivirus*), l'artérite virale équine (equine viral arteritis, *Arteriviridae*), l'infection par le virus Hendra (Hendra virus, *Henipavirus*), l'anémie infectieuse des équidés (equine anemia infectious virus,

Lentivirus), le charbon bactéridien (*Bacillus anthracis*), la trypanosomose (*Trypanosoma evansi)* , la piroplasmose (*Babesia caballi* ou *Theileria equi*) et le purpura hémorragique (infection respiratoire à *Streptococcus equi subsp. equi* ou suite à un épisode de grippe équine, d'anémie infectieuse équine, de rhodococcose, d'entérite, d'abcès streptococcique, de gourme ou suite à une vaccination contre la gourme, la grippe équine ou le tétanos (Thibert, 2007 ; Fiche technique de l'OIE, http://www.oie.int/fr/sante-animale-dans-le-monde/fiches-techniques/).

1.7. Pathogénie et aspects cliniques de la peste équine

1.7.1 Pathogénie

Même si l'AHSV peut être isolé à partir de beaucoup d'organes, le tropisme cellulaire du virus semble limité aux cellules endothéliales (Laegreid *et al.*, 1992), à certaines lignées de macrophages et aux réticulocytes au niveau des organes lymphoïdes (Carrasco *et al.*, 1999). La réplication virale au niveau des cellules endothéliales entraîne des lésions cellulaires avec altération de leur jonction et une augmentation de la perméabilité capillaire. Chez les chevaux, les signes cliniques caractéristiques de l'AHSV sont le résultat de ces lésions causées aux appareils respiratoire et circulatoire, provoquant des œdèmes et des hémorragies dans différents organes et tissus (Skowronek *et al.*, 1995). La pathogénie de l'AHSV comprend deux virémies : la virémie primaire débute par l'infection du nœud lymphatique drainant le site de la piqûre du vecteur et est suivie par la dissémination de l'infection dans les poumons, la rate et d'autres organes lymphoïdes. La multiplication du virus dans ces organes donne la virémie secondaire. Le temps entre l'infection et la virémie secondaire peut varier de 2 à 21 jours mais dure en général moins de 9 jours. Chez les chevaux, la virémie dure environ de 4 à 8 jours avec un maximum de 28 jours tandis que chez l'âne africain ou le zèbre, la virémie peut persister jusqu'à 40 jours. Les zèbres constituent le réservoir principal de l'AHSV et ne développent pas ou très peu de signes cliniques. La virémie chez les zèbres peut durer jusqu'à 40 jours avec un pic de virémie apparaissant au 4ème jour après l'infection (Barnard *et al.*, 1994).

1.7.2 Réponse immunitaire

La réponse immunitaire innée est la première ligne de défense contre les virus, l'ARN double brin et simple brin constituant deux motifs moléculaires spécifiques (PAMP) majeurs reconnus par les récepteurs cellulaires spécialisés (PRR). La liaison de ces molécules sur les récepteurs active notamment la production d'interférons de type I (IFN α/β) et d'autres cytokines pro-inflammatoires qui maîtrisent l'infection. À leur tour, en se liant sur les récepteurs, les cytokines activent la cascade de signalisation Jak/STAT permettant à l'organisme d'établir un état antiviral dans les cellules infectées et avoisinantes non infectées et régulant la réponse immunitaire adaptative générée par les lymphocytes B et T.

Jusqu'à ce jour, aucune donnée sur la réponse immunitaire liée aux voies de l'IFN type I, n'est disponible pour l'AHSV. Cependant, plusieurs études récentes ont générés des données sur l'induction des IFN de type I par le virus de la FCO (Vitour *et al.*, 2015). Dans les cellules non hématopoïétiques le virus de la FCO active les ARN hélicases RIG-1 (retinoic acid-inductible gene-I) et MDA5 (melanoma differentiation-associated gene 5) et la réplication virale est nécessaire à cette activation, alors qu'au niveau des cellules dendritiques plasmocytoïdes primaires ovines, l'induction de la production des IFN α/β par le virus de la FCO requiert la protéine MyD88 mais pas la réplication virale. Cette différence entre les voies de signalisation entre les deux types cellulaires pourrait être expliquée par une activation de récepteurs différents selon le moment de l'infection. D'autre part, des évidences sur le rôle de NS3 dans le blocage de l'initiation de la réponse innée dans les cellules non hématopoïétiques en inhibant la voie de signalisation des récepteurs RIG-I a été démontrée. Cependant, les mécanismes d'action précis de NS3 restent à élucider de même que le rôle des 2 autres protéines NS4 et NS5 qui pourraient également être impliquées dans cette inhibition (Stewart *et al.*, 2015 ; Doceul *et al.*, 2014 ; Chauveau *et al.*, 2013 ; Ratinier *et al.*, 2011).

Les marqueurs sérologiques précoces correspondent principalement à VP5, VP6 et NS2 et minoritairement à VP3 et NS3. Les anticorps neutralisants dirigés contre VP2 apparaissent plus tard (environ 3 semaines après l'infection) ainsi que les anticorps spécifiques de VP7 (environ 15 jours après l'infection) (Martinez-Torrecuadrada *et al.*, 1997). En plus des anticorps neutralisants, la réponse immunitaire cellulaire joue un rôle important dans le contrôle de l'infection. Les cellules cytotoxiques (CD8+) répondant *in vitro* aux antigènes du virus de la FCO, ont été décelées dans le sang de bovins ou moutons pendant la 1ère semaine suivant l'infection et ont atteint un pic deux semaines post-infection (Roy, 2007).

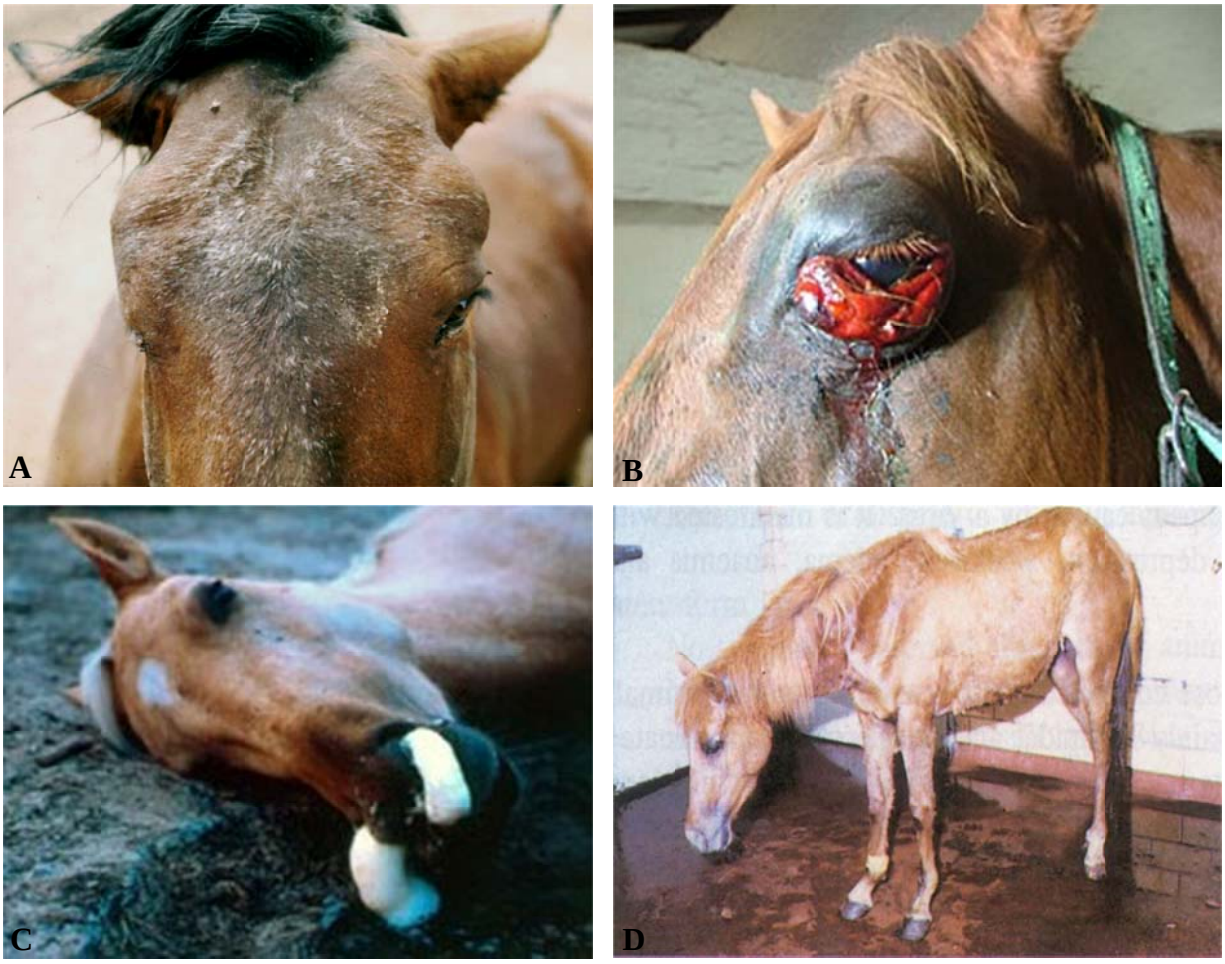

Figure 6 : signes cliniques présents chez le cheval lors d'une infection par l'AHSV.
A : œdème supra-orbitaire (maladie fébrile) **; B :** œdème supra-orbitaire avec conjonctivite hémorragique (forme cardiaque) **; C :** productions spumeuses causée par un œdème pulmonaire (forme pulmonaire) ; **D :** abattement sévère et œdème cou et poitrail (forme cardiaque). Photos Institut de santé, Pirbright et U.S. Department of Agriculture.

Comme pour le virus de la FCO, la prolifération de cellules T CD8+ virus-spécifique, parmi les cellules mononucléées sanguines (PBMC) a été mise en évidence lors de leur infection avec l'AHSV suggérant qu'elles pouvaient conférer une protection contre le virus (Pretorius *et al.*, 2012). Pour le virus de la FCO, les lymphocytes T cytotoxiques (CTL) virus-spécifiques inhibent la réplication virale au niveau des fibroblastes cutanés. Les récepteurs des CTL reconnaissent d'abord les protéines non-structurales suivi de VP3 et pour finir des protéines VP7, VP2 et VP5 (Schwartz-Cornil *et al.*, 2008).

1.7.3 Signes cliniques

Il existe 4 formes cliniques différentes de l'infection par l'AHSV (Mellor et Hamblin, 2004) qui débutent après une période d'incubation de 3 à 15 jours correspondant à l'apparition de la virémie secondaire :

- la maladie fébrile : elle atteint principalement le zèbre et l'âne africain, chez qui la peste équine passe le plus souvent inaperçue ou lors d'une infection par une souche moins virulente. Les signes cliniques sont une fièvre modérée durant 5 à 8 jours et un œdème de la fosse supra-orbitaire parfois accompagnés de congestion de la conjonctive. Les animaux guérissent de cette forme subaiguë et il n'y a pas de létalité constatée avec cette forme clinique (Figure 6 A).

- la forme cardiaque ou subaiguë : le premier signe est une fièvre s'installant progressivement et qui peut persister pendant plusieurs semaines. Lorsque la baisse de la température est amorcée, l'œdème sous-cutané apparaît principalement au niveau de la tête, de l'encolure, du poitrail et de la fosse supra-orbitaire. Les conjonctives peuvent être congestionnées et des pétéchies peuvent apparaître au niveau des yeux ainsi que des ecchymoses sur la face ventrale de la langue. On observe parfois des coliques en fin de maladie et le taux de létalité est observé 3 à 10 jours après le développement des œdèmes sous-cutanés (environ 50 %). (Figure 6 B et D).

- la forme pulmonaire ou aiguë : cette forme peut être fulgurante au point que l'animal meure sans signes cliniques précurseurs ni présence de fièvre. Une fièvre élevée (39-41°C) est notée, accompagnée d'un abattement marqué, de détresse respiratoire et d'une dyspnée avec

un jetage nasal mousseux très abondant suite à l'œdème pulmonaire. Une toux forte, spasmodique et douloureuse secoue l'animal. Le pronostic est alors très réservé et le taux de létalité peut dépasser les 95 % (Figure 6 C). Cette forme aiguë pulmonaire est rencontrée chez l'animal sensible infecté par une souche très virulente.

- la forme mixte : cette forme est un mélange entre la forme cardiaque et la forme pulmonaire. La forme mixte est la forme la plus observée chez les chevaux infectés. Les signes cliniques apparaissent en ordre différent selon les cas. Le taux de létalité approche les 70 % et la mort survient en 3 à 6 jours après le début de la fièvre.

1.7.4 Lésions nécropsiques

Les lésions macroscopiques relevées à l'autopsie sont dépendantes de la forme clinique développée chez le cheval atteint. Avec la forme cardiaque, les lésions prédominantes sont des exsudats gélatineux au niveau des tissus sous-cutané et musculaire et des nœuds lymphatiques. De l'hydropéricarde, des hémorragies et des pétéchies sont présents sur les surfaces de l'épicarde et l'endocarde et sur les séreuses du colon et du caecum. Comme pour la forme pulmonaire, de l'hydrothorax et de l'œdème pulmonaire peuvent être observés mais l'œdème est cependant beaucoup moins marqué dans cette forme.

La forme pulmonaire présente les lésions les plus visibles à l'autopsie. Ce sont surtout de l'œdème interlobulaire des poumons et de l'hydrothorax qui sont observés. Un exsudat gélatineux jaune envahit les espaces interlobulaires. L'arbre bronchique, la trachée, le larynx et les cavités nasales sont remplis par un liquide blanc mousseux. De l'ascite peut être observé au niveau abdominal ou de l'hydrothorax au niveau thoracique et l'estomac, le foie, la rate et les reins présentent de la congestion à des degrés divers (Mellor et Hamblin, 2004). De l'hémorragie présente au niveau de la région fundique de l'estomac est pathognomonique de l'infection par l'AHSV chez le cheval développant la forme pulmonaire de la maladie.

La forme mixte combine les lésions des formes cardiaque et pulmonaire. Les lésions microscopiques sont le résultat de l'augmentation de la perméabilité des parois des vaisseaux capillaires et de la conséquente détérioration de la circulation sanguine. Les lésions histologiques observées sont en rapport avec les lésions macroscopiques observées (congestion, œdème, pétéchies et hémorragies) (Zientara, 2010).

1.8. Diagnostic de l'infection par le virus de la peste équine

Malgré des signes cliniques assez pathognomoniques, le diagnostic de la peste équine est essentiel pour faire la distinction avec d'autres maladies virales équines telles que principalement l'encéphalose équine, l'anémie infectieuse des équidés ou l'artérite virale équine. Il existe des méthodes de diagnostic moléculaire et sérologique pour la mise en évidence de l'AHSV. En général, l'échantillon analysé est le sang total prélevé sur tube EDTA (acide éthylène diamine tétraacétique) pendant la phase fébrile de l'infection. Le virus peut aussi être isolé à partir de différents organes comme le foie, la rate, les poumons et les nœuds lymphatiques. L'isolement viral se fait soit par inoculation intracérébrale à des souriceaux nouveau-nés ou par injection d'œufs embryonnés ou en culture cellulaire. Des tests sérologiques spécifiques sont commercialisés.

1.8.1 Diagnostic moléculaire

Les tests moléculaires (réaction de polymérisation en chaîne, PCR) permettent la détection de l'ARN du virus de la peste équine lors d'une PCR conventionnelle ou une PCR en temps réel. Différentes séquences conservées entre les différents sérotypes ont été utilisées comme cibles après rétro-transcription dans des PCR conventionnelles. Il s'agit des séquences segments 3, 5, 7 et 8 codant VP3, NS1, VP7 et NS2 (Mizukoshi *et al.*, 1994; Zientara *et al.*, 1998; Aradaib, 2009). Une PCR conventionnelle ciblant le segment 2 codant VP2 a été développée et permet la distinction entre les différents sérotypes (Sailleau *et al.*, 2000). Différentes PCR en temps réel ont été développées pour détecter l'AHSV en ciblant soit les séquences du segment 7 (VP7) (Aguero *et al.*, 2008) soit des segments 7 et 8 (VP7 et NS2) (Quan *et al.*, 2010), du segment 5 (NS1) (Rodriguez-Sanchez *et al.*, 2008) ou du segment 8 (NS2) (Monaco *et al.*, 2011) mais ne permettent pas le sérotypage de l'AHSV. D'autres PCR en temps réel sont disponibles pour le sérotypage de l'AHSV et ciblent les séquences du segment 2 (VP2) (Koekemoer, 2008; Bachanek-Bankowska *et al.*, 2014). Enfin, dans le cadre de cette thèse, une PCR en temps réel et en mode duplex a été développée en ciblant les séquences des segments 2 et 6 (VP2 et VP5) et permet la distinction entre les sérotypes 4 et 9 de l'AHSV (de la Grandière *et al.*, en préparation).

1.8.2 Diagnostic sérologique

Les anticorps sont décelés dans le sérum des chevaux infectés à partir de 10 à 14 jours après l'infection. Lors des formes aiguës de l'infection, la progression de la maladie est très rapide

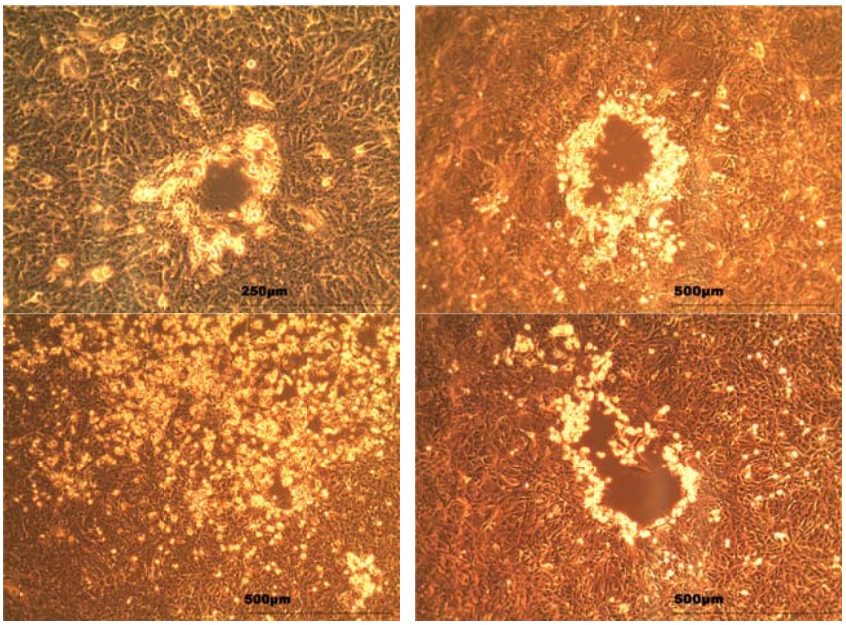

Figure 7 : Effets cytopathogènes observés sur des cellules mammifères infectées (cellules VERO) par le virus de la peste équine et recouvertes par une solution d'agarose (milieu + 0,5 % agarose). Photos prises au microscope optique avec la camera Deltapix (Invenio) 48 à 72 h post-infection.

et les anticorps ne sont pas détectés par les techniques ELISA ou Western Blot avant la mort de l'animal. Quand les animaux infectés survivent au-delà de 9 jours, quelle que soit la forme développée de l'infection, une forte réponse sérologique est observée. L'OIE reconnaît officiellement plusieurs techniques de diagnostic sérologique dont la réaction de fixation au complément, l'ELISA et la séroneutralisation (OIE, Manuel Terrestre, 2012). La séroneutralisation est utilisée pour le sérotypage de l'AHSV et est dès lors intéressante dans les régions endémiques où plusieurs souches virales circulent. Il existe deux kits commerciaux pour la détection sérologique de l'AHSV. Il s'agit d'un kit ELISA de compétition ou indirect (Ingenasa, Ingezim) avec une détection spécifique des anticorps envers VP7 dans le sérum des équidés ou un kit ELISA en double sandwich avec l'anticorps envers VP7 et qui détecte cette fois-ci les antigènes dans les rates d'animaux infectés (Maree et Paweska, 2005).

Un ELISA indirect a également été développé en utilisant comme antigène la protéine NS3 du sérotype 4 de l'AHSV. Cet ELISA développé au moment de l'épidémie espagnole-portugaise, permettait de distinguer les sérums d'animaux infectés, ou vaccinés avec un vaccin à virus modifié, des sérums d'animaux vaccinés avec des vaccins inactivés.

1.8.3 Isolement en culture cellulaire

L'isolement de l'AHSV est possible sur différentes lignées cellulaires de mammifères (cellules de reins de singe vert africain ou VERO, cellules de reins de jeune hamster ou BHK-21, et cellules de singe ou MS) et sur différentes lignées cellulaires d'insectes (cellules d'*Aedes albopictus* ou C6/36, cellules de *Culicoides variipennis* ou K3, et cellules de *C. sonorensis* ou W3).

Des effets cytopathogènes (CPE) peuvent être observés sur cellules de mammifères entre 2 à 8 jours post-infection (Figure 7). En culture de cellules d'insectes, aucun CPE n'est observable. Ce phénomène a été attribué au fait que la voie de signalisation pour l'induction de l'apoptose n'est pas déclenchée par l'infection des cellules d'insectes par l'AHSV (Stassen *et al.*, 2011).

1.9. Vaccination

1.9.1 Vaccins commercialisés

Les premiers vaccins contre l'AHSV étaient des vaccins atténués par plusieurs passages en cerveau de souris. Ils donnaient une bonne protection mais avaient occasionnellement de sévères effets secondaires y compris des cas fatals d'encéphalites chez

les chevaux et les ânes, surtout après la primovaccination. Ce problème fut résolu après passages successifs en culture de cellules (VERO). Les vaccins actuels sont deux vaccins atténués multivalents dont l'un contient les sérotypes 1, 3 et 4, et dont l'autre contient les sérotypes 2, 6, 7 et 8 de l'AHSV. Le sérotype 5 n'est pas compris dans le vaccin à cause de réactions secondaires violentes et le sérotype 9 n'est pas inclus grâce à une forte réaction croisée avec le sérotype 6. La vaccination se compose d'une primovaccination et d'un rappel dans la première année, puis de rappels annuels. Il existe également certains vaccins atténués monovalent, par exemple pour le sérotype 9 en Afrique de l'Est. Les vaccins atténués conviennent pour la vaccination dans les zones endémiques mais ne sont pas autorisés dans les zones indemnes. En effet, des cas de retour de virulence des souches atténuées ou de réassortiment avec la souche sauvage circulant lors d'une épidémie ont déjà été rapportés (Oura *et al.*, 2012; Aklilu *et al.*, 2014).

Au début des années 1990, un vaccin inactivé monovalent contenant le sérotype 4 a été commercialisé. Actuellement, il n'est plus disponible sur le marché à cause de coûts de production élevés et d'une durée d'immunité insuffisante.

1.9.2 Vaccins expérimentaux

La génération de virus recombinant utilisant le virus Vaccinia (MVA) comme vecteur d'expression et exprimant les gènes de différentes protéines de l'AHSV est prometteur pour l' obtention de vaccins efficaces. Une première étude insérant les gènes codant VP2, VP7 et NS3 dans le vecteur Vaccinia a confirmé l'induction d'une réponse immunitaire chez des poneys inoculés par le virus recombinant. Alors que NS3 n'induisait pas d'anticorps, une concentration élevée en anticorps neutralisants a été observée lors de l'immunisation de poneys avec le MVA-VP2 (Chiam *et al.*, 2009). Ces résultats ont été confirmés lors de l'immunisation de souris IFNAR -/- chez qui une immunisation avec le MVA-VP2 protégeait

contre l'inoculation du virus homologue avec notamment l'absence de détection de virémie chez les animaux vaccinés (Castillo-Olivares *et al.*, 2011). De la Poza *et al.* (2013) ont ensuite comparé l'efficacité de MVA recombinant et ADNc recombinant exprimant soit VP2 ou NS1 ou soit VP2 et NS1 chez des souris IFNAR -/-. Ils ont observé que le MVA recombinant protégeait plus efficacement contre l'infection par le virus homologue (AHSV-4) que le vaccin d'ADNc alors que, pour l'infection par le virus hétérologue AHSV-9, la protection induite par les deux types de vaccins était similaire. D'autre part, l'inclusion de NS1 dans la composition vaccinale ensemble avec VP2 conférait une protection optimale contre l'infection par virus homologue et hétérologue. NS1 étant très conservée entre les différents sérotypes de l'AHSV, son incorporation dans le vaccin est importante pour induire une protection croisée avec les autres sérotypes. Des chevaux immunisés avec un MVA recombinant VP2-9 étaient protégés contre l'infection par le virus homologue (AHSV-9), ne développaient pas de signes cliniques et n'ont également pas produit de virémie (Alberca *et al.*, 2014). Les sérums de souris immunisés avec un vaccin MVA recombinant VP2 protégeaient les souris à qui le sérum était administré jusqu'à 48 h avant ou après l'infection par le virus homologue (AHSV-4) (Calvo-Pinilla *et al.*, 2014 ; Calvo-Pinilla *et al.*, 2015). La production de sérums issus d'animaux immunisés pourrait dès lors être utilisée en cas d'urgence lors d'une épidémie.

Le canarypoxvirus a également été utilisé comme vecteur vaccinal pour l'AHSV. Des chevaux immunisés avec un canarypoxvirus recombinant exprimant les deux protéines de capside externe (VP2 et VP5) ont développé des anticorps neutralisants et ont résisté à l'infection par le virus homologue (AHSV-4) (Guthrie *et al.*, 2009 ; Garch *et al.*, 2012)..

1.10 Modèle murin d'infection pour le virus de la peste équine

L'établissement d'un modèle d'étude sur souris est crucial pour l'étude de l'infection d'un virus comme l'AHSV. En effet, outre les problèmes d'éthique qui seraient posés, l'utilisation de chevaux pour une infection *in vivo* rencontre des problèmes de logistique (nécessité d'une animalerie A3 assez spacieuse pour pouvoir détenir des grands animaux, manipulations très lourdes) et donc un coût de mise en place très important.

Peu d'études scientifiques se sont intéressées jusqu'à présent à la mise en place d'un modèle d'étude de l'AHSV sur souris. Dans l'étude de Wade-Evans *et al*. (1998), un premier modèle

a été établi en utilisant les souris immunocompétentes Balb/C et utilisé pour l'étude de la protection conférée par un vaccin sous-unitaire. La seule technique utilisée au cours de cette étude pour déterminer la virémie sanguine est la méthode de dénombrement des particules infectieuses permettant de calculer des doses infectieuses en culture de cellules à 50 % ($TCID_{50}$) et peu d'informations sont donc disponibles. De même, dans l'étude d'O'Hara *et al.* (1998), seule la mortalité liée au sérotype, à la voie d'inoculation et au titre viral est investiguée pour les souris Balb/C. En se basant sur une étude d'infection par le virus de la FCO sur un modèle de souris déficiente en récepteur à l'interféron α (Interferon-α Receptor knock-out, IFNAR -/-), Castillo-Olivares *et al.* (2011) ont mis en place le modèle d'étude animal IFNAR -/- en utilisant le sérotype 4 uniquement. Mais comme pour les études précédentes, la virémie n'est définie que sur base de titrage (en $TCID_{50}$) à certains moments de l'expérience. De plus, de ces 3 études, il ressort une description très succinte des signes cliniques observés chez la souris infectée par l'AHSV. Plus récemment, le modèle souris IFNAR -/- a été utilisé dans plusieurs études testant l'efficacité de vaccins sous-unitaires contre l'AHSV-4 ou l'AHSV-9 (De La Poza *et al.*, 2013; Calvo-Pinilla *et al.*, 2014; Calvo-Pinilla *et al.*, 2015). De plus, la première étude de cette thèse est consacrée à la comparaison de 3 souches de souris pour l'infection par les AHSV-4 et -9 (De La Grandiere *et al.*, 2014).

Objectifs

Les maladies virales transmises par les arthropodes ou arbovirus représentent une menace pour la santé humaine et animale. Les émergences inattendues et la persistance du virus de la FCO et du virus Schmallenberg (SBV), en Europe du Nord et centrale (Gould et Higgs, 2009; Saegerman *et al.*, 2010; Tabachnick, 2010), sont des exemples évidents de la possibilité que des maladies exotiques peuvent émerger et devenir endémiques dans ces régions (Weaver et Reisen, 2009). De plus, l'adaptation de ces virus aux vecteurs *Culicoides* indigènes ont mis en évidence la nécessité d'étudier les mécanismes de mutation et donc d'évolution des populations virales qui permettraient d'expliquer leur adaptation à de nouvelles niches écologiques (Domingo, 2010; Maclachlan et Guthrie, 2010; Backer et Nodelijk, 2011).

Les virus à ARN se répliquent avec un taux de mutation très élevé et montrent de ce fait une diversité génétique significative (Worobey et Holmes, 1999). L'évolution rapide de ces virus à ARN complique la prédiction d'émergence virale de virus comme l'AHSV. Les deux « bottlenecks » auxquels doivent faire face les virus à transmission vectorielle sont d'une part la réplication dans un vecteur arthropode et d'autre part la réplication dans un hôte vertébré, ce qui implique donc une plasticité du virus au niveau du passage dans ces deux systèmes différents. Chez les *Orbivirus*, les mutations ponctuelles ont déjà été analysées en revue (Von Teichman et Smit, 2008; Meiring *et al.*, 2009; Matsuo *et al.*, 2010), alors que le réassortiment a été peu étudié. Pourtant le réassortiment est un important mécanisme d'évolution pour les virus à génome segmenté comme cela est déjà observé avec les virus Influenza A (Zambon, 1999) ou encore avec les *Rotavirus* (Ghosh et Kobayashi, 2011). Le réassortiment peut conduire à des conséquences au niveau de l'épidémiologie du virus : interaction entre les différentes souches virales, les équidés et les vecteurs, et adaptation du virus à un nouvel environnement. D'autres effets possibles du réassortiment peuvent être une modification de la virulence de l'AHSV, du profil antigénique ou du tropisme pour l'hôte ou le vecteur. Les virus réassortants ne peuvent pas être identifiés par les méthodes diagnostiques conventionnelles qui portent sur le segment 2 codant la protéine VP2 responsable du sérotype. D'autres approches diagnostiques sont donc nécessaires pour leur mise en évidence sur le terrain.

Cette thèse a été réalisée dans l'objectif d'approfondir les connaissances sur l'AHSV par la mise au point d'un modèle animal expérimental et de contribuer à l'étude du phénomène de réassortiment pour l'AHSV.

Dans un premier temps, la mise en place et le développement d'un modèle murin s'est révélé un outil nécessaire pour l'étude de l'infection par l'AHSV. La comparaison de l'infection entre trois souches de souris et deux voies d'inoculation différentes a été effectuée dans la première étude de la thèse. Ces trois souches de souris ont été testées pour étudier la pathogénie et les signes cliniques de la maladie après infection par les sérotypes 4 et 9 de l'AHSV. L'influence des voies d'administration (sous-cutanée et intranasale) sur l'issue de la maladie a été étudiée. D'autre part, trois souches de souris ont été utilisées, dont une était une souche de souris déficiente en récepteur à l'interféron α/β (souris IFNAR -/-) et l'influence de la présence ou non de récepteur à l'interféron α/β est discutée en comparant les signes cliniques présentés par ces souris avec ceux des souris immunocompétentes (souris Balb/C et 129 WT).

Deux volets composent la deuxième étude présentée dans cette thèse. Dans le premier volet, le phénomène de réassortiment génétique lors de coinfections entre les sérotypes 4 et 9 de l'AHSV a été étudié. Tout d'abord, des outils de discrimination des virus réassortants potentiels ont été développés. Ensuite des expériences de coinfection *in vitro* ont été réalisées pour l'obtention de virus réassortants. Ces derniers ont été caractérisés par PCR et séquençage. Leurs propriétés de multiplication en culture de cellules ont été comparées aux virus parentaux. Dans le deuxième volet, l'application concrète du modèle murin développé dans la première étude a permis d'évaluer la virulence *in vivo* de virus réassortants obtenus *in vitro* et préalablement sélectionnés grâce aux résultats obtenus dans la première partie de cette étude.

Section Expérimentale

Section expérimentale

Étude 1

Modèle murin expérimental d'infection par le virus de la peste équine

Préambule

L'objectif de cette étude est l'analyse *in vivo* de la virulence de deux différentes souches de l'AHSV et de comparer les conséquences de l'infection sur trois souches de souris différentes. Dans ce but, les sérotypes 4 (AHSV-4) et 9 (AHSV-9 de l'AHSV ont été inoculés par voies sous-cutanée (SC) et en intranasale (IN) à deux souches de souris immunocompétentes (Balb/C 129 Sv/Ev (129 WT)) ainsi qu'à des souris de souche IFNAR -/-, déficientes en récepteur à l'interféron α (avec un background génétique de 129 Sv/Ev).

Chez les souris IFNAR -/-, une mortalité s'élevant à 50 % a été relevée et des signes cliniques significativement plus importants ont été observés en comparaison avec les souris immunocompétentes inoculées par voie SC. Les signes cliniques observés étaient significativement plus prononcés après l'infection par l'AHSV-4, surtout chez les souris immunocompétentes inoculées par voie IN. Le nombre de copies génomiques mesurées dans les échantillons sanguins était significativement plus élevé après l'infection par l'AHSV-4. Dans les organes des souris 129 WT inoculées en IN, le nombre de copies génomiques était significativement plus élevé après infection avec l'AHSV-4. Ensemble et dans les conditions de cette étude, ces différents résultats démontrent une plus grande virulence de l'AHSV-4 par rapport à l'AHSV-9 et la présence de signes cliniques plus graves chez les souris inoculées par la voie IN, du moins pour les 2 sérotypes inoculés. L'étude apporte aussi des évidences indirectes du rôle de la voie interféron de type I au cours de l'infection par l'AHSV.

Cette étude a permis de mettre au point différents modèles murins pour l'étude de l'infection par l'AHSV au sein du laboratoire, et d'établir des critères de sélection du modèle murin à utiliser en fonction des objectifs poursuivis.

Study of the virulence of serotypes 4 and 9 of African horse sickness virus in IFNAR -/-, Balb/C and 129 Sv/Ev mice

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Study of the virulence of serotypes 4 and 9 of African horse sickness virus in IFNAR$^{-/-}$, Balb/C and 129 Sv/Ev mice

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ABSTRACT

African horse sickness virus (AHSV) is a double-stranded RNA virus which belongs to the family *Reoviridae*, genus *Orbivirus*. Recent studies have focused on the interferon-α/β receptor knock-out mice (IFNAR$^{-/-}$) as a small animal laboratory for the development of AHSV vaccines. The aim of this work was to study in vivo the virulence of two strains of AHSV and to compare the outcome of the infection of three mouse strains. To address this, AHSV serotypes 4 (AHSV-4) and 9 (AHSV-9) were inoculated subcutaneously (SC) and intranasally (IN) in two immunocompetent mouse strains (Balb/C and 129 Sv/Ev (129 WT)) as well as IFNAR$^{-/-}$ mice (on 129 Sv/Ev genetic background). In IFNAR$^{-/-}$ mice, fatality up to 50% was measured and significantly more clinical signs were observed in comparison with SC inoculated immunocompetent mice. The observed clinical signs were significantly more severe after AHSV-4 infection, in particular in immunocompetent mice inoculated by IN route. Considering RNAemia, significantly higher viral loads were measured following AHSV-4 infection. In the organs of 129 WT inoculated by IN route, significantly higher viral loads were detected after AHSV-4 infection. Together the results support a higher virulence for AHSV-4 compared to AHSV-9 and a higher clinical impact following infections in IN inoculated mice, at least in the investigated strains. The study also brought indirect evidences for type I IFN involvement in the control of AHSV infection.

1. Introduction

African horse sickness (AHS) is an infectious non-contagious disease for all equids caused by African horse sickness virus (AHSV). AHSV is a non-enveloped virus belonging to the *Reoviridae* family, genus *Orbivirus*. AHSV has a genome composed of ten linear segments of double-stranded RNA (dsRNA). The AHSV genome encodes 7 structural (VP1 to VP7) and 4 non-structural proteins (NS1, NS2, NS3 and NS3a). VP2 and VP5 compose the outer capsid and are involved in cell attachment and virus entry. VP2 together with VP5 determine the antigenic variability of AHSV and until now nine different serotypes of AHSV have been described. The outer capsid covers the inner

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capsid that is composed of two major structural proteins (VP3 and VP7) and three minor proteins (VP1, VP4 and VP6) (Roy, 1996).

AHSV is transmitted by biting midges of the *Culicoides* genus, generally *C. imicola*. Zebras are considered as the natural host for the virus and are an important reservoir for AHSV in South Africa. However, AHSV could be maintained in their absence and in the presence of other equids (Oura et al., 2012). In horses, AHS pathogenesis results from endothelial cell infections (Skowronek et al., 1995) and viraemia can last until 3 weeks post-infection. Both morbidity and fatality are high (up to 95%) with severe economic losses (Mellor and Hamblin, 2004). AHSV is enzootic in sub-Saharan Africa with a particular distribution in East and West Africa depending on the serotypes (Bitew et al., 2011). In the last decades, AHS outbreaks have occurred in Southern Europe with strong epizootic characteristics. AHSV serotype 9 (AHSV-9) was introduced in 1966 in Spain by infected *Culicoides* through Gibraltar Detroit. In 1987, AHSV serotype 4 (AHSV-4) was introduced via infected zebras in the zoo of Madrid (Portas et al., 1999; Wilson et al., 2009).

A major current concern is the risk of re-introduction of AHSV in European countries. Indeed, the combination of different factors such as climatic changes (Tabachnick, 2010), increase of international trade and movement (Martinez-Lopez et al., 2011) and the continuing genetic evolution resulting in new adaptation possibilities (Domingo, 2010) increase the risk of introduction of AHSV in Europe. Furthermore, the recent incursion of two vector-borne viruses, Bluetongue virus (BTV) in 2006 (Saegerman et al., 2010; Thiry et al., 2006) and Schmallenberg virus (SBV) in 2011 (Beer et al., 2013) highlighted the potential of emergence of different arboviruses within this particular geographic region.

Type I interferons (IFN) comprise a large group of molecules. In this family, the IFN α/β genes transcription is induced in response to viral infections via the recognition of pathogens associated patterns. IFN α/β thereafter acts by autocrine and paracrine effects through the IFN α/β receptor (IFNAR) to regulate the expression of a large set of genes that establish an antiviral response in target cells (Biron, 1998). BTV is a great inducer of IFN α/β following virus inoculation in sheep, cattle and mice (Chauveau et al., 2013; Ruscanu et al., 2012). However at present, no similar observations have been made for AHSV. Despite this, interferon-α/β receptor knock-out mice ($IFNAR^{-/-}$) have been used as a receptive and highly sensitive small animal model in the study of the efficacy of AHSV vaccines (Calvo-Pinilla et al., 2014; Castillo-Olivares et al., 2011; de la Poza et al., 2013).

Here we report the AHSV infection of $IFNAR^{-/-}$ mice (on 129 Sv/Ev genetic background) and of two immunocompetent mice (Balb/c and 129 Sv/Ev (129 WT)) in two different experiments. Our results give elements that could indirectly support the role of IFN type I in AHSV infection as already observed during BTV infections. Two different viruses (AHSV serotypes 4 and 9) were used, allowing the observation of remarkable differences in the virulence of the two strains. The results showed the importance of both the selection of the mouse strain the most appropriate for the purpose of a study, and the inoculation route (subcutaneously (SC) versus intranasally (IN) routes) on the onset of the clinical disease in immunocompetent mice.

2. Materials and methods

2.1. Virus and cells

Baby hamster kidney (BHK-21) and Vero cells were grown in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% heat inactivated foetal calf serum (FCS) (Invitrogen), 2% of penicillin–streptomycin combination (PS) (Invitrogen) and 1% of a non-essential amino acids preparation (NEAA) (Invitrogen). The choice of the AHSV serotypes was made in regards with the two past European incursion of the virus (Mellor and Hamblin, 2004). AHSV serotype 4 (AHSV-4) and AHSV serotype 9 (AHSV-9) derived from South African strains 32/62 (GenBank accession numbers: JQ796724.1, JQ796725.1, JQ796726.1, JQ796727.1, JQ796728.1, JQ796729.1, JQ796730.1, JQ796731.1, JQ796732.1, and JQ796733.1) and 90/61 (GenBank accession numbers: KF860036.1, KF860037.1, KF860038.1, KF860039.1, KF860040.1, KF860041.1, KF860042.1, KF860043.1, KF860044.1 and KF860045.1) and were kindly provided by the European reference laboratory for AHSV in Spain (Aguero et al., 2008). Historically, these viruses were passaged three times in mouse brain and two times in BHK-21 before their cloning in the European reference laboratory in Spain. After reception in our laboratory, these viruses were passaged four times in Vero cells and three times in BHK cells. Virus stocks were generated by infection of confluent BHK-21 or Vero cells with these viruses at a multiplicity of infection (MOI) of 0.1 (VERO-based titres). When total cytopathic effect (CPE) was visible, mainly at 48 or 72 h post-infection, supernatants were harvested and centrifuged. The virus was released from the cells by three freezing/thawing cycles. Supernatants were stored at −80 °C until use.

Standard virus titration was performed in Vero cells while viral titres were expressed as $TCID_{50}$/ml (Reed and Muench, 1938).

2.2. Mice

BALB/cByJ (Balb/C) mice were purchased from Charles River France. $IFNAR^{-/-}$ as well as the wild-type 129 Sv/Ev (129 WT) were purchased from B&K Universal United Kingdom.

Two in vivo experiments were performed using 6 week-old female mice. In the first one, $IFNAR^{-/-}$ mice were used in parallel with Balb/C mice, while during a second experiment, $IFNAR^{-/-}$ and 129 WT mice were used. In both experiments 9 groups were created, in which 6 were infected and 3 were mock-infected. Each infected group was composed of 6 mice, while for ethical reasons only 3 mice were used in the mock-infected groups (Fig. 1). Mice were allowed to acclimatize to the biosafety level 3 animal facilities (A3) at Faculty of Veterinary Medicine, Liège, for 1 week before the beginning of the experiments.

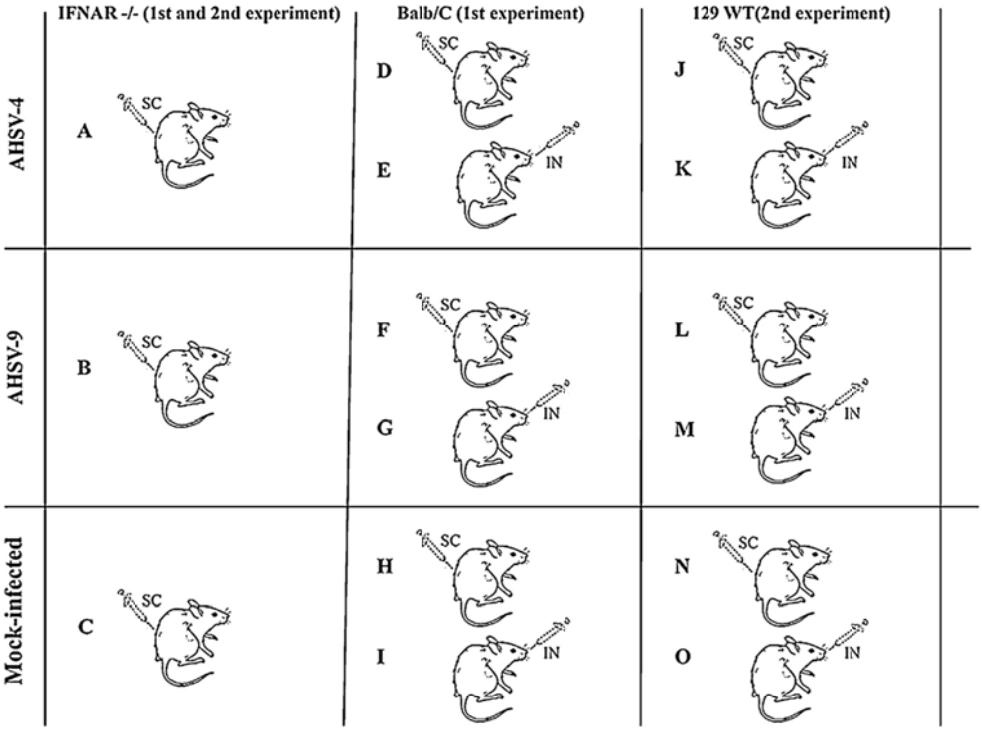

Fig. 1. Composition of the different groups of mice. Two experiments were conducted. During the first experiment, IFNAR$^{-/-}$ (groups A–C) and Balb/C (groups D–I) were tested. For the second experiment, IFNAR$^{-/-}$ (groups A–C) and 129 WT (groups J–O) were used. Each infected group contained 6 mice while mock infected groups were composed by 3 mice. Serotypes 4 and 9 of AHSV were inoculated to the three strains of mice and two different routes of inoculation were tested for Balb/C and 129 WT mice.

All experiments with live animals were performed under the guidelines of the European Community (86/609) and were approved by the site ethical review committee (protocols nos. 1219 and 1362). All efforts were made to minimize suffering.

2.3. Animal inoculation and processing of samples

IFNAR$^{-/-}$ mice were infected SC with AHSV-4 or AHSV-9 (groups A and B) (Fig. 1). Immunocompetent mice (Balb/C and 129 WT) were infected SC with AHSV-4 (groups D and J) or AHSV-9 (groups F and L) and IN with AHSV-4 (groups E and K) or with AHSV-9 (groups G and M). Control mice were inoculated SC (groups C, H and N) or IN (group I and O) with DMEM (Invitrogen). Mice were inoculated using previously titrated virus stocks (1.25×10^6 $TCID_{50}$ for both serotypes) and each inoculum was tested by back-titration in order to assess its infectivity at the time of the experiment. Mice were examined daily for clinical signs until the end of the experiment (21 days p.i.). For each mouse presenting clinical signs, a clinical score was given following standardized criteria previously assigned (supplementary table).

Supplementary table related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2014.10.006.

Animals showing severe clinical signs were euthanized for ethical reasons. Whole blood was collected in EDTA tubes at regular intervals for virus detection and isolation. After euthanasia (performed after anaesthesia with ketamine 50 mg/kg and xylazine 5 mg/kg by intramuscular injection and followed by cervical disruption), several organs (liver, spleen, kidney, lungs and brain) were collected at the necropsy and conserved at −80 °C until RNA extraction or in 4% buffered formalin until histopathological investigations.

2.4. RT-qPCR specific for AHSV segment 7

Total RNA was extracted from whole blood using the Mini Kit QIAamp Viral RNA (Qiagen) according to the manufacturer's instructions, diluted in 60 μl of elution buffer and subsequently stored at −80 °C. Total RNA was extracted from organs with TRI Reagent solution (Applied Biosystems), according to the method recommended by manufacturer, diluted in a final volume of 60 μl of RNAse/DNAse free water and stored at −80 °C. After heat denaturation (5 min at 95 °C), RNA was reverse transcribed with TaqMan Reverse Transcription Reagents (Applied Biosystems). cDNA was amplified by real time PCR on a C1000 Touch Thermal cycler (CFX96 Real Time System, Bio-Rad). The detection of AHSV was performed using a protocol adapted from Aguero et al. (2008) and using primers and probe set directed to a highly conserved sequence within the segment 7 region of the AHSV genome. In addition, a set of primers and probe targeting

murine RNA GAPDH were included as an internal control (IC) for all samples (Gangisetty and Reddy, 2009). Moreover, a synthetic external control RNA (EC-EXTR) having a known and defined concentration was added to the sample prior to extraction, allowing to standardize and monitor the efficiency of the extraction and the RT-qPCR (Vandenbussche et al., 2010). For each sample viral VP7 amplification, as well as murine GAPDH and EC-EXTR detection were performed in duplicate and in separate wells. The results were expressed as mean values of the replicates.

In order to quantify the number of viral genomic copies per µl of blood (RNAemia) or gram of tissue, a plasmid containing the VP7 target sequence amplified during the RT-qPCR was constructed. The specific VP7 PCR product obtained after RT-PCR (84 bp) was purified using a High Pure PCR Product Purification Kit (Roche) and then cloned using the pGEM-T Easy Vector System I (Promega). Plasmid containing the inserted sequence was purified with High Pure Plasmid Isolation kit (Roche) and sequenced using T7 and SP6 primers to determine the orientation of the insert and to verify the integrity and the specificity of the nucleotide sequence. Plasmid DNA quantity was determined using a spectrophotometer (NanoDrop 1000, Thermo Scientific) and was used together with the molecular weight to calculate the copy number of plasmid DNA per µl. 10-fold dilutions from 10^{10} to 10^{0} of plasmid DNA copies/µl were prepared using RNAse/DNAse free water and stored at −80 °C. These VP7 plasmid dilutions were included in duplicate at each qPCR to provide a standard curve.

2.5. Histopathology

Samples from different organs were taken and fixed in 4% buffered formalin and embedded in paraffin for histopathological studies, according to standard laboratory procedures. Tissues sections of 4 µm thickness were stained with haematoxylin and eosin and examined by light microscopy at different magnifications.

2.6. Statistical analysis

For each strain of mice, the survival prediction was assessed based on a logistic regression analysis, in which the fatality was used as explicated variable while weight and days p.i were used as explicative variables. The statistical analyses performed taking in consideration the clinical score (sum of the clinical signs that were present), was assessed by a Poisson regression using the clinical score as explicated variable and the mouse strain, the viral serotype and the day p.i. until fatality as explicative variables. The number of genomic copies in blood and in organs was assessed by a negative binomial regression because of the extra binomial variability. This analysis was performed using the number of genomic copies as explicated variable and the mouse strain, the viral serotype, the route of infection, the blood or organ concerned and the day p.i. as explicative variables. For all tests, P values <0.05 were considered significant (Dohoo et al., 2010).

3. Results

3.1. Clinical disease after AHSV-4 and AHSV-9 infections

Two experiments were conducted successively: the first one comparing AHSV infection in IFNAR$^{-/-}$ and Balb/C mice, and the second one comparing AHSV infection in IFNAR$^{-/-}$ and 129 WT mice, that have a similar genetic background. The back titration of the inocula in both experiments was 1.0×10^{7} $TCID_{50}$/ml for AHSV-4 and 1.6×10^{7} $TCID_{50}$/ml for AHSV-9. All mouse strains were infected SC. AHSV IN inoculation can lead to up to 100% of fatality for some strains (O'Hara et al., 1998). For that reason, the IN route was used only for the two immunocompetent mouse strains (Fig. 1a).

All mock infected mice remained healthy with a regular increase of the body weight (data not shown). The body weight loss occurred along the experiment and after the infection, was predictive of the fatality of both immunodeficient and immunocompetent mice ($P \leq 0.01$). Indeed, the trend of body weight loss in time appears to be a good indicator of the outcomes of the infection.

Considering clinical signs, no significant difference was observed between the two experiments realized with IFNAR$^{-/-}$ mice ($P < 0.05$). Therefore, only the results from the first experiment are showed in Fig. 2(a). In both experiments, IFNAR$^{-/-}$ showed clinical signs starting at 48 h post-infection (p.i.) with a peak at day 6 p.i. (Fig. 2a). These signs were characterized by conjunctivitis, important weight loss (up to 20%), lethargy, hunched posture, neurologic troubles (ataxia, circling gait, paresis, paralysis) and rough hair coat. During the first and the second experiment one mouse from group A (AHSV-4-SC) and one from group B (AHSV-9-SC) respectively, died from the disease. Within the same groups, two more mice had to be euthanized on welfare grounds due to paralysis and ataxia. An overall 50% fatality was observed in infected IFNAR$^{-/-}$ groups in both experiments (Fig. 2a).

Balb/C mice infected SC did not show any clinical signs during the experiment except a slight weight loss (2–5%) (data not shown). In contrast, IN infected Balb/C mice presented a severe weight loss combined with rough hair coat, weakness and dehydration from day 9 p.i. (Fig. 2b). These observations were made in groups infected by both serotypes and 3 mice infected with serotype 4 were euthanized on welfare grounds due to severe dehydration and conjunctivitis.

Less severe clinical signs were observed for the 129 WT groups of mice before fatality was observed (day 11 p.i.). Indeed, SC infected mice did not show clinical signs during the experiment (data not shown). In contrast, four 129 WT mice infected IN with AHSV-4 were euthanized on welfare grounds due to strong dehydration, weight loss, rough hair coat and apathy (Fig. 2c). In this group up to 60% fatality was observed, while for 129 WT infected IN with AHSV-9, identical clinical signs were recorded with a fatality rate of about 30% (Fig. 2c).

Significantly more clinical signs were observed in IFNAR$^{-/-}$ mice than in both immunocompetent mice

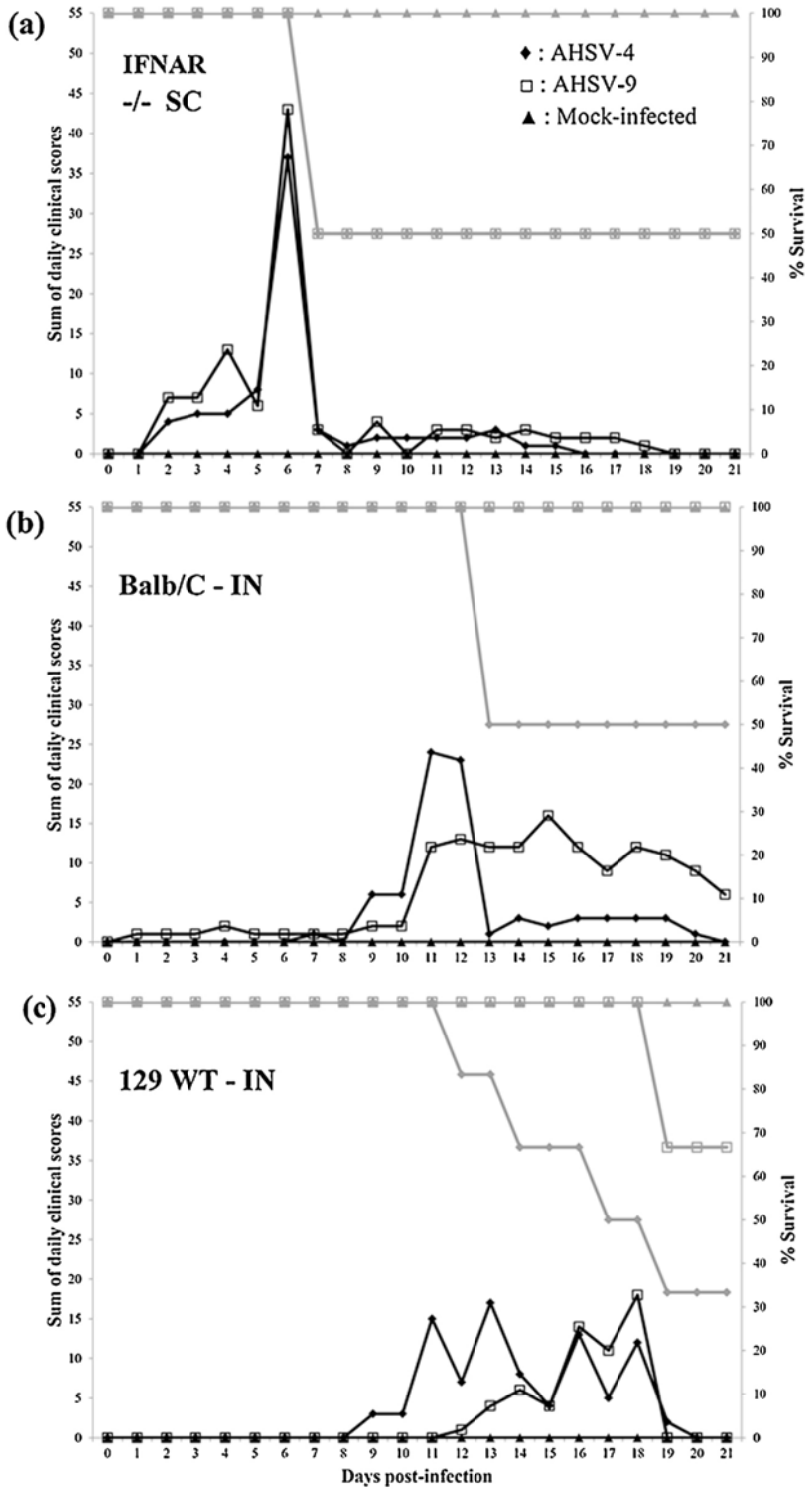

Fig. 2. Sum of daily clinical scores and survival rates. Only groups showing clinical signs are presented, namely IFNAR$^{-/-}$ mice infected SC (a), Balb/C infected IN (b) and 129 WT infected IN (c). In each figure, bold lines represent the sum of daily clinical score attributed to the mice of the same group. Survival rates are represented by simple lines and expressed as the percentages of surviving mice per day.

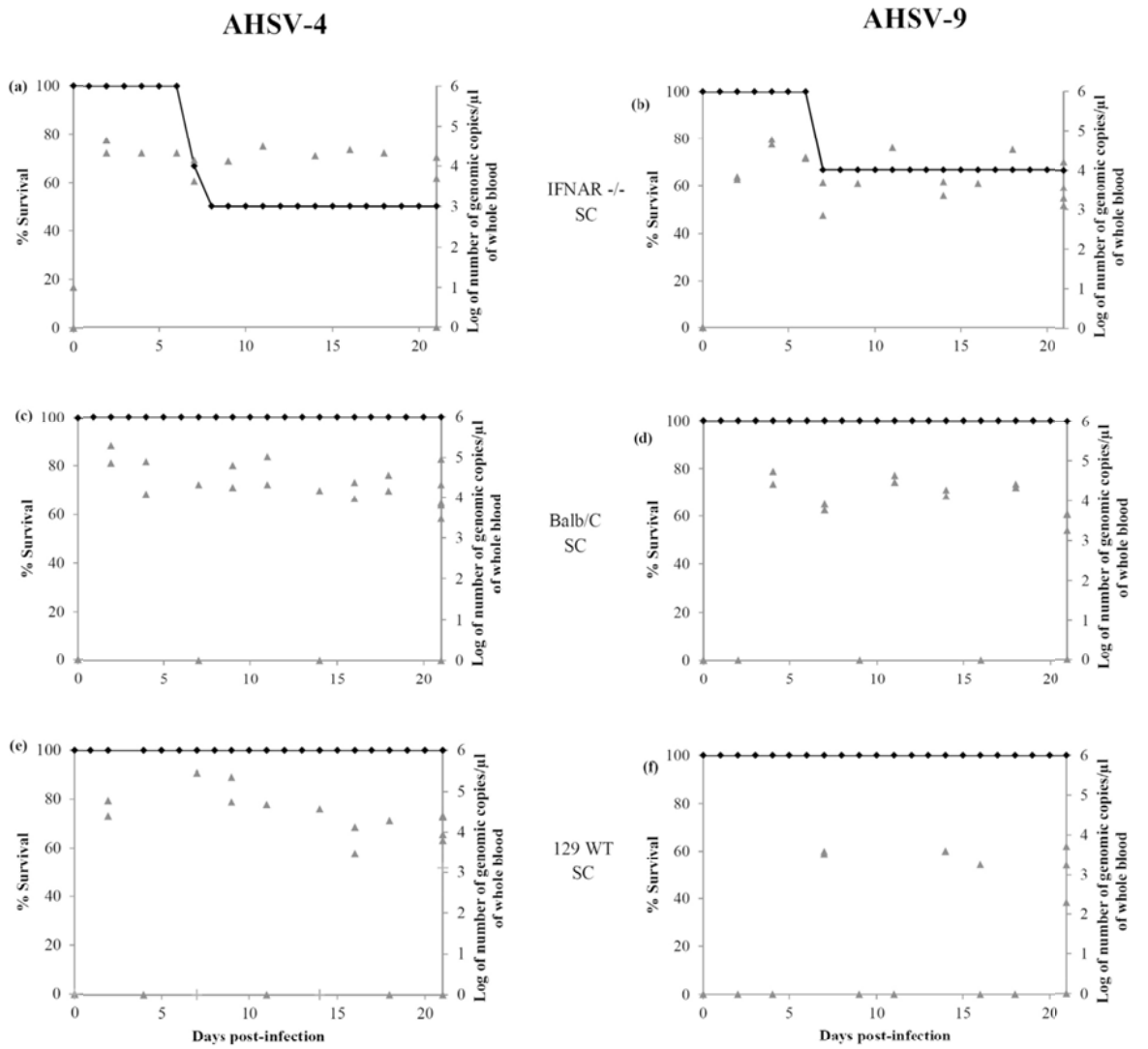

Fig. 3. RNAemia and survival rates of immunodeficient and immunocompetent mice infected SC with AHSV-4 and AHSV-9. RNAemia is represented as symbols (▲) corresponding to the number of genomic copies/µl of blood (expressed in log) detected in one individual mouse. In each group, two mice per day were sampled and each mouse was sampled weekly in accordance with ethical rules. Last samples were taken at day 21 p.i. just before euthanasia. Survival rates are represented by symbols (◆) and expressed as the percentages of surviving mice per day. IFNAR$^{-/-}$ mice are shown in graphs (a) and (b), Balb/C mice in graphs (c) and (d), and 129 WT mice in graphs (e) and (f).

strains ($P \leq 0.001$). In IFNAR$^{-/-}$ mice, no significant difference was observed between the clinical features induced by serotypes 4 and 9 ($P \leq 0.05$). Both immunocompetent mouse strains infected SC developed very low or no clinical signs. On the contrary, immunocompetent mice showed important clinical signs after IN infection justifying euthanasia. In particular, considering the period before euthanasia (from day 0 to day 11 p.i.), clinical signs were significantly more severe in Balb/C mice in comparison to the 129 WT mice ($P < 0.001$). In the same period and in both immunocompetent mouse strains, clinical signs were significantly more severe in groups infected with AHSV-4 than in groups inoculated with AHSV-9 ($P = 0.02$).

3.2. Detection of AHSV RNA from blood samples

Regardless of the mouse strains, no RNAemia was detected in any mock-infected mice. In IFNAR$^{-/-}$ mice, no significant difference was observed between the two experiments and thus only the results from the first one are represented in Fig. 3(a) and (b). SC infection of immunodeficient and immunocompetent mice induced a RNAemia that was detectable until the end of the experiments (3 weeks p.i.). On the contrary, after IN infection, AHSV genome could never be detected, neither in Balb/C nor in 129 WT. The level of RNAemia was lower at day 21 p.i. compared to day 2 p.i. ($P \leq 0.006$) but still detectable in the majority of SC infected mice. Regardless

of the mouse strains, during the 3 weeks p.i., mice infected with AHSV-4 had a significantly higher number of genomic copies per μl of blood in comparison with mice infected by the same route with AHSV-9 ($P \leq 0.001$). The detection of murine GAPDH gene was used as qualitative internal control for all tested samples. A BTV synthetic RNA (EC-EXTR) was added before extraction and was used as qualitative external control exclusively. These two different controls were detected in all samples. In conclusion, we observed similar level of RNAemia for both SC infected IFNAR$^{-/-}$ and immunocompetent mice and infection with AHSV-4 lead to a higher number of genomic copies detected in blood than with AHSV-9 (Fig. 3).

3.3. Detection of AHSV RNA from collected organs

Organs (liver, spleen, kidneys, lungs and brain) were sampled after the euthanasia or the natural death of mice. Considering viral loads in organs of IFNAR$^{-/-}$ mice, no significant difference was observed between the two experiments and thus only the results from the first one are represented in Fig. 4(a) and (b). After RT-qPCR and quantification using VP7-plasmid, high loads of genomic copies per gram were measured in the majority of organs sampled for IFNAR$^{-/-}$ mice (Fig. 4a and b). In IFNAR$^{-/-}$ no significant difference was observed between AHSV-4 and -9 infected individuals and viral loads in organs were significantly lower at day 21 p.i. than at day 6 p.i. ($P < 0.001$). For the same mouse strain, significantly lower viral loads were observed in liver in comparison with the other organs sampled ($P = 0.006$).

In Balb/C mice (Fig. 4c–f), no significant difference in viral load was found between the two routes of inoculation ($P = 0.34$) but the number of organs in which the virus was detected, was significantly higher for mice inoculated IN ($P = 0.048$). As for IFNAR$^{-/-}$ mice, likewise for Balb/C mice, no differences were found between AHSV-4 and AHSV-9. Indeed, after IN inoculation, viral RNA could be detected more frequently in the brain and in the lungs compared to SC inoculation.

In SC infected 129 WT mice, no viral RNA was detected in sampled organs except in the liver of 2 mice infected with AHSV-4 (Fig. 4g and h). The viral load was significantly lower in mice infected SC compared to the IN inoculation ($P < 0.001$). For IN infected 129 WT mice, virus was found in kidneys, lungs and mainly in brain (Fig. 4i and j). Also, AHSV-4 lead to a higher viral load in organs sampled than AHSV-9 ($P = 0.001$). As already observed for IFNAR$^{-/-}$ mice, also in 129 WT mice infected IN, a significantly higher viral load was measured in the first days p.i. (day 11) compared with day 18 ($P = 0.03$) and day 21 ($P = 0.02$).

Murine GAPDH (Ct 27.7–34.4 depending of the organ sampled) and the EC-EXTR (Ct 32–35) were detected in all tested organs.

A summary of all the results of both experiments is reported in Table 1.

3.4. Histopathological findings

No histopathological lesions were found in organs collected from mock infected mice whatever the immune status. For mice euthanized during the 3 weeks p.i. because of severe general conditions (IFNAR$^{-/-}$ and 129 WT infected IN mice), the most frequent lesions were in brain, liver and lungs. In the brain as well as in the meninges, an infiltration of round cells in the periphery of the vessels was observed, suggesting a viral subacute meningoencephalitis (Fig. 5a and b). A severe infiltration of round cells was also detected diffusely in the liver and formed small cell clusters in the periportal spaces and parenchyma. This aspect was typical of a viral subacute multifocal hepatitis (Fig. 5c and d). Inter-alveolar septa were thickened with an infiltration of round cells suggesting a viral interstitial pneumonia. For mice euthanized at the end of the experiment, no histopathological lesions were found, except for one 129 WT mouse infected IN with AHSV-9 that presented a mild interstitial pneumonia.

4. Discussion

Two different serotypes of AHSV, serotypes 4 and 9, being both historically important for Europe and in particular for the Iberian Peninsula, were used in this work. An immunodeficient mouse strain (IFNAR$^{-/-}$) and two different immunocompetent mouse strains Balb/C and 129 WT, with the latest sharing the same genetic background as IFNAR$^{-/-}$ mice, were used. Two separate experiments were conducted with Balb/C and 129 WT, whereas in each experiment IFNAR$^{-/-}$ mice were included. This allowed to repeat twice the infection in the immunodeficient mouse strain and to use it as a comparison with each immunocompetent mouse strain. The following main results were observed: (a) there was a variation of virulence between the two serotypes and a higher virulence for AHSV-4 supported by the observed clinical scoring and survival rates; (b) clinical signs were recorded only for SC infected IFNAR$^{-/-}$ and IN infected immunocompetent mice. Neurological signs (not observed in infected horses) were supported by the presence of meningoencephalitis at the histopathology and viral RNA detection in the brain; (c) RNAemia was still detectable during a long period in SC infected mice such as described in horses, donkeys, mules and zebras (el Hasnaoui et al., 1998; Wilson et al., 2009); (d) the inoculation route was a crucial determinant of the onset of the disease in immunocompetent mice.

A difference in the virulence of serotypes 4 and 9 was already observed in experimentally inoculated horses (Sailleau et al., 1997; Skowronek et al., 1995). Serotype 4 infected horses developed a severe pulmonary form of AHSV, while serotype 9 was less virulent and induced the cardiac or the mixed clinical disease patterns. In a previous work, differences in the virulence of AHSV-4 and AHSV-9 in IFNAR$^{-/-}$ mice were observed (de la Poza et al., 2013). Indeed, in this study the clinical syndrome observed in non-immunized IFNAR$^{-/-}$ mice was milder in mice infected with AHSV-9 than AHSV-4. Nevertheless, the viraemia measured by plaque assay was comparable for both serotypes and no data was reported for the detection of viral RNA by qRT-PCR. Considering our experiments, remarkable differences were found in the virulence of serotypes 4 and 9. In Table 1, the different experimental

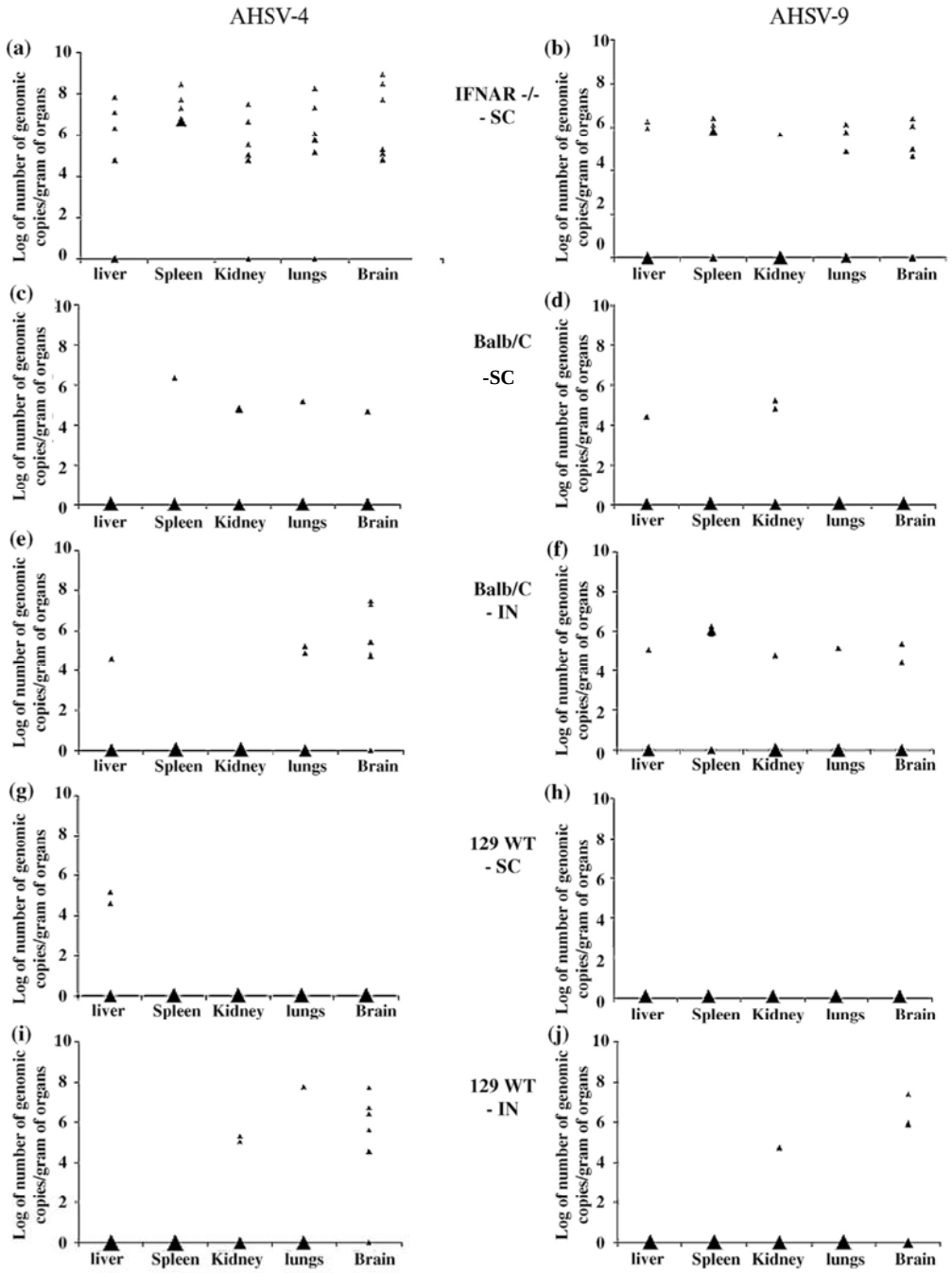

Fig. 4. Virus detection in liver, spleen, kidneys, lungs and brain of IFNAR$^{-/-}$ Balb/C and 129 WT mice. AHSV-4 and AHSV-9 detection in several organs of infected IFNAR$^{-/-}$, Balb/C and 129 WT mice. IFNAR$^{-/-}$ mice were inoculated via subcutaneous route (SC). Balb/C and 129 WT mice were inoculated SC and intranasally (IN). $N = 1$: ▲; $N = 2$: ▲; $N = 3$: ▲; $N = 4$: ▲; $N = 5$: ▲; $N = 6$: ▲. Values of organs sampled from mice euthanized during the experiments are represented in grey. Values of organs sampled at the end of the experiments (day 21 p.i.) are represented in black. Virus was extracted from the indicated tissues at euthanasia (for mice with severe clinical signs) or at the end of the experiment. Results are expressed in log of genomic copies calculated per gram of organ sampled.

parameters evaluated are summarized. Whatever the experimental condition, the combined ratio (F) was always higher for AHSV-4. Furthermore, if each ratio from A to E was individually removed from the combined ratio (F), AHSV-4 had constantly a higher combined ratio compared to AHSV-9.

In particular, three main differences between AHSV-4 and AHSV-9 were found statistically significant: (i) the

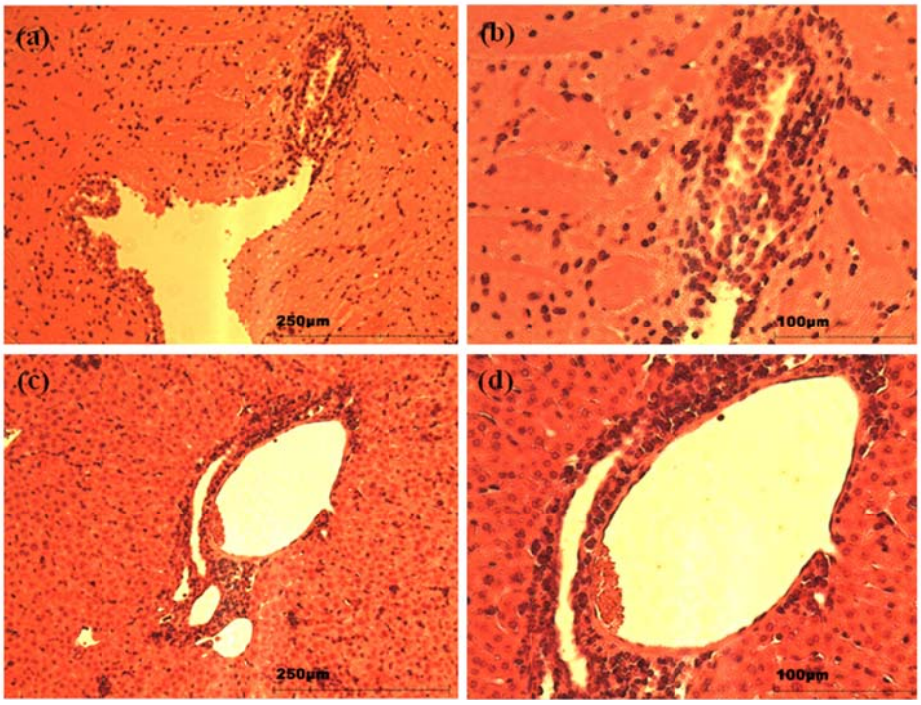

Fig. 5. Histopathological lesions observed in brain and liver of IFNAR$^{-/-}$ mice infected with AHSV-4. Representative examples of the microscopic lesions found in brain and liver tissues of infected IFNAR$^{-/-}$ mice infected with AHSV-4 (sampled at day 6 p.i.) are shown. The brain tissues (a) and (b) presented severe infiltration of inflammatory round cells in parenchyma and in the periphery of the vessels. The liver (c) and (d) presents also an infiltration of inflammatory cells around portal vessels suggesting subacute multifocal hepatitis.

Table 1
Review of the parameters evaluated in the different experimental conditions. All parameters are expressed as a ratio.

Experimental conditions						Ratio					
Group	Experiment	Mice	Route	Serotype	*N*	Fatality[a] (a)	Clinical score[b] (b)	Body weight[c] (c)	Viral RNA in blood[d] (d)	Viral RNA in organs[e] (e)	Combined ratio[f] (f)
A	1 and 2	IFNAR$^{-/-}$	SC	4	6	0.500	0.072	0.083	1.000	0.867	2.522
B				9	6	0.333	0.035	0.110	1.000	0.467	1.945
C				Mock	3	0.000	0.000	0.000	0.000	0.000	0.000
D	1	Balb/C	SC	4	6	0.000	0.001	0.051	0.833	0.167	1.053
F				9	6	0.000	0.010	0.055	0.833	0.100	0.999
H				Mock	3	0.000	0.000	0.000	0.000	0.000	0.000
E	1	Balb/C	IN	4	6	0.500	0.100	0.148	0.000	0.267	1.014
G				9	6	0.000	0.068	0.144	0.000	0.300	0.512
I				Mock	3	0.000	0.000	0.005	0.000	0.000	0.005
J	2	129 WT	SC	4	6	0.000	0.006	0.032	0.833	0.067	0.938
L				9	6	0.000	0.004	0.000	0.500	0.000	0.504
N				Mock	3	0.000	0.000	0.000	0.000	0.000	0.000
K	2	129 WT	IN	4	6	0.667	0.056	0.081	0.000	0.267	1.070
M				9	6	0.333	0.030	0.037	0.000	0.133	0.534
O				Mock	3	0.000	0.000	0.000	0.000	0.000	0.000

[a] Fatality is the ratio between the number of dead animals and the number of animals tested.
[b] Clinical score is the ratio between the cumulative clinical score observed in a group of mice and the maximum possible clinical score.
[c] Body weight is the ratio between the difference between the average initial body weight minus the average of the minimal body weight and the average of the initial body weight for each group.
[d] RNA in blood is the ratio between the number of viremic animals and the total of animals tested.
[e] RNA in organs is the ratio between the number of viremic organs and the total of organs tested in the group.
[f] Combined ratio is the sum of ratios (a)–(e).

RNAemia expressed as genomic copies, with significantly higher values measured for AHSV-4, as well in immunodeficient as in immunocompetent mice; (ii) the development of clinical signs, with significantly higher clinical scores recorded between 0 and 11 days p.i. with AHSV-4 in both immunocompetent strains; and (iii) the viral RNA detected in organs of IN infected 129 WT, significantly higher with AHSV-4. However, AHSV-4 was not superior for each parameter evaluated separately. Indeed, for IFNAR$^{-/-}$ and Balb/C mice infected SC, body weight loss was more important for AHSV-9 than AHSV-4 (Table 1, body weight ratio). AHS in horses is characterized by four clinical forms of disease: horse sickness fever, cardiac form (sub-acute), pulmonary form (peracute) and mixed form (the most frequently observed) between cardiac and pulmonary forms. Clinically affected mice developed neither pulmonary, nor cardiac nor mixed clinical disease pattern, indicating that they are less suitable model for the experimental study of AHSV pathogenesis. In clinically affected mice, a chronological pattern was observed in the development of clinical signs, which always started with conjunctivitis, weight loss, apathy and ended with the occurrence of neurological signs in IFNAR$^{-/-}$ mice. We observed ataxia, paralysis of one or more limbs and circling gait. These neurological signs are not observed in horses. They could be explained by an adaptation of the viruses used in these studies through successive intracerebral passages performed in suckling mice for viral isolation (O'Hara et al., 1998). IN inoculation was found to be responsible for the onset of fatality in Balb/C (O'Hara et al., 1998). However, the outcomes of this study are hardly comparable to our results because of the different origins of viral strains. With immunocompetent mice, the occurrence of clinical signs was only observed after IN infection. Two hypotheses could explain this observation. First the neurotropism acquired by the intracerebral passages of the viruses in mice (O'Hara et al., 1998) and second the retrograde neuroinvasion through the olfactory pathway. The acquisition of neurotropism after intracerebral passage in mice is supported by previous report (Lubroth et al., 2007) and the strains used in the present experiments have been passaged three times in mice at the Reference Laboratory before distribution. For West Nile Fever virus (WNV) as well as for other neuroinvasive arboviruses, an old controversy lies on the possibility of retrograde neuroinvasion of the brain (Monath et al., 1983). In this way, the infection of olfactory neurons was proposed as an entry mode for highly virulent neurotropic WNV strains in the central nervous system (Murray et al., 2010). Obviously, the fact that olfactory neurons are not protected by the blood–brain barrier could explain an easier access to the brain for the virus. In this way, the effect of the inoculation route should be especially considered as well as the genetic background of the mouse strain regarding innate immunity and the neuroinvasive potential of the virus. After IN infection viral RNA could be detected in some of the sampled organs (liver, kidney, spleen etc.) despite the absence of RNAemia. The most likely hypothesis is the lack of detection of a transient RNAemia due to limitations in the frequency of blood collection from the same mouse.

After SC infection, only IFNAR$^{-/-}$ infected mice showed clinical signs (starting at day 2 p.i.), whereas both immunocompetent mice underwent a subclinical infection despite a constantly high RNAemia. The comparison between SC infected IFNAR$^{-/-}$ and immunocompetent mice allows to postulate the role of the IFN type I in the course of AHSV infection. After SC infection, similar high levels of RNAemia were found, but while immunocompetent mice did not develop clinical signs, IFNAR$^{-/-}$ developed these as soon as at day 2 p.i. No data about the role of IFN type I in AHSV infection are available up to now but data exist for another *Orbivirus*, namely BTV (Chauveau et al., 2013; Ruscanu et al., 2012) and other viruses belonging to the *Reoviridae* family (Deal et al., 2013; Johansson et al., 2007). BTV infection induces a strong production of IFN type I. In BTV infected sheep, the peak of IFN production was correlated with the peak of viraemia and then associated with a strong decrease in virus titres (Foster et al., 1991). More recently, the BTV non-structural protein NS3 was shown to interfere with the IFN type I production (Chauveau et al., 2013). IFNAR$^{-/-}$ mice present higher susceptibility to viral infections (Bereczky et al., 2010; Wernike et al., 2012), related to absence of IFN α/β receptor expression and, consequently, a deficiency in IFN type I-related innate immunity. Results from BTV studies and those obtained from our study support the hypothesis of the role of IFN type I in the control of AHSV infection, and in particular by reducing the severity of the clinical expression of the disease. A higher severity of disease in IFNAR$^{-/-}$ mice was observed, while 50% of those mice could survive to the infection despite the absence of the IFN innate antiviral activity. This suggests that other immune mechanisms are involved in the control of the infection.

Our study provided extensive results in relation to AHSV serotypes 4 and 9 clinical severity in two immunocompetent and one immunodeficient mouse strains. Serotype 4 can be considered as more virulent than serotype 9, at least for strains genetically related to the one used in this study. The observed differences in virulence were obtained by the use of two serotypes which underwent the same historical laboratory passages. This supports the hypothesis of a genetic diversity between the two serotypes, and thus of genetic markers of virulence that should be investigated.

The IN infection of Balb/C and 129 WT mice could be used as a valid model in the identification of the genetic markers of neurotropism of these AHSV strains as well as for other neuroinvasive arboviruses. IFNAR$^{-/-}$ mice are an excellent model for vaccine efficacy studies due to their high clinical susceptibility to AHSV infection. Nevertheless, immunocompetent mouse strains having a conserved innate antiviral immune response should be taken in consideration for vaccine efficacy studies and also for other purposes such as the study of the evolution of AHSV viral populations.

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Supplementary table for the article "Study of the virulence of serotypes 4 and 9 of African horse sickness virus in IFNAR -/-, Balb/C and 129 Sv/Ev mice", available on http://www.sciencedirect.com/science/article/pii/S0378113514004829

Supplementary table : **Evaluation and scoring of the different clinical parameters.** Description of the scoring of the different clinical signs observed during the two experiments. The clinical signs were evaluated twice a day during the 3 weeks post-infection.

	Scores			
Parameters	0	1	2	3
Activity	Normal	Isolated, abnormal posture	Hunched posture, inactive or hyperactive	Moribund or convulsions
Awareness	Normal	Depression	Little response to manipulations	Unconscious, lethargy
Movement/gait	Normal	Reduction of mobility	Ataxia, paresia of one to three limbs	Circling gait, paresia of 4 limbs, paralysis
Vocalisation	Normal	Squeaks during palpation	Strongly squeaks and struggles during palpation	Abnormal vocalization
Consistency of faeces	Normal	Wet	Flaccid faeces, dirty perineum or unusually dry faeces +/- mucus	Diarrhea during manipulation or lack of faeces during 48 hours or presence of blood
Urine	Normal	/	Abnormal colour or volume	Lack or urine during 24 hours or incontinence with dirty perineum
Water intake	Normal	Increase or decrease of watering during 24 hours	Increase or decrease of watering during 48 hours	Permanent watering or lack of watering during 24 hours
Food intake	Normal	Increase or decrease of feeding during 24 hours	Increase or decrease of feeding during 48 hours	Lack of feeding during 24 hours
Body weight	Normal	Slow growth curve	Chronic weight loss >15 %, or no growth	Acute weight loss > 10 %, chronic weight loss > 15 % or no growth
Body condition	Normal	Thin	Loss of body fat, stunted growth	Muscle wasting
Respiration	Normal	Rapid, discrete	Rapid, abdominal	Hard, irregular, dyspnea
Aspect of coat	Normal	Rough hair coat	Injured or thin coat	Hair loss, automutilation, suppuration
Dehydration	None	Less elastic skin	Persistent skin fold	Persistent skin fold and enophtalmos
Ocular signs	Normal	Ocular discharge, conjunctivitis	Lacrimation	Closed eyelid
Nasal signs	Normal	Wet	Nasal discharge	Coagulated
Other				

Section expérimentale

Etude 2 :

Etude du réassortiment génétique du virus de la peste équine

Préambule

Après avoir mis en place un outil pour l'étude de l'infection par l'AHSV dans la première étude grâce au développement de modèles murins, le réassortiment génétique est au cœur de la deuxième étude de cette thèse. En effet, en plus des mutations ponctuelles et de la recombinaison, le réassortiment génétique est un mécanisme d'évolution supplémentaire chez les virus à ARN segmenté tels l'AHSV. La circulation de plusieurs souches virales peut générer des virus réassortants lorsqu'elles infectent la même cellule.

L'objectif de cette étude est de préciser la nature du réassortiment entre les sérotypes 4 et 9 de l'AHSV au niveau des segments échangés ainsi que la virulence des virus réassortants qui a été étudiée *in vitro* et également *in vivo* en utilisant un modèle murin mis au point lors de l'étude précédente.

Cette étude a été divisée en deux parties : la première partie décrit l'étude du réassortiment génétique des sérotypes 4 et 9 du virus de la peste équine *in vitro*. À la suite des coinfections, des virus réassortants entre les sérotypes 4 et 9 de l'AHSV ont été générés. Six virus réassortants parmi ceux obtenus ont été caractérisés au niveau génétique par la détermination de l'origine des différents segments du génome par qRT-PCR et PCR. Au regard des résultats, l'échange de segments entre les deux sérotypes s'est avéré être multiple pour les six virus réassortants analysés. Malgré une multiplication virale plus lente du sérotype 9 par rapport au sérotype 4, un phénomène de dominance du sérotype 9 sur le sérotype 4 a été observé lors des coinfections en culture de cellules.

Dans la deuxième partie, l'étude de la virulence *in vivo* des virus réassortants obtenus *in vitro* est décrite. Le modèle souris Balb/C mis au point lors de la première étude, a été utilisé pour évaluer la virulence de 3 virus sélectionnés parmi les virus réassortants obtenus, et comparer cette virulence aux virus parentaux selon les paramètres suivants : score clinique, détection du virus dans les échantillons de sang et d'organes. Parmi les trois virus réassortants testés, un virus réassortant était plus virulent que les virus parentaux, confirmant que le réassortiment génétique fait partie des mécanismes d'évolution de l'AHSV qui peut conduire à une modification de sa virulence.

In vitro and *in vivo* characterization of *in vitro* generated African horse sickness reassortant viruses

Soumis pour publication

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INTRODUCTION

The rapid evolution of RNA viruses makes the prediction of viral emergence of such viruses as African horse sickness virus (AHSV) difficult, especially when additional mechanisms like reassortment take place. Arboviruses meet two bottlenecks for their evolution: in fact, the viral cycle of an arbovirus involves replication in an arthropod vector and a vertebrate host, necessitating a plasticity of the virus at the transition between the two different systems. In this context, point mutations have already been investigated but only few studies exist regarding the genetic reassortment within the *Orbivirus* genus (Von Teichman et Smit, 2008; Meiring *et al.*, 2009; Matsuo *et al.*, 2010), in spite of the fact that it is a very important mechanism of evolution for viruses with a segmented genome and has already been observed for other segmented viruses (Zambon, 1999; Matthijnssens *et al.*, 2006). Reassortment can lead to changes in the epidemiology of a virus such as interaction between different strains, horses and insect vectors and virus adaptation to a new environment. Other possible effects of reassortment may be a change in the virulence, in the antigenic profile or the tropism for host or vector of AHSV.

African horse sickness virus is an exotic arbovirus in Europe and, like Bluetongue virus (BTV), belongs to the *Reoviridae* family within the *Orbivirus* genus. The unexpected emergence of BTV in Northern and Central Europe in 2006 (Thiry *et al.*, 2006; Gould et Higgs, 2009; Saegerman *et al.*, 2010; Tabachnick, 2010) when the virus replicated in an indigenous arthropod competent vector (Maclachlan *et al.*, 2007; Wilson *et al.*, 2009; Maclachlan et Guthrie, 2010; Pfeffer et Dobler, 2010; Martinez-Lopez *et al.*, 2011), is an obvious example for the possibility for exotic arboviruses to emerge and become endemic in these areas. AHSV is endemic in sub-Saharan Africa and for some serotypes in West Africa. In recent decades, AHSV has caused severe epidemics in Europe. AHSV serotype 9 (AHSV-9) was introduced in 1966 in Spain via infected *Culicoides* through the Strait of Gibraltar. In 1987, AHSV serotype 4 (AHSV-4) appeared in Spain and in Portugal and remained there for the next four summers, causing epidemics, thus demonstrating the virus's ability of survival for several years outside of South Africa.

The AHSV virion is non-enveloped with a size of about 70 nm. As for BTV, the virion is composed of seven structural proteins (VP1-7) and four non-structural proteins (NS1-3a), arranged in a double layer of capsid which contains 10 segments of double-stranded RNA. VP2 and VP5 proteins form the external part of the virion capsid and VP3 and VP7 proteins

are the major elements of the inner layer of the capsid. The VP2 protein is the most variable and, is responsible for the different viral serotypes identified until now. Some cross-reactions among serotypes are observed, for example between serotypes 1 and 2, 3 and 7, 5 and 8 and 6 and 9, but no cross-reaction with other known *Orbiviruses* is documented (Roy, 1996).

In this study, coinfections with AHSV-4 and AHSV-9 were performed and led to the generation of reassortant viruses. The aim of the study was to analyse the nature of reassortment, with regards to the exchanged segments and to study the virulence of the obtained reassortant viruses *in vivo*. Characteristics of these reassortant viruses were studied and compared to those of the parental strains. Viral growth curves and identification of the origin of the different segments were described. Three reassortant viruses were selected to inoculate Balb/C mice and to compare their virulence *in vivo* to that of the virulence of the parental strains.

Étude 2

Partie 1 :

Étude *in vitro* du réassortiment génétique entre les sérotypes 4 et 9 du virus de la peste équine

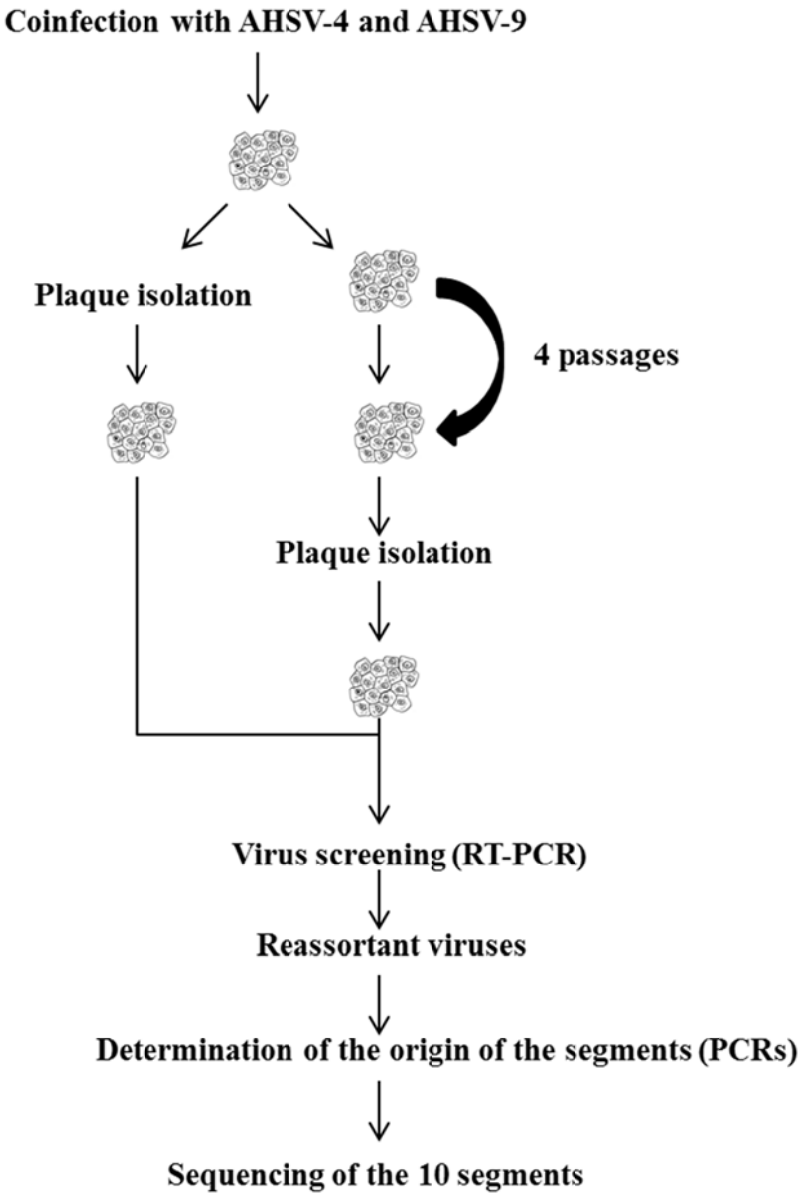


Figure 1 : Coinfection assay protocol. AHSV -4 : Serotype 4 of African horse sickness virus; AHSV-9 : Serotype 9 of African horse sickness virus

MATERIALS AND METHODS

Cells and viruses

Vero cells were grown in Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich) supplemented with 10 % heat inactivated foetal calf serum (FCS) (Invitrogen), 2% of penicillin-streptomycin combination (PS) (Invitrogen) and 1 % of a non-essential amino acids preparation (NEAA) (Invitrogen). Vero cells were incubated at 37 °C in a humidified incubator with 5 % CO_2. The origin of cell passages for the AHSV serotype 4 (AHSV-4) and AHSV serotype 9 (AHSV-9) strains used in this study was previously described (Agüero et al., 2008; de la Grandière et al., 2014). Virus stocks were generated by infection of confluent Vero cells with these viruses at a multiplicity of infection (MOI) of 0.1. When a total cytopathic effect (CPE) was visible, mainly at 48 or 72 hours post- infection, supernatants were harvested and centrifuged. The virus was released from the cells by three freezing /thawing cycles. Supernatants were stored at -80 °C until use.

Standard virus titration was performed in Vero cells while viral titres were expressed as $TCID_{50}$/ml (Reed & Muench, 1938).

Coinfection assays

Vero cells were grown in 12-well plates the day before the coinfection. First, the cells were coinfected in duplicate wells with AHSV-4 and AHSV-9 at an equal MOI of 1 or 5 for 2 h. In a second experiment, synchronous coinfections were performed in duplicate wells with AHSV-4 at MOI of 5 or 10 and with AHSV-9 with a MOI of 1 for 2 h (Fig.1). After 48 h post-infection (p.i.) when CPE was obvious, the supernatants were collected and centrifuged to remove cellular debris. A volume of 10 µl of the viral mixed population was passaged four times on Vero cells and each time in the presence of viral CPE, 10 µl of the supernatant from the previous passage were used to inoculate the cell monolayer. The mixed population of passage 0 and 4 was inoculated onto Vero cells grown in 6-well plates, using five 10-fold successive dilutions (10^{-1} to 10^{-5}). After 2 h of incubation, the inoculum was removed and replaced by 2 ml of agar overlay (0.7 %). When an individual well-isolated plaque was

visible, it was picked through the agar overlay with a 27g needle (Terumo) and suspended in 500 µl of unmodified DMEM. Each suspension was then inoculated onto Vero cells grown in 12-well plates in order to control the appearance of the characteristic viral CPE. Only from the suspensions showing CPE, were supernatants harvested and centrifuged before viral RNA extraction via QIAamp Viral RNA Mini Kit (Qiagen), according to manufacturer's instructions.

Genotyping of new progeny virions isolated from coinfection experiments

After heat denaturation (5 minutes at 95 °C), RNA was reverse transcribed with a Reverse transcriptase Core kit (Eurogentec). The initial characterization of the potential reassortant viruses was performed using two duplex real-time PCR assays in which VP2 and VP5 serotype-specific primers and probes were used (Table 3). cDNA was amplified using Takyon NoRox Probe MasterMix dTTP Blue (Eurogentec) according to the manufacturer's instructions, on a C1000 Touch Thermal cycler (CFX96 Real Time System, Bio-Rad). The reaction mixtures contained 10 µl 2x master mix, 250 nm of each primer and probe, 2 µl of cDNA sample and water for a final volume of 20 µl. The qPCR program was: 10 min at 95 °C and 45 cycles of 15 s at 95 °C and 30 s at 60 °C.

A reassortant virus was identified as a virus with seg-2 from parental AHSV-4 and seg-6 from parental AHSV-9 or a virus with seg-2 from AHSV-9 and seg-6 from AHSV-4. When potential reassortant viruses were detected, they were allowed to amplify *in vitro* (4 passages in Vero cells). One of the potential reassortant viruses was not used for the further genetic characterization as it did not achieve as high viral titres as the six other candidates. Conventional PCRs were developed to characterize the other segments of reassortant viruses and to differentiate between AHSV-4 and -9 origin. For the ten segments, serotype-specific primer sets were designed and are listed in Table 1. Conventional PCRs were performed using a Taq Polymerase (Taq New Englands, BioLab). The reaction mixtures contained 5 µl 10x master mix (New Englands BioLab), 5 µl dNTPs (250 µM), 300 nm of each primer, 5 µl sample cDNA and water to a final volume of 50 µl. The PCR program was: 2 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 56 °C and 45 s at 72°C, and a final elongation of 7 min at 72 °C. Amplicons were loaded onto a conventional gel (2 % of TBE) and migrated for about 45 min at 100 V.

Table 1 : Primers and Probes used for the detection of AHSV-4 and AHSV-9 genome segments

Segment	Oligonucleotide position (strain) *	Sequence (5'-3')	5' fluor
1 (VP1)	1780-1798 (4)	CAATCTAACCATTGCGATC	
	2275-2254 (4)	ACCAAAAATACGCTTGACTTCT	
	1780-1802 (9)	TAACTTAACTATCGCGATT	
	2275-2254 (9)	GCCAAAAATCCGTTTTACCTCC	
2 (VP2)	1769-1789 (4) †	TTAGGACGAAAGTGGGAGGA	
	1875-1856 (4) †	ACATCCTTTCCTGCGCTTAG	
	1836-1854 (4) †	ATGAGCCCCACAGAGTGTTT	FAM
	1753-1774 (9) †	GAGCATTTTGGATCATGAGGA	
	1843-1822 (9) †	TGTCTCTGGTGATGCGTTAAA	
	1785-1806 (9) †	TCGTTGATGATCCTGAGAGGT	Texas Red
3 (VP3)	1445-1465 (4)	ACGTGTAATCCGTTACTGTGA	
	1904-1922 (4)	CACAGTGTCCGGGACTCTG	
	1445-1465 (9)	GCGCATAATTCGTTACTGTGA	
	1904-1922 (9)	TACGGTGTCCGGAACTCTA	
4 (VP4)	377-401 (4)	TTATGCCTTCCAACACGTGACTGTC	
	1181-1158 (4)	CTCAATATGTATATCTTCATATAA	
	377-401 (9)	CTATGCTTTCCAACACGTAACCGTT	
	1181-1158 (9)	TTCAATATGTATATTTTCATACAG	
5 (NS1)	612-631 (4)	TTAAAGGATCTGATCGAAGG	
	824-844 (4)	TCCGTTGCGTCCTCGATCGCG	
	612-631 (9)	CTAAAAGATTTGATTGAAGG	
	824-844 (9)	CCGCAAGCGTCTCCGATCGCA	
6 (VP5)	689-709 (4) †	AAGCGCGGAAGTAATTGAGA	
	779-761 (4) †	CTGTATTGCGCGGGTTGT	
	719-739 (4) †	AGGAGGTACCAATCTTCGGC	FAM
	684-704 (9) †	GATTTAAGCGCCGAAGTCAT	
	794-776 (9) †	CAACTTCAGGCCCCCTTG	
	714-734 (9) †	CTGAGGAGGTGCCAGTCTTT	Texas Red
7 (VP7)	128-149 (4/9)	TAATAGGTACAATGGTTTAACG	
	512-476 (4/9)	TGCTCCTGCATAAGGTCCCGTCTGT	
	105-125 (9)	CGCAATCAATAGGTATAATG	
	444-464 (9)	AATTGATGTATCCACCACGC	
8 (NS2)	291-321 (4/9)	TTTTGAAGCGTTAACACCAGTGCCGATGGCA	
	663-630 (4/9)	ACTCTCATTGCTCTGAACAGAAAGTTTCCTTGGT	
	291-321 (4)	CTTTGAGGCGTTAACACCAGTACCAATGGCT	
	663-630 (4)	GCTTTCATTGCTCTGAACAGAAAGTCTCCTTGGC	
9 (VP6)	867-887 (4)	ATTGCGGAATTTGGAGGTC	
	1078-1058 (4)	TCCCCTTCCCCTTCATGAAC	
	421-441 (9)	CTAAGACCGGAGCAGATCGT	
	623-603 (9)	CTTCGCCTCCTCTCTCTCAG	
10 (NS3)	31-51 (4)	ACAATCGCCAAGAATTATAGC	
	447-426 (4)	TCTTAATATTTCGTCGTCAACG	
	369-388 (9)	TGATTTTTATTAGTGGGTGC	
	481-460 (9)	ACTCTTCATCAACCAATCGTTT	

***** Numbers in parentheses refer to AHSV strain (4 indicates AHSV-4, 9 AHSV-9). Genome positions are provided according to GenBank accession numbers for AHSV-4 (JQ796724.1 to JQ796733.1) and for AHSV-9 (KF860036.1 to KF860045.1) ; † These primers are used in qPCR assays.

The genome segments of reassortant and parental viruses were sequenced, in order to confirm the results of the genotyping of the reassortant viruses according to the conventional and the real time PCRs. Plasmids containing the amplicons obtained after the segment-specific conventional PCRs, were constructed. Each PCR product was purified using a High Pure PCR Product Purification Kit (Roche) and then cloned using the pGEM-T Easy Vector System I (Promega). Plasmids containing the inserted sequence were purified with the High Pure Plasmid Isolation kit (Roche) and sequenced using T7 and SP6 primers to determine the orientation of the insert and to verify the integrity and the specificity of the nucleotide sequence.

Reassortant nomenclature

The nomenclature for reassortant viruses proposed by Meiring et al., 2009 was subsequently adapted for this study. Thus, for example, R4-$9_{2, 10}$ is a reassortant virus with an AHSV-4 backbone with VP2 and NS3 genomic segments originating from AHSV-9. When equal number of segments originated from parental viruses, the backbone of the generated AHSV reassortant was considered as the parental origin of segment 2.

Viral growth curves

For the analysis of the *in vitro* virus growth, $1x10^5$ Vero cells/well were grown in 12-well plates in 1 ml of growth medium the day before infection. The cells were then incubated with 500 µl medium containing the relevant virus in duplicate wells at a MOI of 5 for 2 h at 37 °C (5 % CO2). After 2 h, the inocula were discarded and the cells were washed three times with Phosphate-buffered Saline (PBS) and then incubated with 1 ml of fresh DMEM supplemented with 2 % of FCS, 2 % PS and 1 % NEAA. At 0, 4, 8, 24, 48 and 72 h post-infection, the supernatants were harvested and replaced with 1 ml of PBS. The plates and the supernatants were then stored at – 80 °C. The collected supernatants were clarified by centrifugation (5000 rpm for 15 min) and extracellular viral titres were determined by standard titration and expressed in $TCID_{50}$/ml. After three freezing /thawing cycles, 1ml of PBS were collected from the plates, clarified by centrifugation (5000 rpm for 15 min) and intracellular viral titres were obtained by standard titration and expressed in $TCID_{50}$/ml.

Statistical analyses

All statistical analyses (viral growth curves of reassortant viruses compared to those of parental viruses for both intracellular and extracellular virus titration of viruses) were performed using a linear regression with AHSV-4 as the reference group. For reassortant viruses, the frequency of the origin of the different segments was assessed using a Fisher's exact test. For all tests, P-values of < 0.05 were considered to be significant (Dohoo *et al.*, 2010)

Table 2 : Results of the screening of reassortant African horse sickness viruses by qRT-PCR targeting VP2 and VP5.

Coinfection conditions	Passage	AHSV-4	AHSV-9	Mixed population	Reassortant virus
Equal MOI					
MOI = 1	0	0 %	82 %	18 %	0 %
	4	0 %	100 %	0 %	0 %
MOI = 5	0	10 %	85 %	5 %	0 %
	4	0 %	100 %	0 %	0 %
Different MOI					
AHSV-4 (MOI = 5) AHSV-9 (MOI = 1)	0	43 %	20 %	27 %	**10 %**
	4	0 %	93 %	7 %	0 %
AHSV-4 (MOI = 10) AHSV-9 (MOI = 1)	0	50 %	0 %	50 %	0 %
	4	20 %	32 %	43 %	**5 %**

MOI : multiplicity of infection; AHSV -4 : Serotype 4 of African horse sickness virus; AHSV-9 : Serotype 9 of African horse sickness virus

RESULTS

Production of AHSV reassortant viruses by coinfection of Vero cells with AHSV-4 and AHSV-9

Using synchronous coinfection with an equal multiplicity of infection (MOI), the parental AHSV-9 was predominantly detected (Table 2). No reassortant viruses could be isolated under these experimental conditions after screening of 40 and 73 plaque-isolated viruses (with equal MOI of 1 and 5, respectively). Indeed, when an equal MOI of 1 was used, 82 % of plaque-isolated viruses, characterized directly after coinfection (passage 0), corresponded to the AHSV-9 parental strain, and after four *in vitro* cell passages, 100% of plaque-isolated viruses were identified to be AHSV-9 (Table 2). When an equal MOI of 5 was used, although 10% of plaque-isolated viruses characterized at passage 0 corresponded to the AHSV-4 parental virus, after 4 passages, 100% of plaque-isolated viruses were AHSV-9 parental strain (Table 2). During synchronous coinfections using higher MOIs for serotype 4 than for serotype 9, a total of seven reassortant viruses was obtained (Table 2). During the course of the first condition with MOI of 5 for serotype 4 and 1 for serotype 9, 3 reassortants (from a total number of 119 plaque-isolated viruses) were characterized at passage 0. However, after four passages of the mixed populations, no reassortant virus could be detected and serotype 9 was predominantly identified (93% of tested plaque-purified viruses) (Table 1). During the coinfection with MOI of 10 for serotype 4 and 1 for serotype 9, four reassortant viruses were isolated (from a total number of 82 plaque-isolated viruses) at passage 4 of the mixed populations (Table 1). Using these experimental conditions, only AHSV-4 could be identified at passage 0 from plaque-isolated viruses. The AHSV-9 parental strain could only be detected at passage 0 together with the serotype 4 parental strain in non-isolated plaques, while in isolated plaques only at passage 4 of the mixed populations.

Table 3 : Origin of the different genome segments of the analysed reassortant African horse sickness viruses

Reassortant virus	Origin of the different genome segments									
	Seg1 (VP1)	Seg2 (VP2)	Seg3 (VP3)	Seg4 (VP4)	Seg5 (NS1)	Seg6 (VP5)	Seg7 (VP7)	Seg8 (NS2)	Seg9 (VP6)	Seg10 (NS3)
*R9-$4_{5, 6, 7, 9}$	9	9	9	9	4	4	4	9	4	9
*R9-$4_{1, 4, 6, 7, 9}$ (R1)	4	9	9	4	9	4	4	9	4	9
†R4-$9_{1, 2, 8, 10}$ (R2)	9	9	4	4	4	4	4	9	4	9
†R4-$9_{2, 3, 8, 10}$ (R3)	4	9	9	4	4	4	4	9	4	9
†R4-$9_{2,10}$	4	9	4	4	4	4	4	9	4	9
†R4-$9_{2,8,10}$	4	9	4	4	4	4	4	9	4	9

* R9-4 : reassortant virus between African horse sickness virus (AHSV) serotypes 4 and 9 with a predominant number of segments from AHSV serotype 9

†R4-9 : reassortant virus between serotypes 4 and 9 of AHSV with a predominant number of segments from AHSV serotype 4

The indices report the segment number originating from the less represented parental virus (Meiring *et al.*, 2009).

Genotyping of new progeny virions isolated from coinfection experiments

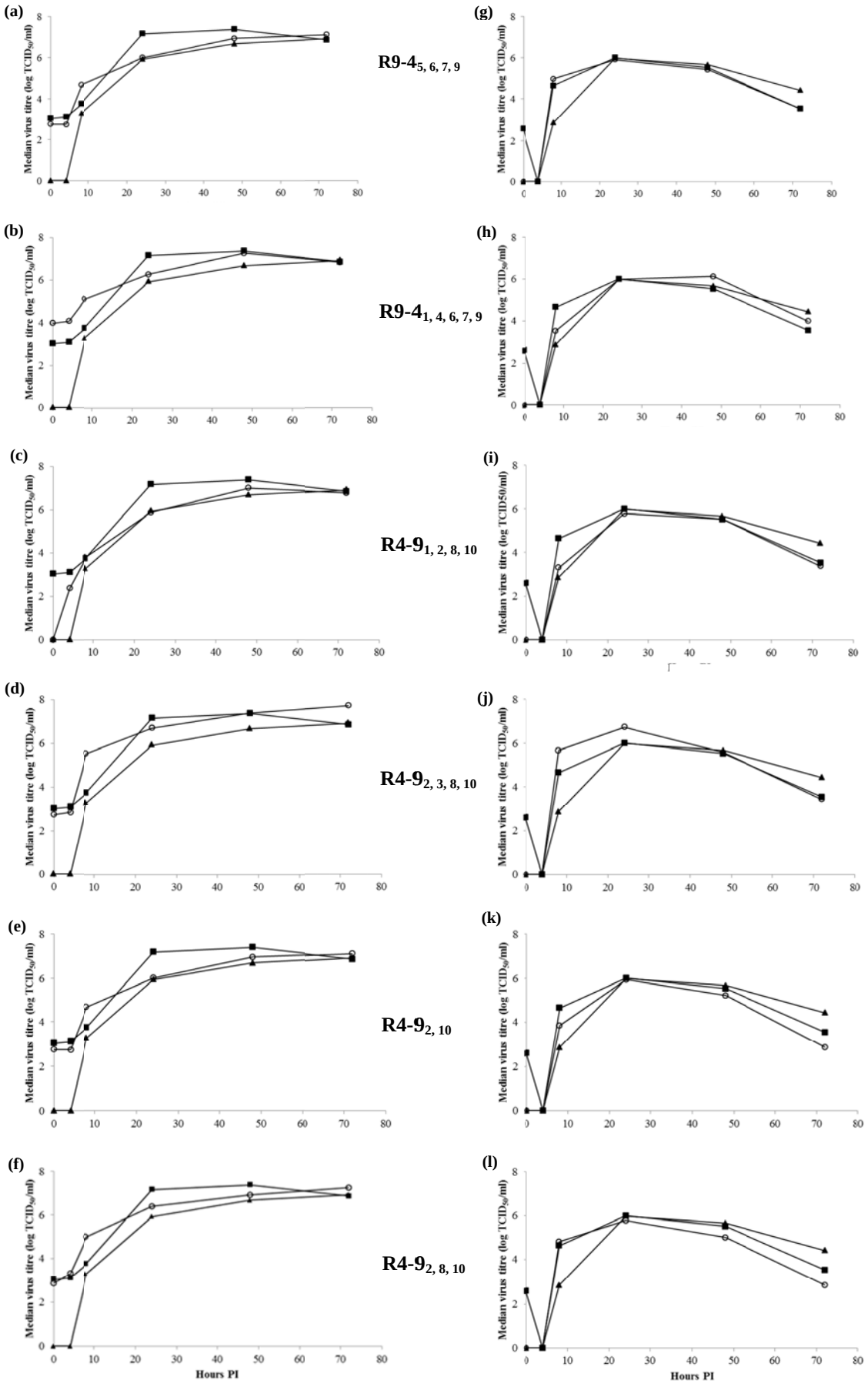
Potential reassortant viruses were plaque-purified and amplified four times in Vero cells. The third passage of six potential reassortant viruses was used for further genetic and biological characterization.

To investigate the eight other segments of the genome, conventional PCRs were used. Because of the high level of homology between the serotypes 4 and 9 regarding segment 7 (VP7; 97.3 % nucleotide sequence homology) and segment 8 (NS2; 96.6 % nucleotide sequence homology), sequencing only allowed determination of their origin (Supplementary Table). The results showed that for the six reassortant viruses investigated, there were multiple exchanges of segments between AHSV-4 and -9 (Table 3). The distribution of the origin of the different segments of the reassortant viruses was not homogeneous (Fisher's exact test, $p<0.001$). The screening by qRT-PCR showed that VP2 always originated from AHSV-9 and VP5 from AHSV-4. VP6 was always derived from AHSV-4 and NS3 from AHSV-9. The origin of the other segments was mostly from AHSV-4 for VP1 (66.7 %) and AHSV-9 for VP3 (66.7 %).

Viral growth curves

Viral growth curves of reassortant viruses were compared to those of parental viruses for both intracellular and extracellular viruses (Fig. 1). For extracellular virus titrations, AHSV-9 and R4-$9_{1, 2, 8, 10}$ had significantly lower viral titres ($p<0.001$) while the five other reassortant viruses had a similar growth curve to that of AHSV-4. Similar observations were made for intracellular virus titrations, with R4-$9_{1, 2, 8, 10}$ having significantly lower viral titres ($p=0.01$) and AHSV-9 lower viral titres ($p=0.05$) than AHSV-4. In addition the intracellular virus titrations of R4-$9_{2, 10}$ and R4-$9_{2, 8, 10}$ showed significantly lower viral titres ($p=0.01$) than AHSV-4.

Figure 1 : Viral growth curves of reassortant viruses and parental viruses. Titration of extracellular viruses (**(a)** to **(f)**) and intracellular viruses (**(g)** to **(l)**) as described in Materials and Methods. AHSV -4 : Serotype 4 of African horse sickness virus; virus AHSV-9 : Serotype 9 of African horse sickness virus; (a) et (g) : R9-$4_{5, 6, 7, 9}$; (b) et (h) : R9-$4_{1, 4, 6, 7, 9}$; (c) et (i) : R4-$9_{1, 2, 8, 10}$; (d) et (j) : R4-$9_{2, 3, 8, 10}$; (e) et (k) : R4-$9_{2,10}$; (f) et (l) : R4-$9_{2,8,10}$


Titration of extracellular virus
Titration of intracellular virus
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
R9-4 5, 6, 7, 9
R9-4 1, 4, 6, 7, 9
R4-9 1, 2, 8, 10
R4-9 2, 3, 8, 10
R4-9 2, 10
R4-9 2, 8, 10
Median virus titre (log TCID50/ml)
Hours PI
AHSV reassortant
AHSV-4
AHSV-9

DISCUSSION

AHSV reassortants were generated during synchronous *in vitro* co-infection experiments with serotype 4 at a higher MOI than serotype 9. The obtained results showed : (a) multiple exchanges of genomic segments with a non-homogenous distribution of the segments depending on the parental virus; (b) differences in the *in vitro* viral growth for some reassortants compared to the parental viruses.

For this study, we decided to screen for potential reassortants based on the determination of the origin of segments 2 and 6 of AHSV. Indeed, the majority of BTV1-BTV8 reassortants have a Seg-2 from BTV-8 and a Seg-6 from BTV-1, making highly probable to detect reassortant viruses only by targeting these two segments (Shaw *et al.*, 2013). Secondly, it was also wanted to study the virulence of AHSV reassortants in which these two segments originated from different parental viruses.

Different MOI were used to perform coinfections. First, equal MOI for both serotypes have been used as previously described for other serotypes of AHSV (O'hara *et al.*, 1998; Meiring *et al.*, 2009) or for BTV (Shaw *et al.*, 2013). In these studies, reassortant viruses were obtained during coinfections with equal MOI. However, in our study, dominance of serotype 9 over serotype 4 was observed during coinfection assays and no reassortant viruses could be obtained in these conditions. The use of a higher MOI for serotype 4 led to the isolation of several reassortant viruses. Using a MOI of 5, reassortant viruses could be detected at passage 0 whereas with a MOI of 10, reassortant viruses were obtained at the fourth passage. Using a MOI of 10, no reassortant virus could be detected at passage 0. It can be suggested that with a ten-fold higher MOI, serotype 4 was rendered artificially dominant thus preventing the exchange of segments with serotype 9. During the four consecutive passages, the conditions enabling reassortment of the genome segments were probably re-established, allowing detection of some reassortants at the fourth passage. The three reassortant viruses obtained at passage 0 corresponded to 10% of the characterised progeny viruses. The other three reassortant viruses obtained at passage 4 corresponded to 5% of the characterized progeny viruses. This indicates that a two-fold increase of the AHSV-4 MOI (from 5 to 10) did not lead to a proportional increase in the number of generated reassortant viruses.

In other studies, performing *in vitro* coinfections of AHSV or BTV, no dominance of serotypes has been described (O'hara *et al.*, 1998; Meiring *et al.*, 2009; Shaw *et al.*, 2013). However, during *in vivo* coinfections with BTV-8 and BTV-1, a predominant detection of BTV-8 was demonstrated in coinfected calves (Dal Pozzo *et al.*, 2013).

Genetic characterization of the six reassortant viruses by qRT-PCR and RT-PCR showed multiple segment exchanges between the two parental viruses complementing with previous observations on AHSV (O'hara *et al.*, 1998) and BTV (Shaw *et al.*, 2013) reassortant viruses. In our conditions of work, the origin of the different segments was almost equally distributed. A genome constellation per serotype was demonstrated among the six generated reassortant AHSV. Some segments originated systematically from serotype 4, namely segments 6 (VP5), 7 (VP7) and 9 (VP6) while on the other hand some segments, such as segments 2 (VP2) and 10 (NS3), originated exclusively from serotype 9. These results suggest a conserved segment constellation during the reassortment events under these conditions. AHSV serotype is characterized both by VP2 and VP5 (Roy, 1996) although the current diagnostic methods rely only on VP2 for serotyping (Sailleau *et al.*, 1997). In this study, the segments encoding these two proteins originated from AHSV4 and AHSV9 for segments 6 and 2, respectively (Table 2), complicating the detection and genotyping of the reassortant viruses when current diagnostic procedures are used.

Supplementary Table : Results from genome sequencing of reassortant African horse sickness viruses and parental African horse sickness viruses. Expressed as percentage of homology (calculated by BioEdit Sequence Alignment Editor).

		32/62*	90/61†	Clone 4	Clone 9	R9-$4_{5, 6, 7, 9}$	R9-$4_{1, 4, 6, 7, 9}$	R4-$9_{1, 2, 8, 10}$	R4-$9_{2, 3, 8, 10}$	R4-$9_{2,10}$	R4-$9_{2,8,10}$
Seg-1 (VP1)	32/62*		90.0	100.0	86.3	87.1	100.0	86.9	100.0	100.0	100.0
	90/61†			86.9	99.0	99.4	86.9	99.6	86.9	86.9	86.9
	Clone 4				86.3	87.1	100.0	86.9	100.0	100.0	100.0
	Clone 9					99.2	86.3	99.4	86.3	86.3	86.3
	R9-$4_{5, 6, 7, 9}$						87.1	99.8	87.1	87.1	87.1
	R9-$4_{1, 4, 6, 7, 9}$							86.9	100.0	100.0	100.0
	R4-$9_{1, 2, 8, 10}$								86.9	86.9	86.9
	R4-$9_{2, 3, 8, 10}$									100.0	100.0
	R4-$9_{2,10}$										100.0
	R4-$9_{2,8,10}$										
Seg-2 (VP2)	32/62*		58.7	100	61.3	62.8	58.2	60.4	61.3	61.3	61.3
	90/61†			62.1	100.0	98.3	97.8	98.9	100.0	100.0	100.0
	Clone 4				61.8	62.8	58.2	60.4	61.8	61.8	61.8
	Clone 9					98.3	97.8	98.9	100.0	100.0	100.0
	R9-$4_{5, 6, 7, 9}$						96.1	96.5	97.0	97.0	97.0
	R9-$4_{1, 4, 6, 7, 9}$							96.7	97.8	97.8	97.8
	R4-$9_{1, 2, 8, 10}$								98.9	98.9	98.9
	R4-$9_{2, 3, 8, 10}$									100.0	100.0
	R4-$9_{2,10}$										100.0
	R4-$9_{2,8,10}$										
Seg-3 (VP3)	32/62*		95.1	100.0	93.9	93.7	93.3	99.4	93.7	99.2	99.6
	90/61†			93.9	100.0	99.4	99.4	93.3	99.8	93.1	93.5
	Clone 4				93.9	93.7	93.3	99.4	93.7	99.2	99.6
	Clone 9					99.4	99.4	93.3	99.8	93.1	93.5
	R9-$4_{5, 6, 7, 9}$						98.7	93.1	99.2	92.9	93.3
	R9-$4_{1, 4, 6, 7, 9}$							92.7	93.1	98.5	99.0
	R4-$9_{1, 2, 8, 10}$								93.1	98.5	99.0
	R4-$9_{2, 3, 8, 10}$									92.9	93.3
	R4-$9_{2,10}$										99.2
	R4-$9_{2,8,10}$										

		32/62*	90/61†	Clone 4	Clone 9	R9-$4_{5, 6, 7, 9}$	R9-$4_{1, 4, 6, 7, 9}$	R4-$9_{1, 2, 8, 10}$	R4-$9_{2, 3, 8, 10}$	R4-$9_{2,10}$	R4-$9_{2,8,10}$
Seg-4 (VP4)	32/62*		74.7	99.9	94.0	94.5	99.9	99.8	99.9	99.9	99.6
	90/61†			94.5	99.4	99.8	94.5	94.4	94.9	94.5	94.3
	Clone 4				93.9	94.4	99.8	99.6	96.8	99.8	99.5
	Clone 9					99.1	93.9	93.8	91.4	93.9	93.7
	R9-$4_{5, 6, 7, 9}$						94.4	94.3	91.9	94.4	94.2
	R9-$4_{1, 4, 6, 7, 9}$							99.6	96.8	99.8	99.5
	R4-$9_{1, 2, 8, 10}$								96.6	99.6	99.4
	R4-$9_{2, 3, 8, 10}$									96.8	96.5
	R4-$9_{2,10}$										99.5
	R4-$9_{2,8,10}$										
Seg-5 (NS1)	32/62*		94.5	97.0	91.4	94.4	94.4	96.4	97.0	97.0	96.6
	90/61†			89.7	99.1	94.9	94.8	90.1	89.7	89.7	89.3
	Clone 4				91.9	94.8	94.8	99.6	100	100.0	99.7
	Clone 9					97.3	97.3	91.4	91.9	91.9	91.4
	R9-$4_{5, 6, 7, 9}$						100.0	94.2	94.8	94.8	94.4
	R9-$4_{1, 4, 6, 7, 9}$							94.2	94.8	94.8	94.4
	R4-$9_{1, 2, 8, 10}$								99.6	99.6	99.1
	R4-$9_{2, 3, 8, 10}$									100.0	99.6
	R4-$9_{2,10}$										99.6
	R4-$9_{2,8,10}$										
Seg-6 (VP5)	32/62*		75.6	100.0	71.6	100.0	100.0	100.0	100.0	100.0	100.0
	90/61†			73.6	100.0	73.6	73.6	73.6	73.6	73.6	73.6
	Clone 4				73.6	100.0	100.0	100.0	100.0	100.0	100.0
	Clone 9					73.6	73.6	73.6	73.6	73.6	73.6
	R9-$4_{5, 6, 7, 9}$						100.0	100.0	100.0	100.0	100.0
	R9-$4_{1, 4, 6, 7, 9}$							100.0	100.0	100.0	100.0
	R4-$9_{1, 2, 8, 10}$								100.0	100.0	100.0
	R4-$9_{2, 3, 8, 10}$									100.0	100.0
	R4-$9_{2,10}$										100.0
	R4-$9_{2,8,10}$										
Seg-7 (VP7)	32/62*		97.3	99.3	95.0	99.7	99.0	99.0	99.0	99.3	993
	90/61†			95.5	99.2	95.8	95.1	95.1	95.1	95.5	955
	Clone 4				95.1	99.7	99.7	99.7	99.7	100.0	100.0
	Clone 9					95.5	94.8	94.8	94.8	95.1	94.8
	R9-$4_{5, 6, 7, 9}$						99.3	99.3	99.3	99.7	99.7
	R9-$4_{1, 4, 6, 7, 9}$							99.3	99.3	99.7	99.7
	R4-$9_{1, 2, 8, 10}$								99.3	99.7	99.7
	R4-$9_{2, 3, 8, 10}$									99.7	99.7
	R4-$9_{2,10}$										100.0
	R4-$9_{2,8,10}$										

		32/62*	90/61†	Clone 4	Clone 9	R9-$4_{5, 6, 7, 9}$	R9-$4_{1, 4, 6, 7, 9}$	R4-$9_{1, 2, 8, 10}$	R4-$9_{2, 3, 8, 10}$	R4-$9_{2,10}$	R4-$9_{2,8,10}$
Seg-8 (NS2)	32/62*		96.6	98.5	94.9	96.5	95.7	94.6	95.4	94.0	94.3
	90/61†			95.3	98.1	96.0	98.1	97.6	98.7	96.9	98.4
	Clone 4				95.1	96.8	96.4	96.4	95.9	96.5	96.5
	Clone 9					98.4	98.4	98.4	99.2	98.5	98.7
	R9-$4_{5, 6, 7, 9}$						98.1	97.2	97.3	96.9	98.1
	R9-$4_{1, 4, 6, 7, 9}$							98.9	99.5	97.4	99.1
	R4-$9_{1, 2, 8, 10}$								99.2	97.0	99.1
	R4-$9_{2, 3, 8, 10}$									97.0	100.0
	R4-$9_{2,10}$										97.8
	R4-$9_{2,8,10}$										
Seg-9 (VP6)	32/62*		72.3	98.1	77.0	89.7	100.0	100.0	100.0	100.0	100.0
	90/61†			74.8	99.0	71.5	75.9	75.8	75.9	75.9	75.9
	Clone 4				NA§	100.0	100.0	100.0	100.0	100.0	100.0
	Clone 9					NA§	NA§	NA§	NA§	NA§	NA§
	R9-$4_{5, 6, 7, 9}$						100.0	100.0	100.0	100.0	100.0
	R9-$4_{1, 4, 6, 7, 9}$							100.0	100.0	100.0	100.0
	R4-$9_{1, 2, 8, 10}$								100.0	100.0	100.0
	R4-$9_{2, 3, 8, 10}$									100.0	100.0
	R4-$9_{2,10}$										100.0
	R4-$9_{2,8,10}$										
Seg-10 (NS3)	32/62*		96.0	99.5	90.3	86.7	89.4	89.4	88.5	89.4	89.4
	90/61†			95.7	98.2	96.5	99.1	99.1	98.2	99.1	99.1
	Clone 4				NA§	NA§	NA§	NA§	NA§	NA§	NA§
	Clone 9					96.5	99.1	99.1	98.2	99.1	99.1
	R9-$4_{5, 6, 7, 9}$						97.3	97.3	96.5	97.3	97.3
	R9-$4_{1, 4, 6, 7, 9}$							100.0	99.1	100.0	100.0
	R4-$9_{1, 2, 8, 10}$								99.1	100.0	100.0
	R4-$9_{2, 3, 8, 10}$									99.1	99.1
	R4-$9_{2,10}$										100.0
	R4-$9_{2,8,10}$										

*32/62 : African horse sickness virus isolate HS 32/62 (AHSV-4), GenBank accession numbers from JQ796724.1 to JQ796733.1

† 90/61 : African horse sickness virus isolate HS 90/61 (AHSV-9), GenBank accession numbers from KF860036.1 to KF860045.1

§ NA : not applicable because primers do not target the same regions for serotype 4 and 9

Étude 2

Partie 2 :

Étude de la virulence *in vivo* des réassortants obtenus *in vitro* à partir des sérotypes 4 et 9 du virus de la peste équine

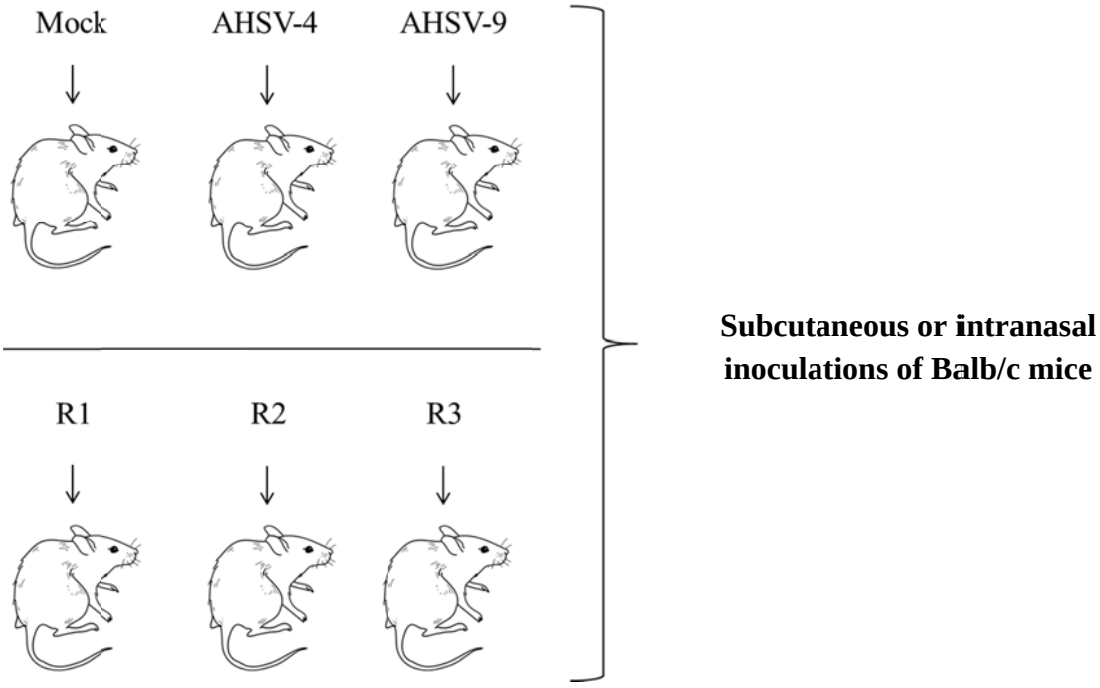


Figure 1 : Schématic representation of animal inoculations with parental or reassortants viruses. AHSV -4 : Serotype 4 of African horse sicknes; virus AHSV-9 : Serotype 9 of African horse sickness virus; R1 : R9-$4_{1, 4, 6, 7, 9}$; R2 : R4-$9_{1, 2, 8, 10}$; R3 : R4-$9_{2, 3, 8, 10}$

MATERIALS AND METHODS

Animal inoculation and processing of the samples

BALB/cByJ (Balb/C) mice were purchased from Charles River France. Two *in vivo* experiments were performed simultaneously using six-week-old female mice. In the first experiment, Balb/C mice were inoculated subcutaneously (SC), while for the second experiment, Balb/C mice were inoculated intranasally (IN). In both experiments six groups were created, in which five were infected and one was mock-infected. Each infected group was composed of six mice, while for ethical reasons only three mice made up the mock-infected groups. Mice were allowed to acclimatize to the biosafety level 3 animal facilities (A3) at the Faculty of Veterinary Medicine, Liège, for one week before the begin of the experiments. All experiments with live animals were performed respecting the guidelines of the European Community (86/609) and were approved by the site ethical review committee (protocol n°1633). All efforts were made to minimize animal suffering.

Control mice were inoculated SC (group A) or IN (group G) with unmodified DMEM. Mice were inoculated SC (250 µl) or IN (50 µl) using previously titrated virus stocks of AHSV-4 (1,36 x10^6 $TCID_{50}$/ml; groups B and H) or AHSV-9 (4.74 x 10^6 $TCID_{50}$/ml; groups C and I) or reassortant virus 1 (R9-$4_{1, 4, 6, 7, 9}$; 8.43 x 10^6 $TCID_{50}$/ml; groups D and J) or reassortant virus 2 (R4-$9_{1, 2, 8, 10}$; 6.32 x10^6 $TCID_{50}$/ml; groups E and K) or reassortant virus 3 (R4-$9_{2, 3, 8, 10}$; 1.36 x10^7 $TCID_{50}$/ml; groups F and L) (Figure 1). Each inoculum was tested by back-titration in order to assess its infectivity at the time of the experiment. Mice were examined daily for clinical signs until the end of the experiment (21 days p.i.). For each mouse presenting clinical signs, a clinical score was given following previously assigned standardised criteria (De La Grandiere *et al.*, 2014). Animals showing severe clinical signs were euthanized for ethical reasons. One mouse from group G and two mice from group H were euthanized for organ collection, at day 14 p.i. having shown no clinical signs. Whole blood was collected in EDTA tubes at regular intervals (days 2, 4, 7, 11, 14, 16, 18 and 21 p.i. for SC infected mice or days 7, 14 and 21 p.i. for IN infected mice) for virus detection. After euthanasia (performed after anaesthesia with ketamine 50 mg/kg and xylazine 5 mg/kg by intramuscular injection and followed by cervical disruption), several organs (liver, spleen, kidney, lungs and brain) were collected at the necropsy and conserved at – 80°C until RNA extraction.

qRT-PCR specific for AHSV segment 7

Total RNA was extracted from whole blood using the Mini Kit QIAamp Viral RNA (Qiagen) according to the manufacturer's instructions, diluted in 60 µl of elution buffer and subsequently stored at – 80 °C. Total RNA was extracted from organs with TRI Reagent solution (Applied Biosystems), according to the method recommended by manufacturer, diluted in a final volume of 60 µl of RNAse/DNAse free water and stored at – 80 °C. After heat denaturation (5 minutes at 95 °C), RNA was reverse transcribed with the Reverse transcriptase Core kit (Eurogentec). cDNA was amplified by qPCR on a C1000 Touch Thermal cycler (CFX96 Real Time System, Bio-Rad). The detection of AHSV was performed using a protocol adapted from Agüero et al., 2008 and previously described (de la Grandière et al., 2014). In addition, a set of primers and probes targeting murine RNA GAPDH were included as an internal control (IC) for all samples (Gangisetty and Reddy, 2009). Moreover, a synthetic external control RNA (EC-EXTR) with a known and defined concentration was added to the sample prior to extraction, allowing standardisation and monitoring of the efficiency of the extraction and the RT-qPCR (Vandenbussche et al., 2010). For each sample, viral VP7 amplification, as well as murine GAPDH and EC-EXTR detection were performed in duplicate and in separate wells. The results were expressed as mean values of the replicates. In order to quantify the number of viral genomic copies per µl of blood (RNAemia) or gram of tissue, a plasmid containing the VP7 target sequence amplified during the RT-qPCR was constructed (De La Grandiere *et al.*, 2014). These VP7 plasmid dilutions were included in duplicate at each qPCR to provide a standard curve.

Statistical analyses

All statistical analyses (ratios between clinical score and the survival time for each mouse, RNAemia and detection of the virus in organs) were performed using a linear regression with AHSV-4 as the reference group (except for the clinical ratio where the mock-infected group of mice was the reference group). For all tests, P-values of < 0.05 were considered to be significant (Dohoo *et al.,* 2010).

Table 2 : Origin of the different genome segments of the analysed reassortant African horse sickness viruses

Reassortant virus	Origin of the different genome segments									
	Seg1 (VP1)	Seg2 (VP2)	Seg3 (VP3)	Seg4 (VP4)	Seg5 (NS1)	Seg6 (VP5)	Seg7 (VP7)	Seg8 (NS2)	Seg9 (VP6)	Seg10 (NS3)
*R9-$4_{5, 6, 7, 9}$	9	9	9	9	4	4	4	9	4	9
*R9-$4_{1, 4, 6, 7, 9}$ (R1)	4	9	9	4	9	4	4	9	4	9
†R4-$9_{1, 2, 8, 10}$ (R2)	9	9	4	4	4	4	4	9	4	9
†R4-$9_{2, 3, 8, 10}$ (R3)	4	9	9	4	4	4	4	9	4	9
†R4-$9_{2,10}$	4	9	4	4	4	4	4	9	4	9
†R4-$9_{2,8,10}$	4	9	4	4	4	4	4	9	4	9

* R9-4 : reassortant virus between African horse sickness virus (AHSV) serotypes 4 and 9 with a predominant number of segments from AHSV serotype 9

†R4-9 : reassortant virus between serotypes 4 and 9 of AHSV with a predominant number of segments from AHSV serotype 4

The indices report the segment number originating from the less represented parental virus (Meiring *et al.*, 2009).

RESULTS

Selection of three reassortant viruses for in vivo *studies*

The three reassortant viruses to be used for the *in vivo* studies were selected based on the results of viral growth curves and the characterization of genome segments. In particular, the viruses R1, namely R9-$4_{1, 4, 6, 7, 9}$, and R3, namely R4-$9_{2, 3, 8, 10}$ had a similar growth curve to that of the parental AHSV-4 with an equal origin of the segments between both serotypes for the first and a major part of their segments originating from AHSV-4 (60 %) for the second ; the virus R2, namely R4-$9_{1, 2, 8, 10}$, had significantly lower titres for both intracellular and extracellular virus and its segments were derived mostly from AHSV-4 (60 %) (Table 1).

Clinical signs and mortality

Two experiments were conducted simultaneously in Balb/c mice: the first one comparing reassortant and parental AHSV strains after subcutaneous (SC) infection, the second one after intranasal (IN) infection. The back titration of the inocula for both experiments was 9.28 x 10^6 $TCID_{50}$/ml for AHSV-4, 4.31 x 10^6 $TCID_{50}$/ml for AHSV-9, 7.78 x 10^6 $TCID_{50}$/ml for R1, 5.37 x 10^6 $TCID_{50}$/ml for R2 and 4.74 x 10^7 $TCID_{50}$/ml for R3.

No mock infected mice showed clinical signs during the three weeks of the experiment. As previously described (De La Grandiere *et al.*, 2014), no clinical signs were observed in all groups of SC infected mice (data not shown).

Clinical signs observed in IN infected mice were very similar to those previously observed (rough hair coat, severe and acute weight loss, weakness and dehydration) (de la Grandière et al., 2014). One mouse IN infected with R1 already lost between 15 and 20% of its body weight at day 7 post infection (p.i.). Both the high weight loss and the presence of other clinical signs led to euthanasia of all mice (Fig. 2). The observation of a euthanasia rate of 100 %, confirmed the higher virulence for R1 in comparison with both other reassortant and parental viruses. In the group IN infected with R2 (group K), the first clinical signs were observed from day 11 p.i. onwards and the final mortality rate observed at the end of the experiment was 33 % (Fig.2). Less clinical signs were reported in the group infected IN with R3 (group L), with only one mouse showing severe clinical signs necessitating subsequent euthanasia at day 10 p.i. (Fig.2).

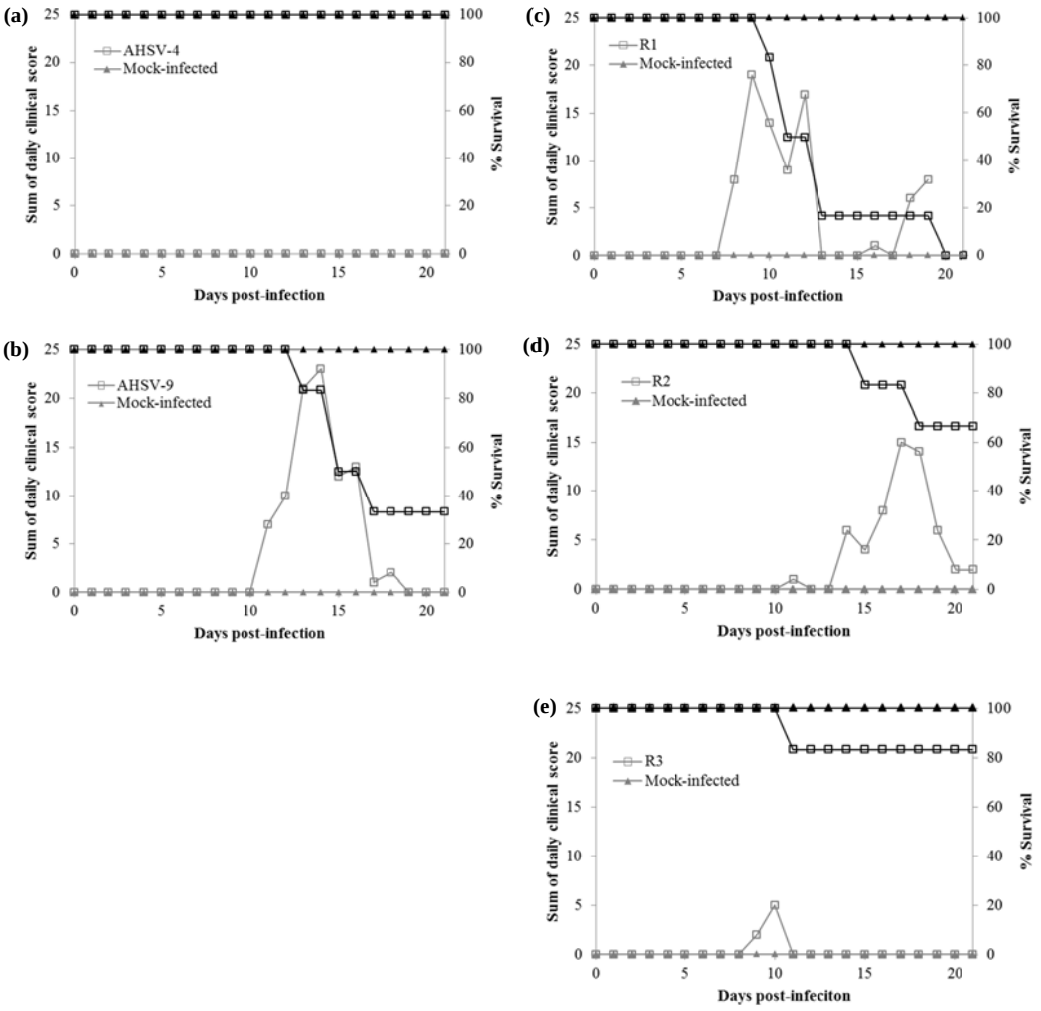


Figure 2 : Clinical scores and survival rate of IN infected mice groups. (a) AHSV -4 : Serotype 4 of African horse sickness virus; **(b)** AHSV-9 : Serotype 9 of African horse sickness virus; **(c)** R1 : R9-$4_{1, 4, 6, 7, 9}$; **(d)** R2 : R4-$9_{1, 2, 8, 10}$; **(e)** R3 : R4-$9_{2, 3, 8, 10}$. Points and curve in grey represent the sum of daily clinical score and points and curve in black represent the survival rate (expressed as a percentage).

Mice infected IN with AHSV-4 (group H) remained healthy with a regular increase of body weight. Two mice in this group without any clinical signs were euthanized at day 14 p.i., to collect their organs for virus detection.

Mice infected IN with AHSV-9 (group I) showed clinical signs starting from day 11 p.i. with the highest sum of clinical scores of all IN infected mice groups. At the end of the experiment, a mortality rate of 66 % was calculated (Fig.2).

In addition, considering the ratio between the cumulated clinical score and the period of life of each mouse, more clinical signs and mortality were significantly observed in groups infected with AHSV-9 ($p<0.001$), R1 ($p<0.001$) and R2 ($p=0.01$) in comparison with the IN mock-infected mice group. This difference was not significant considering the groups infected with AHSV-4 and R3. The clinical signs and mortality rate observed in IN R2 infected group were similar to those observed for IN AHSV-9 infected group. Mice infected with R3 virus showed less clinical signs than other groups infected with reassortant viruses or AHSV-9 but a higher mortality rate in comparison with the AHSV-4 infected group.

RNAemia

Regardless of the inoculation route, no RNAemia was detected in any mock-infected mice. In SC infected mice, RNAemia was regularly detected in all groups until day 18 p.i. except in AHSV-4 and R3 infected mice groups (Fig.3). On the contrary, after IN infection, AHSV genome could never be detected, as previously described (De La Grandiere *et al.*, 2014), with an exception of the detection in the blood sample of one mouse in the IN infected R1 mice group (data not shown), 2 days before its euthanasia (day 9 p.i.). Mice infected with AHSV-9 ($p=0.02$), R1 ($p=0.007$) or R2 ($p=0.001$) had a significantly higher number of genomic copies per µl of blood in comparison with mice infected via the same route with AHSV-4 as a reference. R3 did not have a significantly higher number of genomic copies per µl of blood in comparison with AHSV-4.

Internal (murine GAPDH gene) and external (BTV synthetic RNA) controls (De La Grandiere *et al.*, 2014) were regularly maintained during the analysis of blood samples. AHSV-9, R1 and R2 SC infected groups showed a similar number of RNA genomic copies in blood in comparison with AHSV-4 and R3 SC infected groups in which the detection was comparable but irregular.

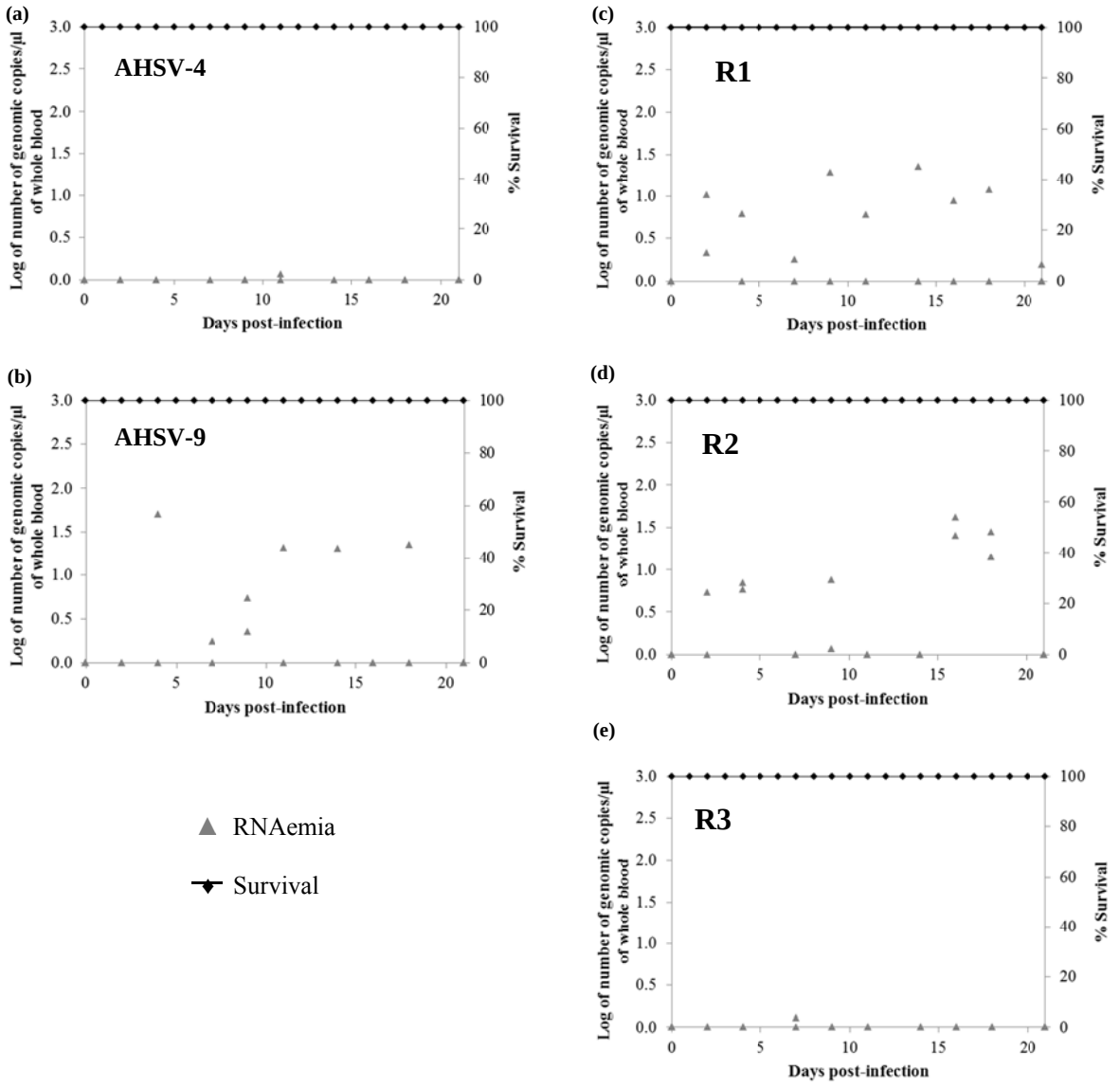


Figure 3 : RNAemia and survival rate in SC infected mice groups. (a) AHSV-4 : Serotype 4 of African horse sickness virus; **(b) AHSV-9** : Serotype 9 of African horse sickness virus; **(c) R1 :** R9-$4_{1, 4, 6, 7, 9}$; **(d) R2 :** R4-$9_{1, 2, 8, 10}$; **(e) R3 :** R4-$9_{2, 3, 8, 10}$. Points in grey represent the number of genomic copies/µl of whole blood; points and curve in black represent the survival rate (expressed in percentage).

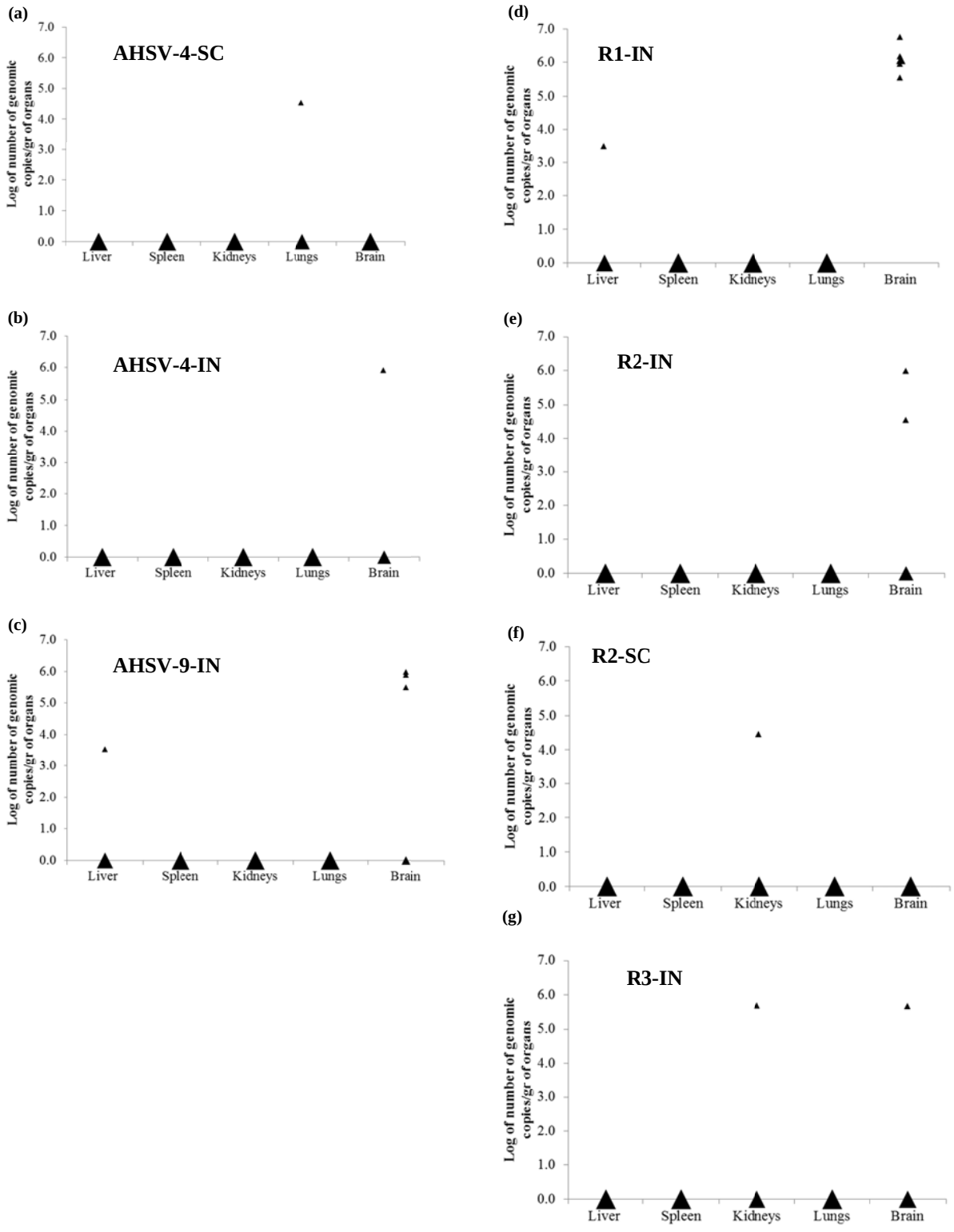


Figure 4 : Detection of the virus in different organs (liver, spleen, kidneys, lungs and brain). AHSV -4 : Serotype 4 of African horse sickness virus (a) and (b); virus **AHSV-9** : Serotype 9 of African horse sickness virus (c); **R1** : R9-$4_{1, 4, 6, 7, 9}$ (d) ; **R2** : R4-$9_{1, 2, 8, 10}$ (e) and (f) ; **R3** : R4-$9_{2, 3, 8, 10}$ (g). N=1: ▲ ; N=2 : ▲ ; N=3 : ▲ ; N=4 : ▲ ; N=5 : ▲ ; N=6 : ▲

Detection of the virus in organs

Organs (liver, spleen, kidneys, lungs and brain) were sampled after euthanasia. Considering viral loads in organs of SC infected mice, virus was exceptionally detected only in the AHSV-4 infected group in lungs (Fig.4a), and from the kidneys of one mouse in R2 SC infected group (Fig.4f).

The one organ, in which viral detection was regular in all IN infected mice, was the brain. Indeed, viral loads were detectable in all brain samples from mice euthanized during the experiment and even in AHSV-4 infected mice euthanized without previously having shown any clinical signs (day 14 p.i.). In correlation with the clinical signs observed, the highest viral load was found in the brain of R1 IN infected mice ($p=0.07$) and over all groups, the brain had a significantly higher viral load than other organs ($p<0.001$). At day 21 p.i. (end of the experiment), no viral load was detected from the others organs in all IN infected mice groups except in one kidney in the R3 IN infected group (Fig.4g). Murine GADPH and the EC-EXTR were detected in all tested organs.

DISCUSSION

AHSV reassortant previously *in vitro* generated, were tested during this *in vivo* experiment. The obtained results showed : (a) differences in the *in vivo* virulence of some reassortants when compared to parental viruses, with one reassortant being more virulent than parental viruses; (b) and a correlation between the severity of clinical signs and the quantification of viral genome in blood and brain.

Three reassortant viruses were selected in order to study their virulence *in vivo* in comparison with the virulence of the parental strains. The SC route was used in order to measure viraemia and the IN route was used to observe clinical signs after viral infection (De La Grandiere *et al.*, 2014).

A higher virulence compared to the parental viruses was observed for one reassortant virus (R1 namely R9-$4_{1, 4, 6, 7, 9}$ IN infected group) demonstrating that reassortment can lead to modifications in the virulence of AHSV. In a previous study with AHSV-3 and AHSV-8 (O'hara *et al.*, 1998) in Balb/c mice, no higher virulence was found for reassortant viruses tested *in vivo* but similar virulence or less virulence was measured in comparison to parental strains. In horses, the natural host of AHSV, reassortment between vaccine strains for AHSV1, -3 and -4 has been demonstrated. However, none of the isolated reassortant viruses induced clinical signs when inoculated to horses (Von Teichman et Smit, 2008), probably because the vaccine strains were already attenuated. In a study using BTV-1 and BTV-8 in IFNAR -/- mice, despite a slower viral growth *in vitro*, reassortant viruses tested *in vivo* showed similar virulence in comparison with both parental strains (Shaw *et al.*, 2013). Results from a study testing *in vitro* virulence of BTV-1, -6 and 8 showed increased rates of CPE and a fast replication curve of reassortants in comparison to parental viruses. Additional study is needed to confirm a suggestion of higher virulence of these reassortant viruses *in vivo* (Coetzee et al., 2014).

A qRT-PCR targeting VP7 was used to compare the levels of viremia from reassortant and parental viruses. RNAemia was correlated with the observation of clinical signs even if the R1 SC group was not different from the R2 and AHSV-9 SC infected groups. Indeed, R1, R2 and AHSV-9 SC infected groups had similar levels of genomic copies in blood but higher numbers in comparison to R3 and AHSV-4 SC infected groups. These data nicely complement previous studies on AHSV (O'hara *et al.*, 1998) and BTV (Shaw *et al.*, 2013) where viral genome equivalent quantities were not estimated.

The detection of the virus from different sampled organs (liver, spleen, kidneys, lungs and brain) was performed using the same qRT-PCR as used for the blood and targeting VP7. As previously described (De La Grandiere *et al.*, 2014), virus was regularly detected in the brains of IN infected mice. In particular, brains of mice euthanized during the experiment and of mice showing clinical signs had higher viral loads. Indeed, in organs from mice euthanized at the end of the experiment (3 weeks post-infection) no viral load could be detected except in the kidneys of one mouse in the R3 IN infected group and R2 SC infected group, and from lungs of one mouse in the AHSV-4 SC infected group. Once again, the higher viral load in the brain was correlated to the higher clinical score found in the R1 IN infected group.

The *in vitro* characterization of the three *in vivo*-tested reassortants of AHSV show a difference in the origin of segment 5 (NS1) for the most virulent, namely R1 (Table 2). No specific property of virulence is associated to Seg-5 in the literature, thus the hypothesis of the combination of all the segments together could more likely explain the higher virulence exhibited by R1. Furthermore, the occurrence of point mutations during the successive passages in VERO cells having led to a modification of virulence could be an additional explanation.

Discussion

Le virus de la peste équine (AHSV) est un virus à transmission vectorielle à potentiel d'émergence en Europe. Ce virus endémique en Afrique sub-saharienne trouve son principal réservoir chez le zèbre mais il est pathogène pour tous les autres équidés. Le zèbre ne présente pas ou peu de signes cliniques mais l'AHSV peut provoquer jusqu'à plus de 95 % de létalité chez le cheval infecté avec des conséquences économiques très importantes pour la filière équine. La circulation de plusieurs souches virales dans les pays endémiques a mené à l'isolement de réassortants et notamment entre souches vaccinale et sauvage. Peu d'études se sont intéressées jusqu'à présent au phénomène de réassortiment génétique de l'AHSV alors que celui-ci peut constituer un mécanisme d'évolution important pour les virus à ARN segmenté comme l'AHSV.

La première étude a abouti au développement d'un modèle murin d'infection par l'AHSV. Trois souches de souris et deux voies d'inoculation ont été testées : des souris déficientes en récepteur à l'interféron α/β ou IFNAR -/-, des souris Balb/C et des souris 129 WT, ces dernières partageant le même background génétique avec les souris IFNAR -/- , ainsi que les voies sous-cutanée (SC) et intranasale (IN) avec des propriétés différentes en ce qui concerne la distribution du virus dans l'organisme. Cette première étude a donc permis l'établissement d'un outil repris et utilisé dans la deuxième étude.

La deuxième étude s'est intéressée au réassortiment génétique de l'AHSV. Dans une première partie, l'obtention et la caractérisation de virus réassortants *in vitro* ont été étudiées. Des virus réassortants ont été générés suite à la coinfection par les sérotypes 4 et 9 de l'AHSV. Une dominance du sérotype 9 par rapport au sérotype 4 a été observée au cours de la coinfection. Après la caractérisation des virus réassortants obtenus *in vitro*, trois virus réassortants ont été sélectionnés pour en étudier la virulence *in vivo* en utilisant un des modèles murins mis en place dans la première étude. Dans cette deuxième partie, la virulence des virus réassortants a été comparée à celle des virus parentaux par la mesure de plusieurs paramètres expérimentaux (scores cliniques et mortalité, ARNémie et détection du virus dans les organes). Les différents résultats de ces deux études seront discutés.

Modèle murin expérimental d'infection par le virus de la peste équine

Deux sérotypes différents de virus de la peste équine, les sérotypes 4 et 9, tous les deux historiquement importants pour l'Europe et en particulier pour la péninsule ibérique, ont

été utilisés dans ce travail. Une souche de souris déficiente en récepteur à l'interféron α (IFNAR -/-) et deux souches différentes de souris immunocompétentes les souris Balb/C et les souris 129 WT, cette dernière partageant le même fond génétique que les souris IFNAR -/-, ont été utilisées.

Deux expériences distinctes ont été réalisées avec des souris Balb/C et 129 WT, alors que dans chaque expérience, des souris IFNAR -/- ont été utilisées. L'utilisation des souris IFNAR -/- dans les deux expériences a permis de répéter deux fois l'infection dans cette souche de souris et de la comparer avec chaque souche de souris immunocompétentes. Les principaux résultats observés sont : (a) une variation de la virulence entre les deux sérotypes et une virulence plus importante du sérotype 4 de l'AHSV ont été démontrées par un score clinique plus élevé chez les souris inoculées par AHSV-4 et également un taux de survie plus faible; (b) des signes cliniques ont été observés uniquement chez les souris IFNAR -/- infectées par voie SC et chez les souris immunocompétentes infectées par voie IN. Les signes neurologiques (non observés chez les chevaux infectés) ont été confirmés par la présence d'une méningo-encéphalite à l'histopathologie et la détection d'ARN viral dans le cerveau; (c) l'ARNémie était détectable pendant une longue période dans le sang des souris infectées en SC tel que décrit pour les chevaux, les ânes, les mulets et les zèbres (El Hasnaoui *et al.*, 1998; Wilson *et al.*, 2009); (d) la voie d'inoculation est un déterminant crucial de l'apparition de la maladie chez les souris immunocompétentes.

Une différence de la virulence des sérotypes 4 et 9 a déjà été observée chez les chevaux inoculés expérimentalement (Skowronek *et al.*, 1995; Sailleau *et al.*, 1997). En effet, dans ces études, les chevaux infectés par le sérotype 4 ont développé une forme pulmonaire sévère de la peste équine, tandis que le sérotype 9 a été moins virulent et a induit la forme cardiaque ou la forme mixte de la maladie. Dans une étude utilisant des souris IFNAR -/-, des différences dans la virulence entre AHSV-4 et -9 ont également été observées (De La Poza *et al.*, 2013). En effet, dans cette étude, les signes cliniques observés chez les souris IFNAR -/- non vaccinées était plus modérés que chez des souris infectées par l'AHSV-9 par rapport à celles infectées par AHSV-4. Néanmoins, la virémie mesurée par la méthode de plages de lyse était comparable pour les deux sérotypes sans données sur la détection de l'ARN viral par qRT-PCR.

Dans la première étude de cette thèse, des différences significatives ont été observées au niveau de la virulence des sérotypes 4 et 9. Tout d'abord, au niveau de l'ARNémie exprimée en log du nombre de copies génomiques, les valeurs sont significativement plus élevées pour le sérotype 4 que pour le sérotype 9, aussi bien lors de l'infection de souris IFNAR -/- que de souris immunocompétentes. Ensuite, les scores cliniques relevés entre J0 et J11 post-infection chez les deux souches de souris immunocompétentes étaient plus élevés pour le sérotype 4. Enfin, la charge virale mesurée dans les organes des souris 129 WT inoculées par voie IN, était significativement plus élevée pour le sérotype 4. La peste équine est caractérisée chez les chevaux par quatre formes cliniques de la maladie: la maladie de la forme fébrile, la forme cardiaque (subaiguë), la forme pulmonaire (suraiguë) et la forme mixte (la plus fréquemment observée). Les signes cliniques présents chez la souris infectée ne font partie ni de la forme cardiaque ni de la forme pulmonaire ou mixte, indiquant les limites du modèle murin comme modèle d'étude de la pathogénie de la peste équine. Même si, lors de chaque expérience, une souris IFNAR -/- a été retrouvée morte sans présenter de signes cliniques préalables comme cela peut être observé chez le cheval souffrant de la forme pulmonaire de la maladie. De même les signes nerveux développés par les souris IFNAR-/- ne sont pas présents chez le cheval infecté. Les souches virales d'inoculation ont pu acquérir un neurotropisme au cours de leurs passages successifs en cerveau de souriceaux, ce qui permet d'expliquer ces signes nerveux.

Chez les souris développant des signes cliniques, une chronologie dans l'apparition de ceux-ci a pu être mise en évidence : d'abord les souris présentent une conjonctivite et/ou un pelage rugueux, ensuite une perte de poids pouvant aller jusqu'à 15 ou 20 %, de l'apathie et enfin des signes neurologiques observés uniquement chez les souris IFNAR -/-. Ceux-ci étaient une ataxie, une paralysie d'un ou plusieurs membres ainsi qu'une démarche en cercle. Ils ne sont pas observés chez les chevaux. Ils pourraient être expliqués par une adaptation des virus utilisés dans ces deux expériences, à travers des passages successifs sur cerveaux de souriceaux nouveau-nés, méthode utilisée pour l'isolement viral (O'hara *et al.*, 1998). L'inoculation IN est la voie qui conduit à l'apparition de mortalité chez les souris Balb/C. Cependant, les résultats de l'étude de O'Hara et al. (1998) sont difficiles à comparer avec les résultats obtenus dans cette thèse, à cause de l'origine différente des souches virales utilisées. Avec les souris immunocompétentes, l'apparition de signes cliniques a été observée seulement après infection IN. Deux hypothèses pourraient expliquer cette observation : d'abord le

neurotropisme acquis par les souches virales après leurs passages en cerveau de souriceaux (O'hara *et al.*, 1998; Lubroth *et al.*, 2007) et ensuite la neuro-invasion par voie rétrograde au départ du nerf olfactif. Pour le virus de la fièvre du Nil occidental (WNV), ainsi que pour d'autres arbovirus neurotropes, une controverse repose sur la possibilité de neuroinvasion rétrograde du cerveau (Monath *et al.*, 1983). De cette façon, l'infection de neurones olfactifs a été proposée comme un mode d'entrée pour les souches du WNV neurotropes et très virulentes dans le système nerveux central (Murray *et al.*, 2010). De toute évidence, le fait que les neurones olfactifs ne soient pas protégés par la barrière hémato-encéphalique pourrait expliquer un accès plus facile du virus au cerveau. De ce fait, le choix de la voie d'inoculation a une importance capitale de même que la souche de souris utilisée (immunocompétente ou IFNAR -/-) et le potentiel neuroinvasif du virus inoculé. Après l'infection, l'ARN viral peut être détecté dans certains organes (foie, les reins, la rate, etc.) malgré l'absence d'ARNémie. L'hypothèse la plus probable est l'absence de détection d'une ARNémie transitoire en raison de limitations du point de vue éthique dans la fréquence de la collecte de sang chez la même souris.

Après l'infection par voie SC, seules les souris IFNAR -/- présentaient des signes cliniques (à partir de J2 post-infection), alors que les deux souches de souris immunocompétentes ont présenté une infection subclinique en dépit d'une ARNémie constante et élevée. La comparaison entre les souris IFNAR -/- et les souris immunocompétentes infectées en SC permet de postuler le rôle de l'IFN de type I au moment de l'infection par l'AHSV. Après l'infection en SC, les niveaux d'ARNémie élevés sont similaires alors que les souris immunocompétentes n'ont pas développé de signes cliniques comme le souris IFNAR -/- dès le J2 post-infection. Aucune donnée sur le rôle des voies IFN de type I dans l'infection par l'AHSV n'est disponible jusqu'à présent, mais il existe des données pour un autre *Orbivirus*, à savoir le BTV (Ruscanu *et al.*, 2012; Chauveau *et al.*, 2013) et d'autres virus appartenant à la famille des *Reoviridae* (Johansson *et al.*, 2007; Deal *et al.*, 2013). L'infection virale induit une forte production d'IFN de type I. Chez les moutons infectés par le BTV, le pic de la production d'IFN a été corrélé avec le pic de la virémie mesurée et ensuite associée à une forte diminution de titres de virus (Foster *et al.*, 1991). Plus récemment, il a été démontré que la protéine non structurale NS3 du BTV interfère avec la production d'IFN de type I (Chauveau *et al.*, 2013). Les souris IFNAR - / - ont une plus grande sensibilité aux infections virales (Bereczky *et al.*, 2010; Wernike *et al.*, 2012) qui est liée à l'absence de récepteurs à l'IFN α/β et, par conséquent, elles présentent une déficience de l'immunité innée. Les résultats des

études avec le BTV et ceux obtenus à partir l'étude de cette thèse supportent l'hypothèse du rôle de l'IFN de type I dans le contrôle de l'infection virale, et en particulier dans la réduction de la sévérité de l'expression clinique de la maladie. Une expression plus sévère de la maladie chez les souris IFNAR - / - a été observée, tandis que 50% de ces souris ont pu survivre à l'infection, malgré l'absence de l'activité antivirale innée liée aux IFN de type I. Cela suggère l'implication d'autres mécanismes immunitaires dans le contrôle de l'infection.

En conclusion de la première étude de cette thèse, les résultats fournissent de nombreuses données sur la virulence des sérotypes 4 et 9 de l'AHSV chez deux souches de souris immunocompétentes et une souche de souris IFNAR -/-. Cette première étude a été initiée dans le but de développer un outil pour l'étude du réassortiment génétique de l'AHSV sur base de certains éléments déjà rapportés dans la littérature. Les souris de souche 129 WT n'avaient pas encore été testées pour l'étude de l'infection par l'AHSV, des données de quantification du virus par qPCR dans le sang et les organes n'étaient pas encore disponibles et la comparaison de l'infection chez trois souches de souris différentes n'avait pas encore été réalisée en parallèle.

Le sérotype 4 peut être considéré comme plus virulent que le sérotype 9, au moins pour les souches virales utilisées dans cette étude. Les différences observées dans la virulence ont été obtenues par l'utilisation de deux sérotypes qui ont subi les mêmes passages historiques de laboratoire. Cela confirme l'hypothèse d'une diversité génétique entre les deux sérotypes, et donc de marqueurs génétiques de virulence qui devraient être étudiés. L'utilisation de ces modèles murins pour l'étude de la pathogénie de la peste équine est limitée par l'absence de lésions endothéliales lors des analyses histopathologiques ainsi que par la présence de signes cliniques de type nerveux chez les souris IFNAR -/- et absents chez l'hôte naturel. Le tropisme nerveux acquis par ces souches lors de leur passage en cerveau de souriceaux est une hypothèse pouvant expliquer ces signes neurologiques. D'autre part, la présence de virus en quantité importante dans le cerveau des souris inoculés par voie IN suggère l'invasion du virus par voie rétrograde via le nerf olfactif. Les seuls accidents humains de laboratoire dus à l'AHSV ont d'ailleurs été provoqués par l'inhalation de souches neurotropes lors de leur manipulation en vue de la production de vaccins et avaient provoqué des encéphalites et des choriorétinites chez les travailleurs (Taylor *et al.*, 1992).

L'infection de souris Balb/C et de souris 129 WT pourrait être utilisée comme un modèle valable dans l'identification de marqueurs génétiques de neurotropisme de ces souches

de l'AHSV ainsi que pour d'autres arbovirus neurotropes grâce à la voie d'inoculation IN. Les souris IFNAR -/- sont un excellent modèle pour les études sur l'efficacité du vaccin en raison de leur sensibilité clinique élevée à l'infection par ce virus. Néanmoins, des souches de souris immunocompétentes ayant une réponse immunitaire antivirale innée conservée doivent également être prises en considération pour les études sur l'efficacité du vaccin et aussi pour d'autres fins, telles que l'étude de l'évolution des populations virales de l'AHSV. En effet, les modèles de souris Balb/C et 129 WT comme les souris IFNAR -/- permettent une multiplication efficace du virus. Ce sont donc des modèles intéressants pour la génération de virus réassortants *in vivo* et peuvent compléter l'analyse du réassortiment *in vitro*.

Étude *in vitro* du réassortiment génétique entre les sérotypes 4 et 9 de l'AHSV

Des virus réassortants ont été obtenus au cours de coinfections *in vitro* synchrones utilisant un MOI supérieur pour le sérotype 4 par rapport au sérotype 9. De multiples échanges des segments entre les deux sérotypes ont été observés chez chaque virus réassortant isolé. Des différences dans les courbes de multiplication virale de certains virus réassortants ont pu être mises en évidence lorsqu'elles étaient comparées aux courbes de réplication des virus parentaux.

La décision de se baser sur les segments 2 (VP2) et 6 (VP5) pour développer une qRT-PCR de criblage des surnageants de culture cellulaire issus des coinfections a été prise pour plusieurs raisons. D'une part, ces segments codent les deux protéines de capside externe de l'AHSV et ont un rôle dans l'attachement du virus sur les cellules et son internalisation. Obtenir des virus réassortants, dont ces deux segments en particulier étaient originaires, d'un virus parental différent était intéressant pour en étudier la virulence. D'autre part, une étude récente sur un virus très analogue à l'AHSV, à savoir le virus de la FCO, a démontré que, lors d'expériences de coinfections entre les sérotypes 1 et 8 du virus de la FCO, la majorité des virus réassortants isolés avait des segments 2 et 6 provenant chacun d'un virus parental (Shaw *et al.*, 2013). Ces résultats rendent donc très probable l'identification des virus réassortants grâce à une qRT-PCR ciblant ces deux segments. À l'initiation de ce travail, les séquences du génome complet de l'AHSV-4 et -9 n'étaient pas disponibles sur GenBank, rendant difficile, le développement de qPCR de criblage sur les 10 segments. D'autre part, le criblage de virus infectieux issus d'une centaine de plages de lyse pour chaque condition expérimentale, nécessitait le développement d'un outil de discrimination rapide.

Cependant, cette décision *a priori* peut restreindre la détection d'autres virus réassortants, sachant que 1024 combinaisons sont théoriquement possibles lors du réassortiment aléatoire. Dès lors, les nouvelles méthodes disponibles actuellement comme le séquençage à haut débit (Next-Generation Sequencing ou NGS) sont à envisager pour des études futures.

Différents MOI ont été utilisés pour réaliser les coinfections *in vitro*. Tout d'abord, des MOI identiques pour les deux sérotypes ont été testés comme cela avait été réalisé lors d'études utilisant d'autres sérotypes d'AHSV (O'hara *et al.*, 1998; Meiring *et al.*, 2009) ou le virus de la FCO (Shaw *et al.*, 2013). Dans ces différentes études, des virus réassortants ont pu être générés dans les conditions expérimentales de coinfection *in vitro* synchrones utilisant un MOI identique pour les deux virus inoculés. Cependant, dans l'étude de cette thèse, aucun virus réassortant n'a été obtenu en utilisant des MOI identiques car une dominance du sérotype 9 sur le sérotype 4 était présente dans ces conditions. En augmentant le MOI du sérotype 4 par rapport au sérotype 9, plusieurs virus réassortants ont pu être générés. Lorsque le sérotype 4 était inoculé avec un MOI de 5, des virus réassortants ont pu être identifiés au cours de l'isolement viral réalisé tout de suite après la coinfection. Par contre, avec un MOI de 10 pour le sérotype 4, des virus réassortants ont pu être mis en évidence seulement au cours de l'isolement réalisé après 4 passages du surnageant de la culture cellulaire issu de la coinfection. Ces résultats suggèrent que le sérotype 4 avec un MOI 10 fois supérieur au MOI du sérotype 9, était rendu artificiellement dominant et n'a donc pas permis l'échange de segments avec le sérotype 9. Les 4 passages successifs ont probablement permis au sérotype 9 de récupérer progressivement le titre viral plus élevé, aboutissant alors à l'échange de segments entre les deux sérotypes et la génération de virus réassortants. Les trois réassortants identifiés au passage 0 avec le MOI de 5 correspondent à 10 % des surnageants de culture cellulaire issus des coinfections tandis que les 4 virus réassortants isolés au passage 4 avec le MOI de 10 correspondent à 5 % du total des criblages par qRT-PCR. Cette observation indique que la génération de virus réassortant n'augmente pas de façon proportionnelle avec le MOI utilisé. La dominance du sérotype 9 et l'utilisation de MOI différents apportent un biais important à connaître puisqu'il peut jouer dans l'obtention de virus réassortants dans les conditions naturelles.

Dans d'autres études sur le réassortiment de l'AHSV (O'hara *et al.*, 1998; Meiring *et al.*, 2009) ou le virus de la FCO (Shaw *et al.*, 2013), aucun phénomène de dominance d'un sérotype par rapport à un autre n'est décrit pendant les expériences de coinfection *in vitro.*

Cependant, dans une étude de coinfection *in vivo* utilisant les sérotypes 1 et 8 du virus de la FCO, une détection prédominante du sérotype 8 a été mise en évidence dans des échantillons provenant de veaux coinfectés (Dal Pozzo *et al.*, 2013). La caractérisation des six virus réassortants analysés par qRT-PCR et PCRs conventionnelles a montré que l'échange de segments entre les deux sérotypes était de nature multiple et hétérogène dans la répartition de leurs origines. L'échange multiple de segments a également été mis en évidence pour l'AHSV (O'hara *et al.*, 1998) et le virus de la FCO (Shaw *et al.*, 2013). Certains segments étaient systématiquement issus du sérotype 4, à savoir les segments 6 (VP5), 7 (VP7) et 9 (VP6) tandis que d'autres provenaient toujours du sérotype 9, les segments 2 (VP2), 8 (NS2) et 10 (NS3). Le segment 2 provenait systématiquement du sérotype 9 et conférait donc ce sérotype au virus réassortant, cependant, l'exploration des autres segments montrent que pour la majorité des virus réassortants analysés, le « backbone » était de sérotype 4 (66 %).

Ces résultats suggèrent l'existence de constellations conservées dans le génome selon les sérotypes pendant les événements de réassortiment. Une étude récente sur le réassortiment génétique pour le BTV, a mis en évidence des associations de segments durant les échanges entre les différents virus en conditions naturelles (Nomikou *et al.*, 2015). Ce résultat contraste avec les résultats obtenus par Shaw et al., 2013, qui ont réussi à démontrer par génétique inverse que le réassortiment pouvait impliquer tous les segments sans association spécifique de ceux-ci pendant les échanges. Ces résultats suggèrent l'existence de contraintes réelles dans l'assemblage de segments génomiques dans le cadre d'obtention de virus réassortants lors de coinfection. Ces observations peuvent être étendues aux rotavirus humains où l'existence de constellations de segments conservés a été mise en évidence. Ceci est expliqué par une évolution virale avec une adaptation à l'espèce, en l'occurrence l'homme (Patton, 2012). Pour l'AHSV, d'autres études sont nécessaires pour dégager le rôle des différents segments à partir de ces constellations génomiques et déterminer si l'avantage sélectif ou non conféré par cette association de segments est lié à un effet individuel d'une protéine ou à un effet coopératif de différentes protéines entre elles.

Étude de la virulence *in vivo* des virus réassortants issus des coinfections *in vitro*

Après avoir généré et caractérisé des virus réassortants *in vitro*, trois virus réassortants ont été sélectionnés pour être inoculés à des souris Balb/C. Les résultats principaux sont :

l'observation de différences au niveau de la virulence *in vivo* des virus réassortants avec les virus parentaux, avec un virus réassortant plus virulent, et la mise en évidence d'une corrélation entre la sévérité des signes cliniques relevés et les résultats de détection du virus dans les échantillons de sang et d'organes.

Suite aux résultats de la première étude de cette thèse (De La Grandiere *et al.*, 2014), les voies SC et IN ont été utilisées pour inoculer les souris avec les 3 virus réassortants générés et sélectionnés *in vitro*. En effet, alors que la voie SC apporte l'avantage de permettre la détection du virus dans les échantillons sanguins pendant une longue période (jusqu'à 21 jours post-infection), les souris inoculées par voie IN développent des signes cliniques dont la sévérité peut amener à procéder à leur euthanasie et du virus peut être détecté à partir de leurs échantillons d'organes. Les deux voies d'inoculation sont donc complémentaires.

Des résultats différents ont été obtenus au cours de cette deuxième étude par rapport à ce qui avait été observé dans la première étude. En effet, les conclusions de la première étude aboutissait à une virulence plus élevée pour l'AHSV-4 par rapport à l'AHSV-9 alors que l'inverse a été relevé au cours de la deuxième étude. Pourtant, le même protocole expérimental ainsi que les mêmes méthodes de détection du virus ont été utilisés pour les deux études. L'historique des passages des souches virales utilisées est légérement différent, avec un nombre inférieur de passages en culture cellulaire pour les souches virales utilisées dans la deuxième étude. Au cours des passages supplémentaires, des mutations ont pu engendrer une virulence plus importante pour l'AHSV-4 par rapport à l'AHSV-9 pour l'étude 1 ; dans ce contexte, il serait pertinent d'envisager un séquençage complet des inoculums de départ afin d'expliquer la différence de virulence observée pour l'AHSV-4 entre les deux études.

Une plus grande virulence a été observée pour un des virus réassortants testés en comparaison avec les virus parentaux ; il s'agissait du R9-$4_{1, 4, 6, 7, 9}$ ou R1 dans le groupe de souris inoculé en IN avec ce dernier. Cette observation démontre bien que le réassortiment génétique peut modifier la virulence de l'AHSV. Dans une précédente étude ayant généré des virus réassortants entre les sérotypes 3 et 8 de l'AHSV, aucune différence de virulence *in vivo* n'a été constatée lorsque ces virus réassortants étaient inoculés en IN à des souris Balb/C (O'hara *et al.*, 1998). Chez les chevaux, hôtes naturels pour l'AHSV, du réassortiment génétique a pu être mis en évidence lors de vaccination avec des vaccins multivalents. Des

virus réassortants entre les sérotypes 1, 3 et 4 ont été isolés mais aucun de ces derniers n'a montré une différence de virulence lorsqu'ils étaient inoculés à leur tour à des chevaux (Von Teichman et Smit, 2008). De même, dans l'étude ayant généré des virus réassortants entre les sérotypes 1 et 8 du virus de la FCO et ce, malgré une courbe de croissance ralentie *in vitro*, aucun des virus réassortants inoculés à des souris IFNAR -/- n'a montré de différence de virulence par rapport aux virus parentaux (Shaw *et al.*, 2013). Enfin, des différences dans les courbes de multiplication virale et dans la production de CPE ont été observées pour des virus réassortants obtenus lors de coinfections avec les sérotypes 1, 6 et 8 du virus de la FCO lorsqu'ils étaient comparés aux virus parentaux (Coetzee *et al.*, 2014). La caractérisation du virus réassortant R1 qui a démontré une plus grande virulence *in vivo* que les virus parentaux, met en évidence une différence dans l'origine du segment 5 codant la protéine NS1. Cependant, aucune propriété de virulence spécifique n'est associée au segment 5 dans la littérature, donc la virulence plus importante de R1 doit plutôt être associée à la combinaison des différents segments composant son génome plutôt qu'à NS1 seul. De plus, des mutations ponctuelles ont pu se produire pendant les passages successifs en culture de cellules VERO ou lors de la réplication dans la souris et pourraient également contribuer à expliquer une différence dans la virulence *in vivo.*

Une qRT-PCR ciblant le segment 7 (VP7) a été utilisée pour comparer le niveau de virémie des virus réassortants et parentaux. La RNAémie était correlée aux signes cliniques observés dans les groupes correspondants inoculés en IN. Cependant, le nombre de copies génomiques mesurées était identique dans les groupes inoculés en SC avec R1, R2 et le sérotype 9 de l'AHSV, tout en étant significativement plus élevé que dans les groupes inoculés en SC avec R3 et le sérotype 4 de l'AHSV. Les autres études sur le réassortiment de l'AHSV et du virus de la FCO n'ont pas procédé à la détection du virus dans le sang (O'hara *et al.*, 1998; Shaw *et al.*, 2013).

La détection du virus dans les échantillons provenant des différents organes prélevés (foie, rate, reins, poumons et cerveau) a été effectuée en utilisant la même qRT-PCR que pour la RNAémie. Comme décrit dans la première étude (De La Grandiere *et al.*, 2014), le virus était le plus souvent détecté à partir des échantillons de cerveau des groupes de souris inoculées en IN. En particulier, les souris euthanasiées en cours d'expérience suite à la sévérité des signes cliniques présentés, sont celles dont le cerveau avait la charge virale la

plus importante. En effet, dans les organes des souris euthanasiées à la fin de l'expérience (3 semaines post-infection), aucune charge virale n'a pu être mise en évidence excepté dans les reins d'une souris inoculée en IN avec R3 et une souris inoculée en SC avec R2 et dans les poumons d'une souris inoculée en SC avec le sérotype 4. Pour le cerveau aussi, la charge virale la plus élevée a été mesurée dans le groupe inoculé en IN avec R1 et est donc aussi correlée aux signes cliniques relevés dans ce groupe.

Le modèle d'infection expérimental de souris, même avec la souche immunocompétente Balb/C, a démontré son efficacité en permettant de comparer la virulence des virus réassortants par rapport aux virus parentaux et à mettre en évidence la plus grande virulence d'un virus réassortant.

Conclusions – Perspectives

Les résultats présentés dans cette thèse alimentent les connaissances sur un virus possédant un potentiel d'émergence en Europe, tel l'AHSV. Ils participent à consolider un état de préparation souhaitable pour être prêt à enrayer une éventuelle épidémie en cas d'introduction réussie du virus.

Au cours de la première étude, différents modèles murins ont été testés pour l'infection par le virus de la peste équine. Différentes applications et perspectives peuvent être envisagées dès le moment où ce modèle animal est disponible. Les études récentes de l'infection par l'AHSV n'utilisent que les souris IFNAR -/- car elles présentent l'avantage de développer des signes cliniques et une virémie détectable pendant une longue période. Cependant, il semble que le modèle souris immunocompétente constitué aussi bien par des Balb/c que des 129 WT, présente des caractéristiques semblables à savoir le développement de signes cliniques et de la virémie en fonction de la voie d'inoculation choisie et ce modèle peut donc être également utilisé.

Une première application du modèle a été réalisée au cours de la deuxième étude dans laquelle la virulence de virus réassortants obtenus *in vitro* a été étudiée sur des souris immunocompétente Balb/C. Cette étude a démontré que le réassortiment génétique pouvait conduire à une modification de virulence. Il était intéressant de pouvoir tester la virulence *in vivo* des virus réassortants obtenus *in vitro*. En effet, les expériences *in vitro* impliquent l'infection d'un type particulier de cellule alors que le modèle animal est un système éminemment complexe et l'extrapolation des résultats *in vitro* vers la situation *in vivo* peut être difficile. Néanmoins, les manipulations *in vitro* restent une étape indispensable pour l'étude du virus.

Une première perspective pour une autre utilisation du modèle souris est de procéder à une expérience de coinfection *in vivo* dans le but de générer des virus réassortants directement *in vivo*. Différents paramètres comme le MOI ou encore le moment d'inoculation (coinfection synchrone ou non) devront être réglés pour arriver à obtenir des virus réassortants. Les temps de prélèvement des échantillons sanguins et/ou d'organes devront être adaptés pour permettre l'isolement viral.

Ensuite, l'AHSV étant un arbovirus, il serait très intéressant d'étudier la compétence et la capacité vectorielle des vecteurs indigènes belges en utilisant les modèles souris mis au point dans la première étude. Jusqu'à présent, les culicoïdes sont un frein à ce type d'expérience à cause de la difficulté que représente leur élevage mais la compétence d'autres

vecteurs potentiels comme les moustiques pourraient être d'abord testée et servirait de mise au point à appliquer dès que l'élevage des culicoïdes sera disponible.

La deuxième étude avait pour sujet l'étude du réassortiment génétique entre les sérotypes 4 et 9 de l'AHSV. Des virus réassortants ont pu être générés *in vitro* et suggèrent que le réassortiment génétique n'est pas un évènement rare pour l'AHSV, du moins entre les deux sérotypes utilisés dans cette étude. De plus, lorsque trois virus réassortants ont été inoculés *in vivo*, une virulence modifiée a pu être mise en évidence. Ces résultats soutiennent le rôle du réassortiment dans l'évolution des populations virales des *Orbivirus* en conditions de circulation de plusieurs sérotypes dans une même région. Dans ces conditions épidémiologiques et comme cela a déjà été démontré pour le virus de la FCO (Maan *et al.*, 2010), le réassortiment génétique pourrait également survenir pour l'AHSV en conditions de terrain. Cela impliquerait d'adapter les techniques de diagnostic courantes pour l'AHSV pour pouvoir détecter la présence de virus réassortants. En effet, jusqu'à présent le sérotypage pour l'AHSV est réalisé en se basant sur des techniques de détection qui ciblent le segment 2 (VP2) et ne permettent donc pas de mettre en évidence la variabilité génotype et phénotypique existant au sein des différents sérotypes. Cependant, la détermination de l'origine de VP2 reste capitale pour le choix des stratégies de vaccination et de contrôle.

Le réassortiment génétique pourrait être utilisé pour investiguer plus en profondeur le rôle des différents gènes dans la virulence de l'AHSV. En fait, peu de données sont disponibles sur les facteurs de virulence de l'AHSV et, même si certaines données générées par des études sur le virus de la FCO peuvent être transposables à l'AHSV, il est important de connaître les déterminants de la virulence spécifiquement pour l'AHSV. Ce type d'étude mettrait à profit les méthodes développées dans cette thèse, à savoir le modèle animal et la production de virus réassortants *in vitro.*

Résumé - Summary

RÉSUMÉ

Les maladies à transmission vectorielle représentent un enjeu important en termes de santé publique et animale. Le virus de la peste équine (African horse sickness virus ; AHSV) est un virus à double brin d'ARN segmenté, appartenant à la famille des *Reoviridae* et au genre *Orbivirus*. L'AHSV se différencie en 9 sérotypes distincts et est transmis par la piqûre d'un vecteur, principalement de l'espèce *Culicoides imicola*. Chez les chevaux, l'AHSV cause une morbidité sévère s'accompagnant d'un taux de létalité pouvant atteindre 95 % ainsi que de lourdes conséquences économiques.

L'établissement d'un modèle d'étude en souris est nécessaire pour plusieurs champs d'étude de ce virus, comme l'investigation de la pathogénie de ce virus, l'étude de la virulence, l'étude d'efficacité de nouveaux vaccins. Dans la première étude de cette thèse, trois modèles murins, soit une lignée de souris déficientes en récepteur à l'interféron α (A129 KO ou IFNAR -/-), et deux lignées immunocompétentes (Balb/C et 129 WT) ont été testées. Les virus de sérotypes 4 et 9 de l'AHSV ont été utilisés pour les inoculations des souris ; ces deux sérotypes ont été à l'origine des épidémies observées en Espagne en 1966 et à la fin des années 80 en Espagne et au Portugal. Les voies d'inoculation sous-cutanée (SC) et/ou intra-nasale (IN) ont été choisies et un groupe de souris témoin (mock-infected) a été utilisé pour les trois modèles testés. Des échantillons de sang ont été prélevés de chaque souris infectée et témoin à intervalles réguliers. Les organes (foie, rate, reins, poumons et cerveau) ont été prélevés à la fin de l'expérience ou après euthanasie. Tous les échantillons, sang et organes, ont été analysés par qRT-PCR avec comme cible le segment 7 codant la protéine VP7 de l'AHSV, cette dernière étant la protéine de structure la plus conservée entre les différents sérotypes. Les deux sérotypes (4 et 9) de l'AHSV ont été détectés par qRT-PCR jusqu'à 3 semaines post-infection (correspondant à la fin de l'expérience) dans le sang de toutes les souris infectées par voie SC. Le virus de sérotype 4 atteint des niveaux de virémie légèrement plus élevés par rapport au virus de sérotype 9. Le pic de virémie a été mesuré entre les jours 2 et 4 post-infection pour les trois lignées de souris. Les souris Balb/C et 129 WT infectées par voie IN n'ont à aucun moment de l'expérience montré de virémie détectable par qRT-PCR. La mise au point de ce modèle sur souris permet de disposer d'un outil efficace et nécessaire pour l'étude de l'infection par l'AHSV, afin de caractériser *in vivo* la virulence de ce virus et de suivre l'évolution des populations virales pendant la multiplication virale *in vivo*.

Dans la deuxième étude, le phénomène de réassortiment génétique pour l'AHSV a été investigué. Dans un premier temps des coinfections *in vitro* ont été réalisées et ont permis de générer des virus réassortants entre les sérotypes 4 et 9 de l'AHSV. Malgré une vitesse de réplication plus lente pour le sérotype 9, une dominance sur le sérotype 4 a été observée et a nécessité l'adaptation des conditions de coinfections. La caractérisation des virus réassortants par qRT-PCR et PCR a montré de multiples échanges de segments et des constellations génomiques de segments en fonction du sérotype. Trois virus réassortants obtenus *in* vitro ont été inoculés à des souris Balb/C afin de comparer leur virulence *in vivo* avec celle des parentaux. Un virus réassortant a montré une virulence plus importante que les virus parentaux et les autres virus réassortants. Une corrélation entre le développement des signes cliniques et les résultats de détection de charge virale (nombre de copies génomiques d'ADN_c) dans les échantillons de sang et d'organes a été mise en évidence. Ces résultats supportent l'hypothèse que le réassortiment peut conduire à une modification de la virulence de l'AHSV qui pourrait avoir des répercussions sur de l'épidémiologie du virus.

En conclusion, un modèle murin expérimental a été mis au point dans la première partie de cette thèse. Il a prouvé son efficacité par son utilisation lors de l'évaluation de la virulence des virus réassortants générés au cours de la deuxième partie de la thèse. Les virus réassortants de cette étude possèdent tous des segments 2 (VP2) et 6 (VP5) d'origine parentale différente. Cette constatation montre les limites des méthodes de sérotypage conventionnelles qui portent exclusivement sur l'identification du segment 2 et qui ne permettent donc pas la mise en évidence de virus réassortants.

SUMMARY

Vector-borne diseases are a major threat for public and animal health. African horse sickness virus (AHSV) has a segmented genome composed of ten double-stranded RNA segments and belongs to the family *Reoviridae* and genus *Orbivirus*. The virus has nine known antigenically distinct serotypes and is transmitted by culicoides biting midges, principally *Culicoides imicola*. African horse sickness causes severe morbidity and mortality of up to 95 % in horses leading to severe economic losses.

The establishment of an experimental mouse model is needed for the investigation of the pathogenesis of this infection, the study of its virulence and the efficacy of vaccines. Three mouse models, interferon-α receptor knock-out mice (IFNAR -/- or A129 KO) and immunocompetent mice (Balb/C and 129 WT), were tested. The virus used for inoculation of mice belongs to the two serotypes, serotype 4 and 9, which have been known to cause epidemics in Europe, The virus was inoculated via subcutaneous route (SC) and/or intranasally (IN). Samples of whole blood were taken for both infected and knock-out mice at regular intervals. Organs (liver, spleen, kidney, lung and brain) were sampled at the end of the experiments, when the most affected mice were euthanized. All these samples were tested by a qRT-PCR, targeting African horse sickness genome segment 7, the most conserved segment between serotypes. Both serotypes (4 and 9) of African horse sickness were detected by qRT-PCR until three weeks post-infection (corresponding with the end of the experiments) in blood from SC infected mice. Serotype 4 showed a higher peak of RNAemia than serotype 9. The peak of RNAemia was measured between days 2 and 4 post-infection for the three strains of mice. No RNAemia was detected in blood from IN infected mice during the 3 weeks of the experiment. The setting up of this mouse model has developed a tool for efficient *in vivo* study to characterize the *in vivo* virulence of this virus, to monitor the evolution of viral populations during *in vivo* replication cycles.

In the second study, genetic reassortment for AHSV was investigated. First, *in vitro* coinfections were performed and reassortant viruses between AHSV serotypes 4 and 9 were generated. Despite a slower viral growth curve for serotype 9 in comparison to serotype 4, dominance of serotype 9 over serotype 4 was observed during coinfection assays and required adaptations of the conditions for coinfections assays. Genetic characterization of the six

reassortant viruses by qRT-PCR and RT-PCR showed multiple segment exchanges and a genome constellation per serotype was demonstrated. Three reassortant viruses were selected in order to study their virulence *in vivo* in comparison with the virulence of the parental strains in Balb/C mice. A higher virulence compared to the parental viruses and other reassortant viruses was observed for one reassortant virus. A correlation between severity of clinical signs and virus detection in blood and organs was found. These data support the modification of virulence by genetic reassortment with possible consequences in virus epidemiology.

In conclusion, in the first part of this thesis, an experimental mouse model was developed. It has proven its effectiveness by its use in the assessment of the virulence of reassortant viruses generated during the second part of the thesis. All the reassortant viruses from this study possess segments 2 (VP2) and 6 (VP5) originating from different parental viruses. This result shows the limitations of conventional serotyping methods which focus exclusively on the identification of segment 2 and therefore do not allow the detection of reassortant viruses.

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Annexes

Préambule

Cette thèse a été réalisée dans le cadre de deux projets financés par le service public fédéral santé publique, sécurité de la chaîne alimentaire et environnement (INDEVIREQ 1.0 et 2.0). Un des premiers thèmes de ces projets était le virus de la peste équine avec la mise au point de méthodes de diagnostic, d'un modèle murin, l'étude du réassortiment génétique et la détermination qualitative des risques d'introduction du virus en Belgique. Le deuxième thème du premier projet s'est intéressé en plus à l'évaluation du risque d'introduction des virus d'encéphalites équines américaines de l'Est (EEEV), de l'Ouest (WEEV) et vénézuélienne (VEEV). Dans ce cadre, deux autres volets ont été étudiés et correspondent aux deux annexes.

Tout d'abord une recherche bibliographique des voies d'introduction potentielle de ces virus en Belgique a été effectuée et a permis l'étude qualitative du profil de risque d'introduction de ces virus. De même un recensement bibliographique des espèces de vecteurs d'hôtes potentiels et de vecteurs compétents pour ces virus présents en Belgique a été effectué et est venu compléter l'évaluation de risque au niveau de l'exposition de ces espèces aux virus une fois introduits en Belgique. L'ensemble des résultats obtenus est rassemblé dans la première annexe et correspond à un article intitulé : ***Risk profiling of introduction and dissemination of Eastern, Western and Venezuelan equine encephalitis viruses in Belgium.***

Ensuite des campagnes de piégeage des vecteurs autour et dans des fermes équines ont été réalisées pendant deux années consécutives de manière à faire l'inventaire des espèces de vecteurs présentes près des chevaux et d'identifier les espèces potentiellement vecteurs pour les 3 virus. Ce travail a été effectué en collaboration étroite avec l'unité d'entomologie fonctionnelle et évolutive de la Faculté de Gembloux-Agro Bio Tech. Dans ce cadre, des pièges ont été placés à proximité de fermes équines afin de capturer les adultes moustiques et des prélèvements des points d'eau ou d'environnements humides ont été réalisés afin de récolter les larves de moustiques. Une fois les échantillons ramenés au laboratoire, les identifications morphologiques et moléculaires des vecteurs ont été effectuées. Les résultats obtenus sont présentés dans la deuxième annexe et constituent un article intitulé : ***Diversity and ecology survey of mosquitoes potential vectors in Belgian equestrian farms: a threat prevention of mosquito-borne equine arboviruses***.

Annexe 1

"Risk profiling of introduction and dissemination of Eastern, Western and Venezuelan equine encephalitis viruses in Belgium."

En préparation

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Abstract

Assessment of the introduction and spread of Eastern, Western and Venezuelan equine encephalitis viruses in Belgium was performed by establishing a risk profiling. First a scenario tree was built to identify the main routes of potential introduction and spread of vectors and hosts. After considering the epidemiological status of countries and biological factors affecting the risk of introduction, the different transportation forms were discussed. The probabilities and uncertainties associated with each step of the introduction and spread risk profiling were qualified by expert opinion. The probability of introduction of these three viruses was estimated from very low to moderate but all elements of their cycle are present in Belgium. This risk profiling is valid for the current period but may help decision makers for implementing measures preventing the introduction of these arboviruses.

Introduction

Arthropod-borne diseases transmitted by arboviruses are often associated with human outbreaks and represent a serious public health issue (Figueiredo, 2007). They are distributed worldwide and represented nearly 30% of all emerging infectious diseases in the last decade (Jones et al., 2008). The majority of these are RNA viruses of medical and veterinary importance belong to the families *Flaviviridae* (genus *Flavivirus*), *Bunyaviridae* (genus *Nairovirus, Orthobunyavirus, Phlebovirus,* and *Tospovirus*), *Togaviridae* (genus *Alphavirus*), *Rhabdoviridae* (genus *Vesiculovirus*), *Orthomyxoviridae* (genus *Thogotovirus*), and *Reoviridae* (genus *Orbivirus* and *Coltivirus*) (Figueiredo, 2007, Vasconcelos et al., 2005, Go et al., 2014).

In humans, arboviral infections are usually asymptomatic or presented with a mild influenza-like illness but in some cases they can be very severe and life threatening. However, the importance of several arboviruses as human and veterinary pathogens has increased, causing respiratory illness, arthritis, febrile illness, encephalitis, hemorrhagic syndrome, shock, and death (Hollidge et al., 2010). Humans and domestic animals (horses and pigs) usually are dead-end hosts to most of arboviruses as a result of an incidental "spillover" when they can become infected after entering enzootic areas (Anishchenko et al., 2006). Nevertheless, in some cases, horses and human can act as amplifying hosts by presenting with high viremia, enough to infect the arthropod vector (Nathanson, 2007, Unnasch et al., 2006, Anishchenko et al., 2006, Coffey et al., 2013, Hollidge et al., 2010, van den Hurk et al., 2009, Brault et al., 2002, Weaver et al., 1999).

The number of arboviruses-related outbreaks increased dramatically during the years due to ecological changes in the natural habitat of the virus leading to changes in their life cycles, contributing to the adaptation to new host and reservoirs (Peters, 2007, Figueiredo, 2007). Indeed, arboviruses outbreaks have been reported in several regions e.g. West Nile Fever virus (WNV) in North America, Mediterranean area, Romania, Central Europe and Russia (Weaver and Reisen, 2010, Fyodorova et al., 2006, Savage et al., 1999); Rift Valley Fever virus (RVFV) expanding in Africa and in the Arabian Peninsula (Anyamba et al., 2006, Madani et al., 2003); Chikungunya Fever virus (CHIKV) in North of Italy and France (Thiboutot et al., 2010, Staples et al., 2009, Rezza et al., 2007, Gould et al., 2010); Bluetongue-8 (BTV-8) in Netherlands, Belgium, Germany, France and Luxembourg and others European countries (Wilson and Mellor, 2009, Toussaint et al., 2006, Thiry et al., 2006, Wilson et al., 2007); Schmallenberg virus (SBV) first in Germany and Netherlands and

then spreading through Europe (Hoffmann et al., 2012, Sailleau et al., 2013, van den Brom et al., 2012) and Dengue in Croatia, France, USA and Japan (Gjenero-Margan et al., 2011, Gould et al., 2010, Centers for Disease and Prevention, 2010, Takasaki, 2011).

Equine encephalitis Alphavirus are part of these already occurred or potential arbovirus infections, especially Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV) and Venezuelan equine encephalitis virus (VEEV). They belong to the genus *Alphavirus* of the family *Togaviridae*. The virions are 60 to 70 nm in diameter, enveloped containing spikes in the surface. The genus comprises 28 virus species classified antigenically into 7 complexes (Zacks and Paessler, 2010, Travassos da Rosa et al., 2001, Powers et al., 2001, La Linn et al., 2001, Griffin, 2007). They can be divided into two groups: those which cause encephalitis and are known as New World Alphaviruses and those which cause systemic human disease with rash and arthralgia, been known as Old World Alphaviruses (Greenlee, 2014, Griffin, 2007). EEEV, WEEV and VEEV are the representative species of the New World Alphavirus group whereas the Old World group is represented by Sindbis virus (SINV), Chikungunya (CHIKV), O'nyong-nyong virus (ONNV), Ross River virus (RRV), Barmah Forest virus (BFV) and Semliki Forest virus (SFV) and Mayaro virus (MAYV). However, RRV, SINV and CHIKV have been occasionally associated with encephalitis (Go et al., 2014). It should be also pointed that even MAYV is classified as Old World Alphavirus, it was described in Central America and is mostly found in the Amazon Basin (Terzian et al., 2015, Munoz and Navarro, 2012, Mourao et al., 2012). Among the viruses described above, the Equine Encephalitis, represented by three zoonotic RNA Alphaviruses named WEEV, EEEV and VEEV, could represent a new threat to Europe causing deaths in humans and horses (Durand et al., 2013). The introduction of these viruses can be caused by the intense traffic of goods and animals, increasing the risk of introduction of infected vectors and reservoirs (Tatem and Hay, 2007). Recently hotspots for the introduction of zoonotic arboviruses in Europe were identified. Accordingly, Belgium presents high risk areas to the introduction of EEEV, VEEV and JEV (Durand et al., 2013).

The aim of this study was to provide a qualitative risk profiling of EEEV, WEEV and VEEV introduction in Belgium. These three viruses were chosen because they are representative of the New World Alphavirus group and different elements of their cycle are already present in Europe.

This study identified the vulnerabilities that can lead to the introduction of these three viruses.

Hazard identification

EEEV, WEEV and VEEV belong to *Togaviridae* family, genus *Alphavirus*. Alphaviruses are small, spherical, enveloped, positive sense ssRNA (Kononchik et al., 2011). The genome is composed by positive single-strand RNA, linear of approximately 11 kb in size containing two ORFs and it is organized in non-structural proteins (nsP1 to nsP4) at the 5' end and in structural proteins (C, E3, E2, 6K, and E1) at the 3' end (Simmons and Strauss, 1972, Kuhn, 2007, Hollidge et al., 2010). The RNA encodes four non-structural proteins involved in virus replication and pathogenesis and five structural proteins that compose the virion (Weaver et al., 2012). EEEV, WEEV and VEEV are considered such as New World viruses and their infected hosts may succumb with encephalitis (Powers et al., 2001). The viruses are transmitted by blood sucking arthropods (most of the time mosquitoes), and replicate in both arthropod and vertebrate hosts (Jose et al., 2009).

a. Eastern equine encephalitis

EEEV is probably the most pathogenic of the encephalitic alphaviruses and can be distinguished into two strains: the South American and the North American strains, the latter being more pathogenic for humans. These two strains may themselves be differentiated antigenically and genetically. Among the four groups of EEE viruses, group I is endemic in North America and the Caribbean, and is responsible for most human diseases. The other three groups (IIA, IIB and III) are primarily responsible for diseases in horses in South and Central America. The genotypes of the viruses from the North and South American EEEV strains have significant differences in their transmission cycle and their virulence (Arrigo et al., 2010, Arrigo, 2010). EEEV causes sporadic infections in the human population during the summer months and occasionally causes epidemics. Enzootic transmission cycle in North America involves birds and *Culiseta melanura*. The majority of the mosquito vectors implicated in the transmission of the South American EEEV strains, belong to *Culex* (*Melanoconion*) species, which have a broad host range (Arrigo, 2010). Unlike the North American strains, susceptibility of a vertebrate species as a preferred host for the South

American strain of EEEV has not been clearly established. Viral isolations and seroprevalence show that wild birds, rodents, marsupials and reptiles are susceptible to infection (Arrigo, 2010). Humans and horses are infected during outbreaks through bridge vectors but they do not develop a sufficient level of viremia to infect new vectors (Hassan et al., 2003).
Recently the South America strain was proposed to be classified as a distinct species called Madariaga Virus due to its genetic and biological characteristics. Evolutionary patterns of EEEV in North versus South America suggest ecological differences and taxonomic revision (Arrigo et al., 2010).

a. Western equine encephalitis

WEEV is found in the Western part of North and South America. The WEEV is a recombinant between Sindbis virus and EEEV (Weaver et al., 1997). WEEV has two transmission cycles. The main transmission cycle occurs in North America and remains between a vector, *Culex tarsalis* and a bird host. The birds include the *Passeriformes* order, pheasants and domestic fowl which have the highest level of antibodies against WEEV (Hardy, 1987). The secondary cycle of transmission occurs in South America and involves *Aedes melanimom* and a mammal and/or a rodent host (Pfeffer and Dobler, 2010). This cycle is less known but still seems to have an important role in the transmission of WEEV. Similarly, some species of reptiles such as turtles could play a role in the overwintering of the virus.

b. Venezuelan equine encephalitis

VEEV is an emerging and re-emerging neotropical pathogen of great importance for animal and public health. The VEEV virus complex includes at least 14 different subtypes and is divided into epizootic (types IA, IB and IC) and enzootic strains (type ID, IE, IF and II-IV) (Aguilar et al., 2011). A wide range of vectors may be involved in the transmission cycle and, as a consequence, VEEV epizootic strains can be opportunistic about their choice of potential vectors. Enzootic strains are almost exclusively transmitted by mosquitoes of the genus *Culex* and subgenus *Melanoconion* (Weaver et al., 2004b, Deardorff and Weaver, 2010). Wild rodents seem to be the main reservoir hosts for most endemic strains because they are frequently infected in the nature, have high rates of immunity, develop a moderate to high viremia after experimental inoculation and less or no apparent clinical signs (Weaver et al., 2004b, Weaver et al., 2004a, Deardorff et al., 2009, Pfeffer and Dobler, 2010)

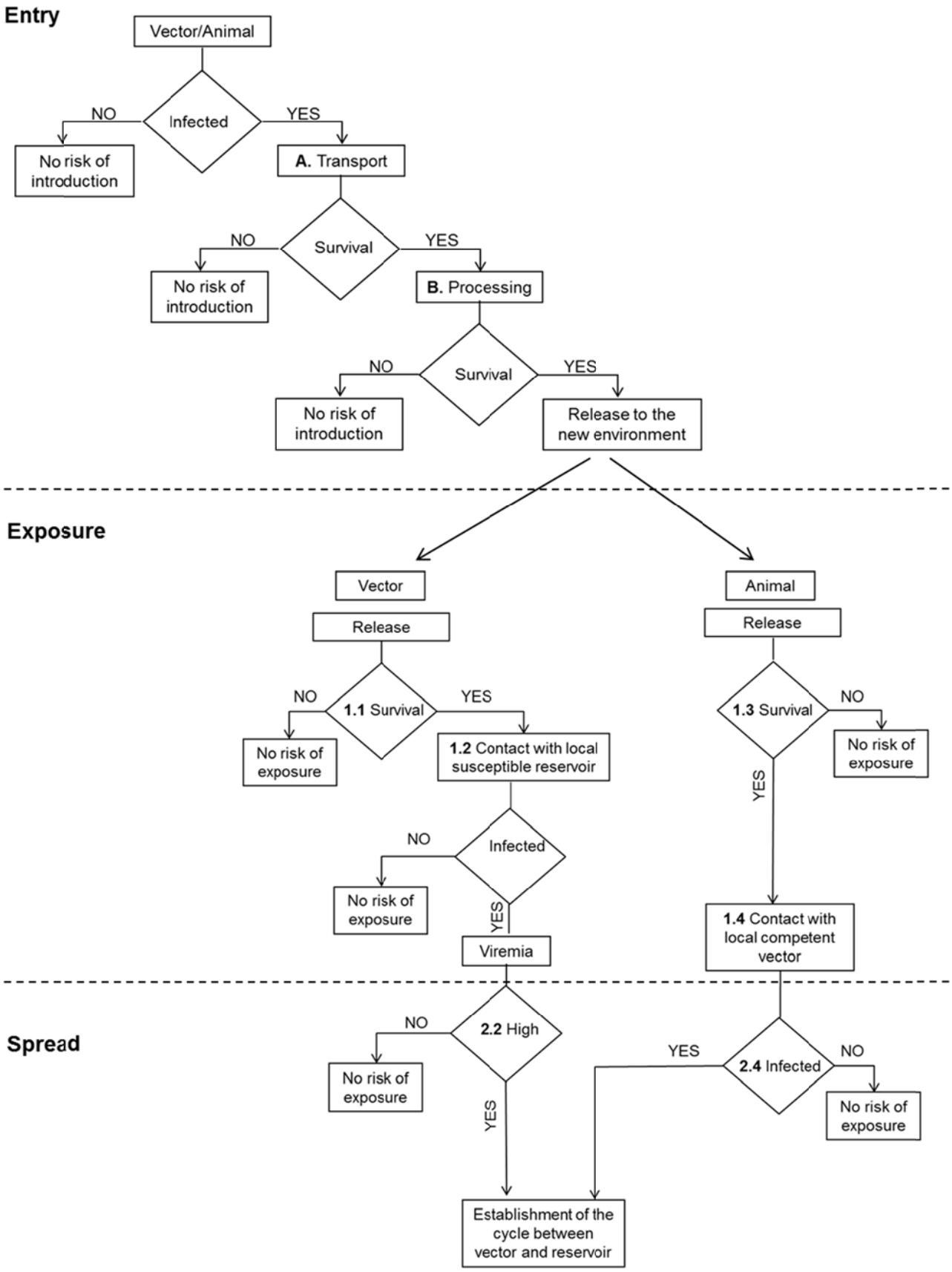

Figure 1 : Scenario tree for the introduction and exposure of EEEV, WEEV and VEEV into Belgium by the transportation of infected vector or host from endemic countries. A, B, 1.1 to 1.4 and 2.2 to 2.4 refere to the steps described in Table 4.

VEEV uses bats and shorebirds (herons) as alternative host reservoirs, with evidence of the involvement of bats in the spread of epizootic and enzootic infection (Seymour et al., 1978a, Seymour et al., 1978b, Ubico and McLean, 1995, Ferro et al., 2003, Weaver et al., 2004a, Weaver et al., 2004b). Horses are efficient amplifying hosts for epizootic strains of VEEV and a strong association exists between the importance of viremia in horse and the size of outbreaks (Weaver et al., 2004b, Pfeffer and Dobler, 2010). Finally, any major outbreak has never been reported in the absence of an infected equine population. Humans are also amplifying hosts for the virus but their lower exposure to mosquito bites makes them less efficient amplifying hosts than horses (Weaver et al., 2004b).

Risk profiling

a. Qualitative import risk assessment method

A qualitative import risk assessment (IRA) based on the recommendations of OIE terrestrial code was used here for the assessment of the risk profiling of introduction of these three viruses in Belgium. This risk assessment method includes 4 steps: entry assessment, exposure assessment, consequence assessment and risk estimation. In this work, the risk profiling focused on the two first steps of the risk assessment, namely introduction and exposure/spread. The risk of entry is identified as the probability of introduction of an infected vector or animal host in Belgium because they are the two main elements of the arbovirus cycle. Many parameters are considered such as the lifecycle of the vector or the conditions during transport. Exposure starts with the arriving of an element of the equine encephalitis cycle in Belgium (vector or animal host). The importance of the point of arrival of the infected element or the season of importation is discussed in this part. Virus spread is evaluated as the establishment of the viral cycle and virus dissemination in Belgium.

To describe the events that could lead to the introduction of the viruses, a scenario tree was build (Figure 1). Definitions of the different levels of probability and uncertainty for the different steps from the introduction to the spread of the viruses, are given in Tables 1 and 2.

Table 1. Definition of the different qualifications of probability (adapted from EFSA, 2010 and OIE 1999)

Qualification	Definition
Negligible	Probability of occurrence sufficiently low to be ignored or occurrence only possible in exceptional circumstances
Very low	The event would be very unlikely to occur but not excluded
Low	Occurrence of the event is a possibility in some conditions
Moderate	Occurrence of event is a possibility
High	Occurrence of event is clearly a possibility; expected to occur

Table 2. Definition of the different levels of uncertainty (EFSA, 2010)

Qualification	Definition
Low	Solid and complete data are based on convergent results of scientific studies or on a reliable data collection recognized system.
Medium	Some but not complete data available or limited number of scientific studies; authors report conclusions that vary or converge from one another.
High	Few or scarce data available or data collection system with a limited reliability; evidence is not provided in references but rather in unpublished reports, based on observations, or personal communications; authors reports conclusions that vary considerably between them.

b. Legislation for animal importation in Belgium (Europe)

Legislations for horse importation are found in the Terrestrial Animal Health Code of OIE (chapter 12.4 for EEEV and WEEV and chapter 12.11 for VEEV). The recommendations concern only the importations of horses and no specific legislation exists for other animal species. However, for birds, there are specific legislations concerning other diseases that could be useful for the prevention of these three viruses. Indeed, for WNV, strict rules (e.g. quarantine) are applicable for exporting countries depending on their disease status. Similar recommendations are described in the Terrestrial Animal Health Code for avian influenza and New castle disease viruses. CITES treaty resolutions provide strict measures for the importation of exotic species in Europe. The CITES text is applicable to threatened species but also for other exotic species.

All these regulations exist in the case of legal trade. The evaluation of illegal trade is very difficult to perform because the exact numbers are not known. However, the illegal trade could be an important source of introduction of the above mentioned diseases and is expressed here as uncertainty.

c. Entry

1. Country factors

The EEEV North American viral subtype I is endemic from Canada to Florida and outbreaks occur from June to November corresponding with the peak of vector activity (*Cs. Melanura*). South American strains of EEEV (IIA, IIB and III) are widely distributed in Central and South America (Figure 2) and epizootics can occur all year round in these countries. For both strains, the first signs of a new outbreak appear with the first equine clinical case. For that reason, horses could act as sentinel host for EEEV early detection in the surveillance program (Go et al., 2014, Weaver et al., 1999, Greenlee, 2014). Every year, during summer and autumn, presence of EEEV outbreaks in animals and/or humans, are notified to health authorities in the USA mostly in the East Coast.

WEEV have been detected from Canada to Argentina mainly in the western regions of Americas (Figure 2). The number of human cases was very high in the years 1940 to 1950 and has since heavily declined without a clear explanation (Forrester et al., 2008, Zhang et al., 2011).

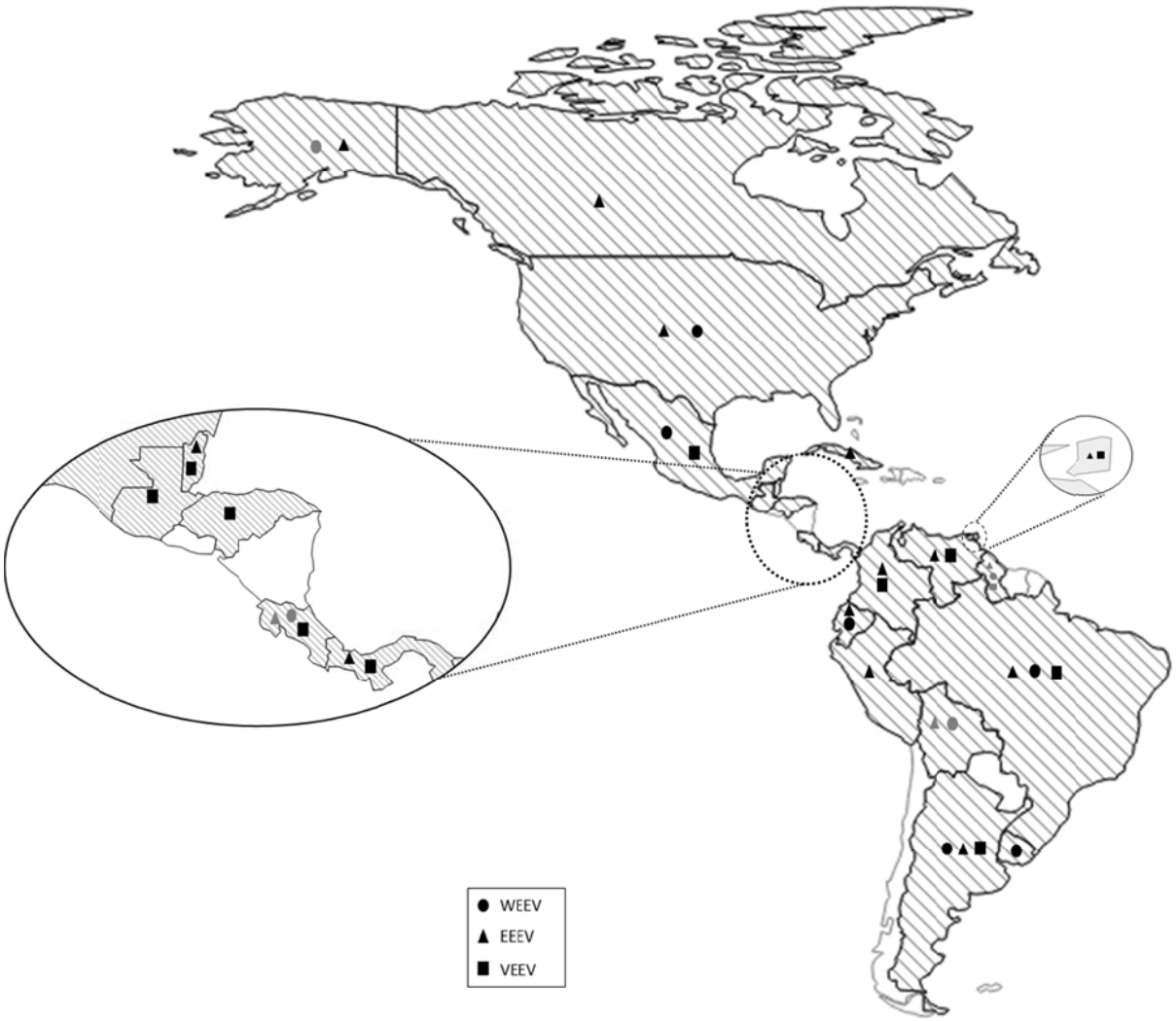

Figure 2 : Geographic distribution of EEEV, WEEV and VEEV in North, Central and South America. Triangles, squares and circles in black colour indicate confirmed cases while in light grey colour indicate suspected cases of American equine encephalitis virus infections. **(**Map build with Quantum GIS –QGIS Development Team, 2012. QGIS Geographic Information System. Open Source Geospatial Foundation Project. http://qgis.osgeo.org).

This decrease of infections could be due to a reduction in the virulence of currently circulating viruses in comparison with those circulating previously, to a better irrigation of agricultural land, and to an enhanced policy to fight against vectors (Barker et al., 2010). In comparison with EEEV, WEEV outbreaks occurred every 10 years with sometimes fatal issues in humans (Delfraro et al., 2011).

VEEV was isolated for the first time in 1938 in South America (Venezuela) (Weaver et al., 2004b). Seven antigenic complexes including enzootic and epizootic strains of VEEV were already isolated in several countries in Central (until South of Florida) and South America (Figure 2). The origin of epizootic strains is suggested by different hypotheses: maintenance of epizootic strains in a continuous cycle and maintenance in mammalian hosts (horse or alternative host), re-emergence of epizootic strains after administration of an incompletely inactivated vaccine and maintenance of endemic strains in the host reservoir population with periodic emergence of VEEV via mutation of those enzootic strains (mutation in the glycoprotein E2).

2. *Biological factors*

Introduction of American equine encephalitis viruses in Belgium can occur with the introduction of an infected vector or an infected host (Figure 1). Both circumstances are described here. These three viruses have a wide range of potential vectors with the majority of vectors belonging to the *Culicidae* family. The *Culicidae* life cycle takes place in four stages (egg, larvae, pupae and adult) and its length depends on intrinsic factors such as species variability and extrinsic factors such as temperature and humidity. Depending on species, each stage of the cycle could instigate an overwintering of the vector. Thus, when considering the risk of introduction of the vector, all stages must be taken into consideration and not only the adult mosquito according to the virus. Transovarial transmission is very difficult to identify and has not been described for the vectors belonging to *Culicidae* family and thus there is no overwintering of the viruses in such vectors (Burkett-Cadena et al., 2011). However, for *Culiseta Melanura*, a vector of EEEV, the eggs can become infected externally by passing through the infected oviducts (Morris and Srihongse, 1978). Eggs could be transported in used tyres as already observed for the (re-)introduction of *Aedes albopictus* or *japonicus* in Belgium (Boukraa et al., 2013, Schaffner et al., 2004).

A classical cycle of *Culicidae* lasts about 15 days from egg stage to adult with variations depending on the species and the environmental conditions. An adult mosquito has a life span of 15 days to 1 month depending on the gender (longer life for a female) and also according to environmental and species conditions. The female dies after 2-3 blood meals, being necessary for egg laying. The presence of the male is important because of its role in fertilization and thus it is considered rather for the risk of exposure (continuation of the cycle). A mated female ingests more blood than unmated female and therefore is a greater risk in viral transmission.

3. *Commodity factors*

Different scenarios must be considered for animal host introduction. Indeed, legal trade, illegal trade and accidental transport are three distinct situations. In legislation, only horse's importations are submitted to quarantine when they are imported from an endemic country (OIE, terrestrial manual, 2010). For other animal hosts, many efforts exist for the well-being of the imported species and for the preservation of endangered species (CITES conventions) but no prevention exists for diseases. The impact of illegal trade is difficult to estimate but is not negligible in regards with the risk of introduction of American equine encephalitis viruses. For these small animal species, the risk could be increased by the fact that the animal remains asymptomatic or show very few clinical signs. However, the introduction of these viruses by an infected host is limited by the very short duration of viremia (2-4 days). The duration of the quarantine (21 days) is thus sufficient to prevent the introduction (OIE, chapter 12.4 and 12.11). Although the transport time is short, it is still necessary that the host meets competent vector during viremia.

3.1 Transport

Several factors are involved in the emergence of diseases into a new territory. Among these factors the increasing number and velocity of people and animal movement between countries and continents play a crucial role in the introduction of diseases. Particularly for vector-borne diseases, transport of infected vector and infected animals, and susceptible vector should be considered. The introduction can occur by all kinds of transport: aircrafts, boats, land (rail and road) and wind.

Aircraft Transportation

Nowadays the transport by aircraft is the most important route of introduction due to the ability to transfer quickly both arthropods vectors and potentially infected animals. The rapidness of air transportation increases the chances of vector survival (Gratz et al., 2000) and consequently the risk of both new potential and viral infected vector introduction. Indeed, arthropod vectors are capable to remain for long periods inside the passenger cabins, passenger luggage, cargo holds and in the wheel bays even in flights between tropical and temperate regions (Russell, 1987). For instance, in the USA, 73% of pest detections occur in international airports, among these the fruit fly *Ceratitis capitata* can be easily found in the aircraft baggage, the plant pathogens in the air cargo and mosquito-vectors in the aircrafts cabins even in long flights (Tatem and Hay, 2007, Tatem et al., 2006, McCullough et al., 2006, Liebhold et al., 2006, Lounibos, 2002). Study carried on by Russell (1987) showed that the domestic mosquito (*Culex quinquefasciatus*), the house fly (*Musca domestica*), and the flour beetle (*Tribolium confusum*) could survive, under test conditions, from one to seven hour flights in the wheel bays between tropical and temperate airports (Russell, 1987). Risk assessment analysis of the introduction of WNV vectors mosquitoes in Barbados (Douglas et al., 2007), Galapagos (Kilpatrick et al., 2006), Hawaii (Kilpatrick et al., 2004) and United Kingdom (Brown et al., 2012) described that aircrafts represent the greatest threat to the introduction of the arthropods vectors. In case of transport of animals by air, there is a chance that infected animals are transported presenting clinical signs. For instance, for the equine encephalitis viruses this scenario could happen due to the relative long incubation period for EEEV (4-10 days), WEEV (5-10 days) and VEEV (2 -6 days).

Ship transportation

The entrance of an exotic disease through ship should be considered as important route as aircraft, for instance the Port of Antwerp, in Belgium, together with Rotterdam (Netherlands) and Hamburg (Germany), is considered one of the most active ports of the world.

The particularity of ship transportation is the long duration of the journey, which might allow transmission between an infected animal to a susceptible arthropod vector, as well as the establishment of invasive vector species into the ship environment. This situation could theoretically lead to vertical transmission within vector population and the infection of rodents present in the ship.

Indeed, vertical transmission has been demonstrated in *Aedes aegypti* mosquitoes infected with Dengue virus (Joshi et al., 2002) and CHKV (Agarwal et al., 2014) and suggested for WEEV (Fulhorst et al., 1994).
Moreover, the importance of shipping mosquitoes and viruses as a source of introduction has already been shown. The introduction of vectors via boats was previously described in the 30's, where *Anopheles gambiae* mosquitoes, the major malaria vectors, were introduced into Brazil via ship from Senegal (Gratz et al., 2000) and more recently, introduction was reported in Marseille (France) and Ghent (Belgium) with the detection of *Anopheles* mosquitoes and patients positive to malaria. These patients were residents from a port area where there was no previous description of the presence of the malaria vector, assuming that the mosquitoes were released in the ship dock of the port (Peleman et al., 2000, Delmont et al., 1994).

3.2 Processing

Once the infected animal or vector survives the transportation they should be able to survive the processing. The process of disinsection includes treatments with chemical insecticides /aerosol and quarantine in the importing country (Figure 1).

Chemical insecticides /aerosol

The process of aircraft disinsection is foreseen by international legislation according to regulations published in the International Health Regulations (IHR) from the World Health Organization (WHO) (Yu et al., 2014). IHR describes the meaning of disinsection as "the operation in which measures are taken to kill the insect vectors of human disease present in ships, aircraft, trains, road vehicles and means of transport, and containers" (Berger-Preiss et al., 2006, WHO, 1983).

Aircraft disinsection

The measures of aircrafts disinsection to avoid the entrance of insect vectors into countries began in the 20s and until today only 20 countries have adopted it. The disinfection will be applied in some flights depending on flight origin and route (Rayman, 2006).
For flight disinfection, WHO recommends procedures for passenger cabins and cargo areas (Russell, 1987) using aerosol spray preparations with different natural or synthetic chemicals like pyrethrins, resmethrin, *d*-phenothrin or permethrin (Berger-Preiss et al., 2006).

According to WHO, two groups of procedures must be followed: (1) spraying before or during flight using insecticide aerosol and (2) residual treatment. In the first group, the methods applied are the (a) "blocks away" where the interior of the aircraft is sprayed with insecticide aerosol before take-off with the passenger already boarded and the doors closed (Gratz et al., 2000, WHO/HQ, 1995, Sullivan et al., 1972, Sullivan et al., 1964); the (b) "pre-flight spraying" which consists of spraying the aircraft prior to passengers boarding and the (c) "top-of-descent spraying" which is an in-flight spraying before the aircraft reaches the airport (WHO/HQ, 1995). Despite the WHO recommendations and the methods that are recognized as reliable treatments (Berger-Preiss et al., 2006), the "blocks away" and "top-of-descent" methods, applied in the presence of passengers, may present a limited effect if its application is not well performed, once mosquitoes can keep alive inside the aircrafts (in the overhead luggage space or even inside the passenger's luggage) (Gratz et al., 2000, Russell and Paton, 1989, WHO, 1985, Goh et al., 1985).

An alternative method described as "pre-embarkation", suggests the spraying of the *d*-phenothrin aerosol under the seat rows and in a second step at the height of approximately 1.60 m by moving from frontside to backside of the cabin, in the absence of passengers and crew. The presence of *d*-phenothrin in the air was sufficient to kill flying insects like house flies (*Musca domestica*) and mosquitoes (*Aedes aegypti*, *Anopheles stephensi* and *Culex pipiens*) within 20 min. The method was 100% effective against insects in horizontal surfaces up to 24 h after spraying (Rayman, 2006, Berger-Preiss et al., 2006).

The residual treatment will be applied in internal spaces of the aircraft (excluding the food preparations areas) to prevent the infestation by insects that have reached the aircraft and be sheltered in these areas (WHO/HQ, 1995).

For cases of infestation by rodents and cockroaches, the fumigation methods may be applied leaving the aircraft out of service for seven to 15 hours (Gratz et al., 2000). These methods present pro- and contra- arguments regarding the real necessity of their application. For example, the USA discontinued the spraying routine since 1979, concerned by the health of the passengers and crew, and convinced that outbreaks occurring in countries of arrival are not related to imported vectors. By the other hand, Australia, New Zealand and India still maintain disinsection methods before landing (Berger-Preiss et al., 2004).

Ship disinsection

Although the importance of ship transports for the introduction of arthropods vectors, the methods of disinsection are not well established as well as for aircrafts. For ships, the quarantine and inspections should be done during movements of goods, however it is not an effective way to prevent the transport of vectors on tourist or local ships (Bataille et al., 2009, Causton et al., 2006). The prevention measures adopted should be related to the use of insect traps and the use of only yellow light during the night to avoid the attraction of flying insects to the ship, even so fumigation and disinsection methods applied to aircrafts should be used as models for surveillance and control measures (Bataille et al., 2009, Roque Albelo et al., 2006, WHO/HQ, 1995).

Quarantine

To avoid the entrance of new diseases, the processing control should be done not only in the means of transport controlling the presence of vectors but also to the imported animals that may carry enzootic diseases. The control of animals should follow the World Organisation for Animal Health (OIE) recommendations present in the Terrestrial Animal Health Code. When the concern is related to the risk of introduction of VEEV, EEEV and WEEV through importation of equines, the procedures recommended by OIE determine more specific actions based on the animal health prior the animal departure (OIE, 2014b, OIE, 2014c). Quarantine is applicable for horses in the case of VEEV only because they are a competent amplifying host during epidemics for VEEV only. For OIE, a country is considered free of VEEV if no confirmed case occurred during the past two years. A main recommendation for exporting horses from an infected country is the quarantine of three weeks in the country of origin in addition to 7 days of animal isolation in the importing country. Both these measures combined with other recommendations decrease significantly the risk of introduction by the importation of VEEV infected horses. Indeed, considering that the incubation period (1-5 days) and the infectious period (2 weeks), the three weeks of quarantine only are sufficient to reduce to a minimum the risk of introduction of an infected horse.

In general lines, all animals accepted into a new country must have an International Veterinary Certificate issued by the Veterinary authorities from their country of origin. When an animal is suspected of being affected by or infected with a disease capable of being transmitted to the animals or susceptible vectors in the new territory,

it should be placed immediately in quarantine to clinical observation and perform the laboratorial diagnosis to after that, follow the legal procedures (OIE, 2014a).
Quarantine and isolation are under official veterinary inspection and thus involve a good organization and training of the official veterinary authorities in both exporting and importing countries to detect any clinical signs during those periods.

d. Exposure

1. Biological factors

EEEV, WEEV and VEEV are RNA viruses which imply a high mutation rate due to the lack of a mechanism of correction by their RNA polymerase. In general, the point mutation rate for RNA viruses is one mutation per 10^4 to 10^5 nucleotides. Mutations in the viral genome can result in modification in the virulence and in the host or vector spectrum (Domingo, 2010).
The evolution of these three viruses already counts several mutations with high importance. Indeed, WEEV is a recombinant virus between EEEV and a Sindbis virus and epizootic strains of VEEV result from mutations in the genome of enzootic strains (Greenlee, 2014). For VEEV in particular, studies have implicated mutations in the E2 envelope glycoprotein as factor to become epizootic variants and to change this host range (Weaver et al., 2004a).
Taking into account these considerations about their viral genome, some mutations are expected to occur after the introduction of these viruses in Belgium.

2. Country factors

After the successful introduction of the infected vector / host, the transmission step begins. First, the exotic infected vector / host exotic has to survive in its new environment and second the transmission has to occur (Figure 1).
The vector survival will depend on several factors including the point of arrival and the season. The importance of the point of arrival is related to the accessibility to breeding sites depending on the need of the vector species. Temperature and humidity have an important role for its survival but also for its life cycle. Humidity plays a role in the development cycle since eggs need humidity to hatch and the larvae to become adults. Temperature is involved in the length of the extrinsic period (time between virus ingestion by the vector and sufficient replication in the vector to transmit the virus to a susceptible host). The availability of a sufficient population of susceptible (or competent) hosts in place and time to coincide with

Table 3 : Identification of potential vector species for American equine encephalitis viruses (EEEV, WEEV and VEEV) in Belgium

Vector	Indigenous	Virus
Culiseta morsitans	+	EEEV
Culex pipiens	++++	EEEV
Culex (Mel.) spissipes	+	VEEV
Aedes vexans	++	EEEV
Aedes cinereus	+	EEEV
Aedes aegypti	+	WEEV (ulc*)
Ixodes persulcatus	+	WEEV

*ulc : under laboratory conditions

the introduction and the biting activity of the vectors (Kuno and Chang, 2005) is essential for a successful viral transmission and for vector survival and reproduction. Host populations are variable according to the point of arrival. Some publications suggest that *Culicidae* could be carried by wind over a great distance (Calisher, 1994, Howard et al., 1989) and thus wind by the dispersal of exotic vectors allows the transmission of viruses over several miles around the point of entry and increases the availability of susceptible hosts. In Belgium, a large population of potential hosts is likely available for these three viruses, whether horses or other hosts such as birds, rodents and humans.

At the level of introduced host, the transmission could occur if the host is still developing a sufficient level of viremia to infect a vector. For these three viruses depending on host species, the duration of the viremia lasts from 3-4 days for mostly hosts to 100 days for reptiles under certain conditions. The short period of viremia involves that the vector population is abundant to increase the probability that the host transmits the infection to the vector. The European summer is than the perfect season to account for a maximum of available vectors (Vos, 2012) but climate changes could introduce new favorable conditions during other seasons.

Identification of competent vectors for these three viruses in Belgium is reported in Table 3. *Culex pipiens* is the most distributed mosquito in this country and EEEV has already been isolated from this species. Epizootic strains of VEEV are opportunistic in their use of mosquito vectors (Weaver et al., 2004b) and thus vector is not a limited factor in the spread of VEEV. Furthermore with the global warming, it is important to continue the surveillance and identification of new vector species in Belgium.

Some vertebrate hosts for these three viruses are also present in Belgium but further studies are needed to compare the competency of Belgian species (Supplementary table).

Table 4. Probability and uncertainty of the different steps from introduction to spread of EEEV, WEEV and VEEV viruses in Belgium, qualified by an expert opinion based on a consensus among the authors.

Step		Probability of the event occurring	Uncertainty
Introduction			
Vector	**A. Vector Survival after transport**		
	Aircraft	Moderate	Medium
	Boat	Low	Medium
	Others	Very low	High
	B.Vector survival after processing		
	Chemical	Low	Medium (aircraft) to high (boats and others)
Vertebrate host	**A. Animal survival after transport**		
	Aircraft	Low	High
	Boat	Low	High
	B.Animal survival after processing		
	Chemical	Low	High
	Quarantine (horses)*	Negligible	Low
Exposure			
1.1 Survival of the infected exotic vector in the new environment		Very low to low	Medium
1.2 Exotic infected vector bite endogenous host		Very low to Low	Medium
1.3 Survival of the infected exotic host in the new environment		Low to moderate	Medium
1.4 Exotic infected host is bitten by a endogenous vector		Low	High
1.5 Exotic host is bitten by an exotic vector		Very low	High
Spread			
2.1 Adaptation of the exotic vector species in the new environment		Very low	Medium
2.2 Endogenous host develop viremia		Very low	High
2.3 Adaptation of the exotic host in the new environment		Low	Medium
2.4 Endogenous vector become infected		Very low	Medium
2.5 Exotic vector become infected		Very low	High

*Quarantine probability is the probability that an animal remain viremic after the quarantine period

Discussion: risk profiling

The qualitative approach to estimate the risk of introduction of EEEV, WEEV and VEEV by the importation of live animals and vectors from North, Central and South America into Belgium is presented in Table 4 and follows the steps described in the scenario tree (figure 1). Belgium is in the heart of Europe and could be representative of Western European countries and particularly in relation with air-, boat- and road transport. The objective was firstly to highlight the factors associated with a higher probability (Table 4) and therefore the factors to be controlled during live animals importation or international exchanges, and then to qualitatively estimate the probability of establishing a transmission cycle for these viruses in Belgium.

The entry of a vector is composed by two equally important steps. Indeed, the vector has to survive to the air, boat or other transports, but also have to survive to the different steps of the processing applied in these different types of transport. The probability of vector survival to the transport is estimated very low to moderate depending on the type of transport. Several studies have shown that the survival of vectors in air transport was common, and trade of used tyres is responsible for the introduction of *Aedes albopictus (Takumi et al., 2009, Boukraa et al., 2013)*. Estimation of the probability of vector survival to processing is low, but is dependent on the policy of the airline and also on the respect of the application of the insecticide by the crew. Indeed, no legal rules exist for this processing.

Regarding the introduction of live animals, firstly, the quarantine of horses (OIE Terrestrial Manual) from the Americas is a very effective measure against the introduction of these viruses, the risk of introduction was considered negligible. However, quarantine is applied for the three viruses but only the epizootic strains of VEEV use the horse (and human) as amplifying hosts. For other livestock trades such as poultry, there are no specific rules of importation for these three viruses. However, for the prevention of the introduction of other viruses such as Newcastle and Avian A Influenza viruses, a quarantine of 3 weeks is applied.

This measure could therefore indirectly help for the prevention of EEEV and WEEV viruses entry. Exotic animals are protected by international conventions like CITES with a primary objective to protect these species but little (or nothing) to prevent and control infectious diseases. In the context of legal imports, survival to processing should not be considered, indeed, the aim is to keep the animal alive until the final destination. Regarding the illegal trade or accidental introduction of vertebrate species by transports, the uncertainty is high and it is difficult to perfectly assess the risk of introduction. However, despite the limited amount of available data (Rosen and Smith, 2010), it can be hypothesized this type of import only concerns a minority of importations. The risk of introduction of these species was assessed low, such as their survival to the processing either in the cargo aircrafts or on dock boats. The probability of animal survival to both transport and processing is estimated low and not very low because species like rodents or reptiles (turtles) are considered resistant species and only one infected host individual is enough to have a successful introduction of the virus.

In this study, only the risk of introduction by direct transport from the Americas to Belgium is considered. Therefore it is possible that the risk is higher if indirect transports are taken into account namely the introduction through neighboring countries (road) or passive transport of vector through wind as already shown for other viruses such as BTV-8 and AHSV-9 (Maclachlan and Guthrie, 2010, Johnson et al., 2012). Concerning the introduction of the New World Alphaviruses (EEEV, VEEV and WEEV) in Belgium the wind could be considered less important as they have not been detected in the Old World so far (Go et al., 2014), however it may be an important pathway for transporting vectors into the continent (Kilpatrick et al., 2006).

After surviving to transport and processing, the vector has to survive into a new environment and a new climate. The probability of vector survival is estimated from very low to low depending on the vector species, its stage of development and its ecological preferences. The season of arrival in Belgium is also very important for its survival. Indeed, an adult mosquito survives at 10 ° C and larvae restart their development from 15 ° C. Similarly the probability of vector biting an indigenous vertebrate host is estimated from very low to low depending on trophic preferences of the vector species and host availability in the point of entry of the vector. For example, *Cx pipiens* feeds mainly on birds, *Ae dorsalis* in mammals and *Ae albopictus* is opportunistic in its host choice vertebrate.

The probability of survival for a vertebrate host is higher than for a vector and is estimated from low to moderate. On the other side, the probability that the imported vector bites the imported vertebrate host is estimated very low. This particular case could only happen if they arrive almost together at the same entry point and at the same time.

The first consequence of the successful introduction of the vector is its adaptation to the new ecological environment. In addition, the probability of complete adaptation is estimated very low as observed, for instance for exotic mosquito species recorded in Belgium *Ae.albopictus*, are so far not established even if they have been found by trapping during several years (Boukraa et al., 2013). However climate changes with higher temperatures could increase this probability and even an increase in viral persistence could be observed. Several studies have shown that global warming shortens extrinsic incubation period (EIP) in the vector (Johnson et al., 2012).

The probability that the vertebrate host develops a sufficient viremia to infect a vector is estimated very low. Indeed, despite a broad host range for these viruses, only a few species of birds, rodents and reptiles serve as a reservoir host for the virus, the others being considered as dead- end hosts. An exception should be noted for the VEE virus for which horses and humans being efficient hosts in epidemic conditions.

The adaptation of a vertebrate host to its new environment is considered here more likely (low) that for the vector (very low). Indeed, in the past, several invasive species have already shown their ability to adapt and reproduce in a new environment in Belgium (http://ias.biodiversity.be/species/all). Regarding EEEV, WEEV and VEEV, the period of infectivity last about 14 days (OIE, Terrestrial Manual) with an incubation period of 5-14 days for EEEV and WEEV and 1-5 days for the VEE virus and a viremia period lasting less than a week (except in reptiles with EEEV, viremia up to 100 days post-infection). The probability of infection of an indigenous vector by the infected vertebrate host introduced is estimated very low in regards with the short viremia.

So far the virus has not been identified in Belgium, but it does not mean that attempts of a viral introduction did not occur. These failed attempts, if any, were not observed. Several hypotheses could explain this situation. So far no other alphavirus was isolated in Belgium. This argument, however, could be plausible in other regions (meaning that would possess other alphaviruses). The phenomenon of overwintering is less plausible for exotic vectors than for indigenous vectors. Although these diseases are notifiable diseases, they are subject only to passive surveillance in horses, which limits the possibilities of identification.

The probability of introduction of these three viruses is estimated from very low to moderate (Table 4) but all elements of their cycle are present in Belgium. The use of horses as sentinel around the points of entry such as ports and airports could be used as part of an active localized surveillance such as already done in Americas or Canada (Rocheleau et al., 2013). This would involve training of all public health sectors, starting with veterinarians. In addition to veterinary surveillance, it would be useful to improve the knowledge of medical practitioners to include these diseases in their differential diagnosis of human encephalitis.

This risk profiling is valid for the current period. Climate change could lead to changes in the evaluation of risk factors, including the easiest survival of exotic vectors. Although the economic aspects have not been addressed in this study, the consequences of a successful introduction of these three viruses would be severe in regards with the economic importance of the horse industry in Belgium. The knowledge of the severity of these consequences could help to precisely measure the risk of introduction. Belgium is situated in Western Europe and shares with surrounding European states and the risk factors that have been discussed here. The conclusions of this work could be therefore extended to other European countries possessing a significant equine industry. Furthermore, except for quarantine which is well documented, the level of uncertainty was qualified as medium or high for the other risk factors, requesting therefore specific scientific works dedicated to gain a better knowledge of the various factors involved in the introduction and spread pathways of EEEV, WEEV and VEEV.

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Supplementary Table : Identification of animal host species for American equine encephalitis in Belgium

Class	Order	Family	Sub-family and Genera	Virus
Mammals	*Rodentia*	Muridae		EEEV VEEV
		Sciuridae		WEEV VEEV
	Artiodactyla	*Cervidae*		EEEV
	Lagomorpha	*Leporidae*	Lepus europaeus*	EEEV, WEEV*
	Chiroptera			VEEV
	Perissodactyla	*Equidae*		VEEV
	Primates	*Hominidae*	Human	VEEV
	Didelphimorphia			VEEV
Birds	Passeriformes	*Troglotytidae,Parulidae, Emberizidae, Turdidae, Paridae*		EEEV
		Vireonidae	*Vireo olivaceus#*	EEEV
		Sturnidae	*Sturnus vulgaris*	EEEV
		Passeridae	*Passer domesticus*	EEEV, WEEV
		Fringillidae		WEEV
		Emberizidae	*Zonotrichia leucophrys#*	WEEV
		Icteridae		WEEV
	Cuculiformes	*Cuculidae*		EEEV
	Galliformes	*Phasianidae*	*Phasianus colchicus*	EEEV
			Domestic chick	
	Strigiformes	*Strigidae*	*Strix aluco*	EEEV
	Ciconiformes	*Ardeidae*		EEEV, VEEV
		Threskiornithidae	*Plegadis falcinellus#*	EEEV, VEEV
Reptilia	*Chelonii*	*Cheloniidae*	*Trachemys scripta*	EEEV, WEEV
	Squamata	*Viperidae*		EEEV
		Colubridae		EEEV
		Lacertidae		WEEV
Amphibia	*Anura*	*Ranidae*	*Rana Catesbeiana*	EEEV

* Only for WEEV and # Migratory species accidentally or exceptionally present in Belgium

Annexe 2

Diversity and ecology survey of mosquitoes potential vectors in Belgian equestrian farms: a threat prevention of mosquito-borne equine arboviruses arboviruses

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Abstract

Emergence of West Nile Virus was recently recorded in several European countries, which can lead to severe health problems in horse populations. Europe is also at risk of introduction of mosquito-borne equine alphavirus from Americas. Prevention of these arboviruses requires a clear understanding of transmission cycles, especially their vectors. To characterize mosquito fauna, their ecology and identify potential vectors of equine arboviruses in Belgium, entomological surveys of six equestrian farms located in the Wolloon Region were conducted during 2011-2012. The harvest of mosquitoes was based on larval sampling (272 samples from 111 breeding sites) and monthly adults trapping (CO2-baited traps, Mosquito Magnet Liberty Plus). Among 51493 larvae and 319 adult mosquitoes collected, morphological identification showed the presence of 11 species: *Anopheles claviger* (Meigen), *An. maculipennis s.l.* (Meigen), *An. plumbeus* (Stephens), *Culex hortensis* (Ficalbi), *Cx. territans* (Walker), *Cx. pipiens s.l.* L., *Cx. torrentium* (Martini), *Coquillettidia richiardii* (Ficalbi), *Culisata annulata* (Schrank), *Oc. cantens* (Meigen), *Oc. geniculatus* (Olivier). Molecular identification of *Cx. pipiens* species complex allowed the detection of three molecular forms, Pipiens (92.3%), Molestus (4.6%) and Hybrid (3.1%). Larvae of *Cx. pipiens sl* and *Cx. torrentium* were omnipresent and the most abundant species. Water troughs, ponds and slurry (liquid manure) were the most favorable breeding sites of mosquito larvae. Based upon behavior and ecology of the identified mosquito species, equestrian farms seem to provide a suitable environment and breeding sites for the proliferation of potential vectors of arboviruses and those being a real nuisance problem for horses and neighboring inhabitants.

Keywords: Belgium; *Culex*; Equine arbovirus; Horse; Mosquito; West Nile Virus

Introduction

Viruses transmitted by hematophagous arthropods (arboviruses) represent a threat for animal and human health with the increase of their emergence outside the endemic areas. Environment and climate change, as well as intensification of international trade could favor the re-emergence of vector-borne diseases (Gould and Higgs, 2009). In terms of morbidity and mortality, mosquitoes are the most dangerous animals confronting mankind (Becker et al., 2010). Among the hundreds viruses that can infect humans and animals, several are transmitted by mosquitoes such as some of Togaviridae (Chikungunya, Sindbis, Equine Encephalitis alphaviruses and Ross River virus), Flaviviridae (Yellow fever, Dengue, West Nile Fever, Japanese and St. Louis encephalitis viruses) and Bunyaviridae (Rift Valley fever) (Goddard, 2008). Mosquitoes are considered as the main vectors of some arboviruses that affect horses and that cause serious diseases in humans during epidemics. Eastern Equine Encephalitis virus (EEEV) is an *Alphavirus* that belongs to the family Togaviridae as Western equine encephalitis virus (WEEV) and Venezuelan equine encephalitis virus (VEEV). The EEEV is the most pathogenic arbovirus endemic in the Americas and can be distinguished into two strains: South American and North American, the latter being more virulent for humans. The EEEV causes sporadic infections in the human population during the summer months and occasionally causes epidemics (Zacks and Paessler, 2010). For the horse, clinical signs are non-specific and last 1-2 weeks, after which the outcome is either recovery or death. There is no available treatment and the only protection against the disease remains the vector control and vaccination (Davis et al., 2008). This arbovirus has been isolated from 20 mosquito species including *Aedes vexans* (Meigen), *Ae. albopictus* (Skuse), *Culex pipiens s.l.* L., *Culiseta morsitans* (Theobald), and *Cx. territans* Walker (all: Diptera, Culicidae) that can be found frequently in Europe (Scott and Weaver, 1989 and Pfeffer and Dobler, 2010). In America, the majority of mosquito vectors of EEEV have been identified as belonging to the genus *Culex* (*melanoconion*), which has a wider host range (Arrigo, 2010).

Western equine encephalitis virus (WEEV) is found in the Western part of North America and South America. The WEEV is a recombinant virus between Sindbis virus and EEEV (Weaver et al., 1997). Despite the decrease in the number of cases since the 1950s, WEEV is always a risk to human and equine populations. As for the other encephalitis alphaviruses, WEEV causes non-specific clinical signs of fever, meningitis or encephalitis according to the immune status and age of the infected individual.

No therapy is available and the only way to fight is also the routine immunization of horses and the vector control (Davis et al., 2008 and Zacks and Paessler, 2010). The WEEV has two transmission cycles; the main transmission cycle (North America) is maintained between *Cx. tarsalis* (Coquillett) (Diptera: Culicidae) and host birds. The secondary cycle of transmission (South America) involves *Ae. melanimon* Dyar (Diptera: Culicidae) and host mammals and / or rodents (Pfeffer and Dobler, 2010). *Aedes melanimon* acts as a bridge vector to transmit the disease to humans and horses, as *Ae. dorsalis* Meigen (Diptera: Culicidae) and *Ae. campestris* (Dyar & Knab) (Diptera: Culicidae). Others vector species have been tested in laboratory conditions: *Cx. quinquefasciatus* (Say) (Diptera: Culicidae), *Cx. pipiens,* and *Ae. vexans* (Pfeffer and Dobler, 2010) .

Venezuelan equine encephalitis virus (VEEV) is an important emerging and re-emerging pathogen of horses and humans in South America. During epidemics, the lethality mortality rate varies from 19 to 83% of horses, while for humans, the mortality is less common but important neurological complications are frequent. Epizootic strains are opportunistic for the choice of potential vectors. A wide variety of vectors may be involved as *Psorophora confinnis* (Lynch Arribalzaga), *Ps. columbiae* (Dyar & Knab), *Oc sollicitans* (Walker), or else *Oc. taeniorhynchus* (Wiedemann) (all: Diptera: Culicidae) whose was considered as the primary vector during an epizootic (Weaver et al., 2004 and Aguilar et al., 2011). Enzootic strains are almost exclusively transmitted by mosquitoes of the genus *Culex* subgenus *Melanoconion* (Weaver et al., 2004). *Aedes albopictus* is an efficient vector of VEEE under laboratory conditions (Aguilar et al., 2011).

West Nilevirus (WNV) is an arthropod-borne virus worldwide distributed belonging to Flaviviridae and to *Flavivirus* genus. This arbovirus is maintained in an enzootic cycle with birds that serve as amplification reservoir hosts and mosquitoes as the principal transmitting vectors. Humans and horses are susceptible hosts and can develop clinical illness but are considered dead-end hosts because they do not frequently produce sufficient viremia to infect mosquitoes, and therefore, do not contribute to the transmission cycle (Campbell et al., 2002 and Hayes et al., 2005). West Nilevirus was recognized as a cause of central nervous system infections such as meningitis, encephalitis and myelitis (Campbell et al., 2002). The clinical signs in horses are most commonly ataxia, weakness, and changes in mental state. However, the majority of equines are very susceptible to WNV infection, which can be responsible for encephalomyelitis in a fraction of infected animals, and may evolve into fatal encephalitis (Campbell et al., 2002 and Venter et al., 2009).

Mosquitoes in the genus *Culex* have been widely implicated as primary vectors of WNV (Becker et al., 2010 and Calistri et al., 2010). Despite the low vector competence of these species to transmit the WNV, other factors such as mosquito density, biting preference and seasonal activity makes *Culex* species the most important mosquito genus in WNV transmission (Kilpatrick et al., 2005). *Culex univittatus* (Theobald) (Diptera: Culicidae) is considered the primary vector in Africa (Hubalek and Halouzka, 1999) and *Cx. vishnui* Theobald (Diptera: Culicidae) in Asia. In the northeast America, *Cx. pipiens s.l.* is regarded as the most important vector of WNV and in the southeast the subspecies *Cx. quinquefasciatus* is considered as an important vector. *Culex tarsalis* takes place as the primary vector in the west American (Campbell et al., 2002 and Kilpatrick et al., 2005). In Europe, WNV has been detected from largely ornithophilic mosquitoes especially *Cx. pipiens s.l.*, *Cx. modestus* (Ficalbi), *Cx. torrentium* (Martini), *Coquillettidia richiardii* (Ficalbi), and occasional vectors such as *Ochlerotatus cantans* (Meigen) and *Anopheles maculipennis s.l.* (Meigen) (all: Diptera: Culicidae) (Engler et al., 2013). *Aedes albopictus* is also a competent vector of WNV and has been demonstrated experimentally that can infect horses (Bunning et al., 2002).

Since that WNV was first detected in New York in 1999, more than 26581 horses (period, 1999–2013) were affected in the United States (http://www.aphis.usda.gov/vs/nahss/equine/wnv/wnv_distribution_maps.htm), with many horses being euthanized because of grave prognosis indicating that several infections were fatal (Sebastian et al., 2008). A strong resurgence of horse and human cases of WNV was recorded in Europe in the last decade, including Hungary, Italy, southern France, Portugal, Romania, Spain and more recently Greece (Engler et al., 2013 and Di Sabatino et al., 2014). The main route of introduction of WNV in the regions of Europe with a temperate climate is most likely by infected migratory birds from Africa (Hubálek and Halouzka, 1999 and Calistri et al., 2010). Moreover, WNV could be introduced in Western Europe by birds arriving from central Europe (Koopmans et al., 2008).

West Nile virus circulation in Europe is confined to two basic types of cycles and ecosystems. The first belongs to rural cycle where wetland birds were usually serve as amplification hosts and ornithophilic mosquitoes such as *Cx. pipiens pipiens* as vectors. The second is the urban cycle with synanthropic or domestic birds as amplifying hosts and mosquitoes feeding on both birds and mammals, mainly *Cx. p. molestus* (Hubalek and Halouzka, 1999). Environmental factors, including human activities that enhance vector population densities (irrigation, heavy rains followed by floods, higher than usual temperatures, and formation of ecologic niches

enabling the mass breeding of mosquitoes) allow the reemergence of this mosquito-borne disease (Hubalek and Halouzka, 1999). An understanding of arboviral transmission cycles begins with the correct identification of potential vectors and thorough knowledge of their bioecology in the environment. The goals of this work were to determine diversity, abundance, and seasonal dynamics of potential mosquito vectors for equine arboviruses in equestrian farms in the Walloon Region of Belgium. Additionally, this study is intended to be useful in assessing the risk that those mosquito species and their breeding sites represent for transmission of equine arboviruses in Belgium.

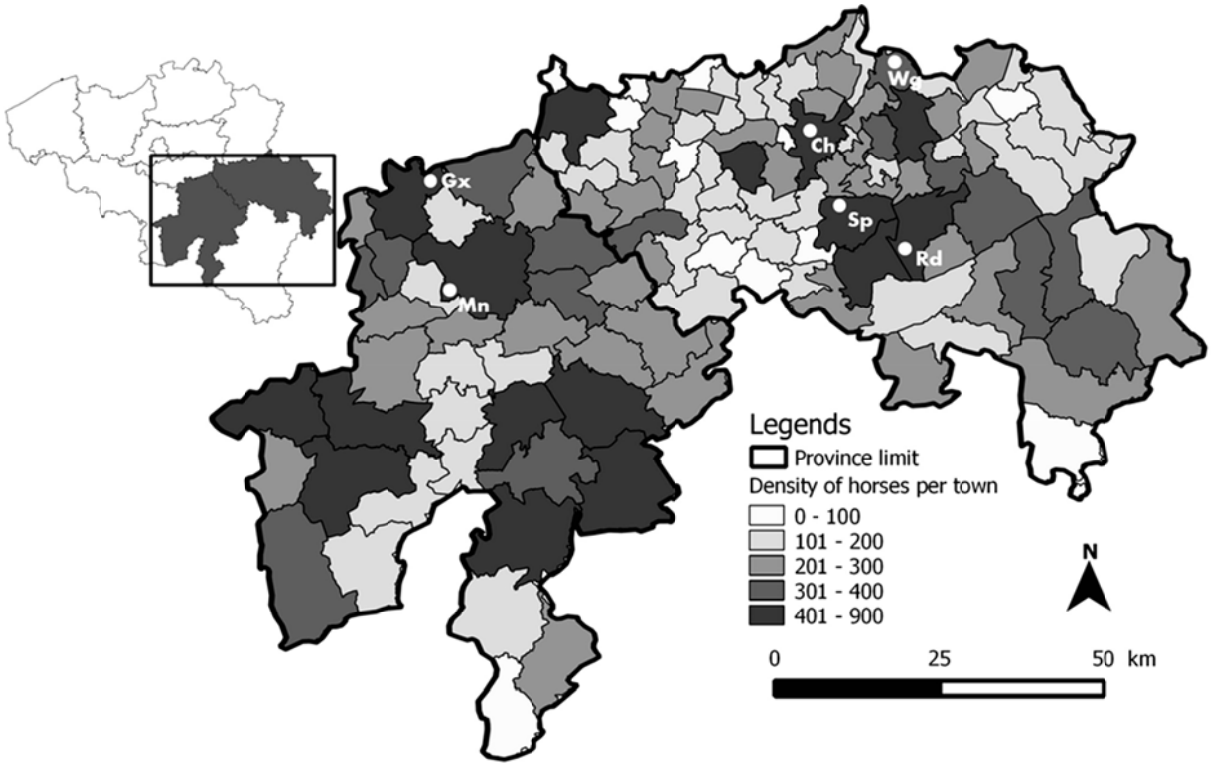

Figure 1. Map of horses density by localities showing the six sampling sites [Warsage (Wg), Chênée (Ch), Sprimont (Sp), La Reid (Rd), Gembloux (Gx), Malone (Mn)] in the two Provinces: Liège (on the right) and Namur (on the left).

Material and methods

Study sites

The present study was performed in 2011-2012, with the aim to investigate the mosquito fauna species and their bioecology in the equestrian farms. The study was conducted in six locations in Belgium belonging to Namur and Liège Provinces (Figure 1). Each of the six equestrian farms selected contain a hundred horses and had two types of environments: horse livestock in the buildings of the farms (stables) and surrounding grasslands. In addition to the presence of many breeding larval sites in and around stables studied, the farms were also close to forests and streams, which give humid environment and provide suitable conditions for the proliferation of mosquito species. Localities of the equestrian farms inspected are: Gembloux (50°34'40"N, 4°44'40"E; 33 m above sea level), Malonne (50°27'06"N, 4°48'15"E; 85 m a.s.l.), Warsage (50°43'38"N, 5°47'57"E; 223 m a.s.l.), Chênée (50°37'00"N, 5°37'58"E; 151 m a.s.l.), La Reid (50°28'51"N, 5°46'38"E; 372 m a.s.l.), and Sprimont (50°32'46"N, 5°40'16"E; 274 m a.s.l.). Weather data (precipitation and temperature) were obtained from local meteorological stations of Royal Meteorological Institute (RMI®) and were presented by Ombrothermic diagram for each of the localities of equestrian farms (Figure 2).

Mosquito collection

Larvae sampling

Larvae sampling is realized in all potential larval habitats reported after inspection of each study site and its surroundings (a circle of radius of 250 ± 50 m). All sites have been investigated 4 times/year, in 2011 (June, July, August and October) and 2012 (June, July, August and September). Larvae were collected by using the dipper method made-up by a metal pan (500 ml). However, the smallness of some breeding sites such as the temporal puddles and the inaccessibility of others sites like used tires had necessitated the use of an aquarium net 12 X 10 cm to inspect them and collect immature stages. We collected in several places of breeding sites to obtain homogeneous samples (4-10 times). In the case of breeding sites which are formed by tires, the sample is composed by the sum of 4-10 tires selected on all tires placed in the same location (heap of tires). Data sheets of physical and biological characteristics were designed for each breeding site, to collect all possible data such as: type (artificial/natural), kind (buckets/tires/water troughs/puddle/pond/streams), sunshine (full/partial/shadow), state water (permanent/temporary, standing/running), water depth and vegetation situation (presence/absence of floating and/or emergent vegetation) (Figure 3).

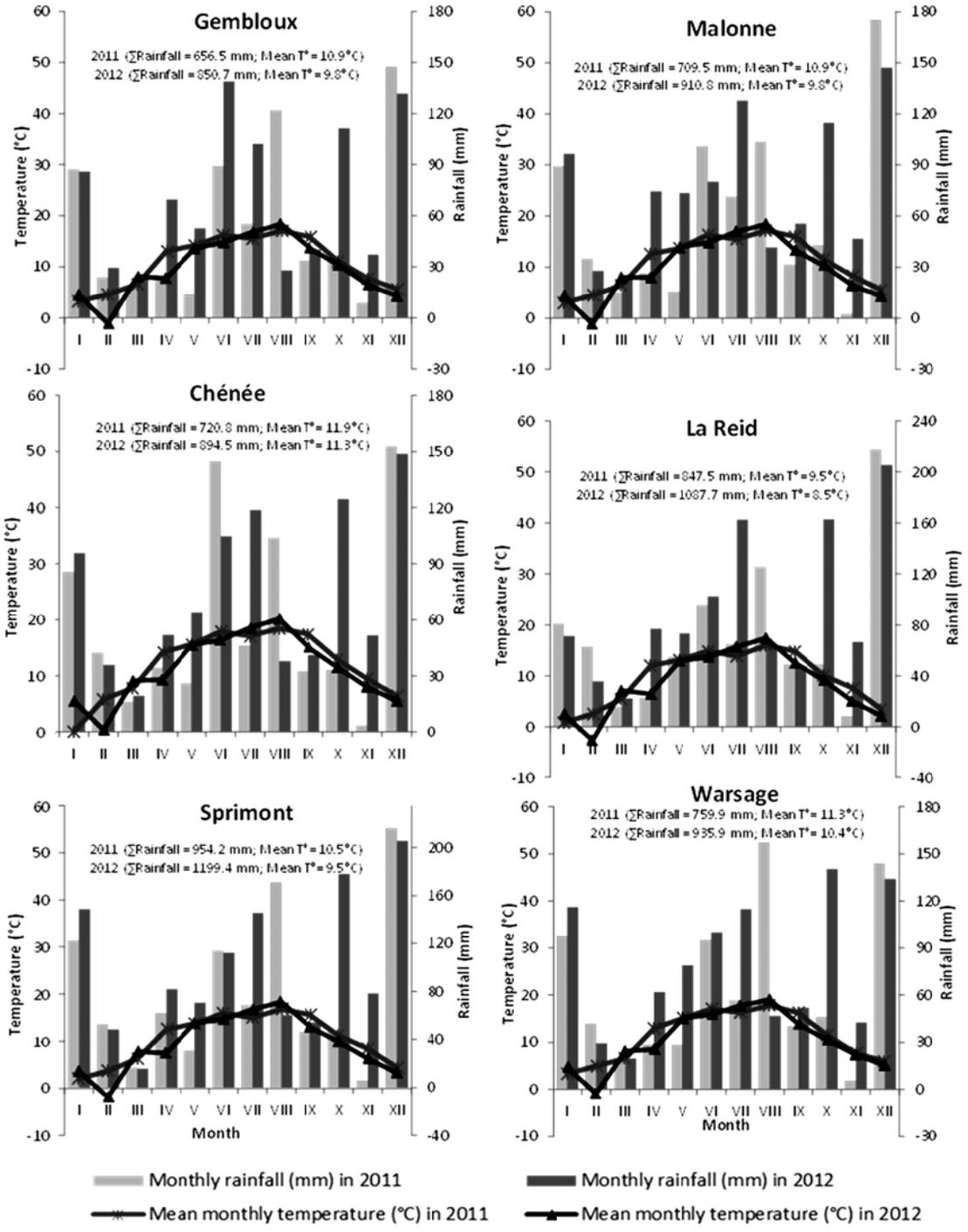

Figure 2. Weather data (ombrothermic diagrams) of each studied station in 2011 and 2012.

Figure 3. Some examples of positive breeding sites of mosquito larvae in the equestrians farms inspected: a, Water troughs; b, Puddle behind stables (farm); c, Tarpaulin cover; d, Obstacle of cross country races (water obstacle); e, Septic of horse washing residues; f, Watercourse (in grassland); g, slurry (or liquid manure); h, Pond (in grassland); i, Drain behind stables; j, Tractor rut; k, Used tires; l, Puddle and horse hoofprint (grassland).

Chemical analysis of the water samples including salinity, pH, conductivity, oxygen and temperature were also measured and recorded directly in the field using a Multiparameters analyzer (C562 Consort nv®). Each larval habitat was geo-referenced (Google Earth Pro, GeoBasis-DE/BKG 2009) and encoded in its data sheets. The samples of the same breeding sites are then mixed, reduced to two liters of each one by sifting the excess water with a dip net of fish and finally placed in individual plastic containers with caps and labeled. A total of 123 and 149 samples were collected from the six equestrian farms in 2011 and 2012 respectively. Once in the laboratory, all larvae and pupae were counted. After counting, a half of each sample (larvae and pupae) was placed in emerging boxes (21 cm X 15 cm X 20 cm) in order to obtain adults males (in particular) that were required to confirm identification by analyzing of the genitalia of some species such as *Cx. pipiens* s.l. and *Cx. torrentium*. The fourth larva stages were recovered from the second half of the sample, and the younger larvae were reared in boxes to collect gradually the fourth stages. These were preserved in 75% ethanol in small flasks (50 ml) labeled with date, study site, number and code of the breeding site.

Adult trapping

Adult mosquitoes were collected monthly in the study sites from June till October in 2012 using the CO_2-baited traps, Mosquito Magnet Liberty Plus® (MMLP). The sampling was realized simultaneously in the six equestrian farms where each trap operated three days in one study site. The traps have been installed on farms and were located less than three meters of the stables in such a way to not disturb the entry and exit of horses. Once in the laboratory, mosquitoes captured in the net of the trap were collected using an entomological mouth aspirator, killed by freezing and stored with silica gel in -20 °C. Same techniques were used for adults who are gradually recovered from the emergence boxes of the larvae sampled in field.

Morphological and molecular identification

In the laboratory, the fourth larval stages and the male genitalia were dissected using a stereomicroscope (20-40 X, magnification), mounted between slide and coverslip and examined under a microscope (100-400 X, magnification). Sampled mosquito species identification was based on morphotaxonomy analysis, by using the electronic identification key of Schaffner et al. (2001) and dichotomous identification keys (Becker et al., 2010).

Because of the remarkable morphological resemblance between *Cx. pipiens* and *Cx. torrentium* the separation at larval stage or adult females is very delicate. For this purpose, morphological identification of these two species has been based much more on the examination of the male genitalia, which carries most discriminating and distinguishing characters. Regarding the species of the tribe of Aedini and their recent reclassifications, we maintained in this article the use of the traditional names of species, with the only exception of the elevating of *Ochlerotatus* to genus (Reinert, 2000). Members of the *Anopheles maculipennis* complex have not been differentiated in this study and are cited as *An. maculipennis s.l.* However, the molecular study was performed to identify the different forms of *Cx. pipiens* complex. Total DNA from 126 individual mosquitoes (4th stage larvae), selected from six more abundant breeding sites of *Cx. pipiens* complex was extracted with the DNeasy Blood & Tissue kit (Qiagen), according to the method recommended by the manufacturer and diluted in a final volume of 70 µl of TE. Purity and quantity of DNA then checked with Nanodrop (NanoDrop ND-1000 Spectrophotometer, Isogen®) and finally stored at -20 °C. A multiplex PCR assay which targeted the region of a microsatellite locus (CQ11) was used with 60 ng of genomic DNA. Sets of primers were composed by the specific reverse primer for Molestus: molCQ11R 5'-CCCTCCAGTAAGGTATCAAC-3', for Pipiens: pipCQ11R 5'-CATGTTGAGCTTCGGTGAA-3', and the common forward primer: CQ11F2 5'-GATCCTAGCAAGCGAGAAC-3' (Bahnck & Fonseca, 2006). Reactions were performed in 50 µl volume containing 2 µl of 60 ng of DNA template, 5 µl of Dream Taq PCR buffer 1X (Fermentas, St. Leon-Rot, Germany), 1 µl of 1 mM of each primer (molCQ11, pipCQ11 and CQ11F2), 1 µl of 0.2 mM dNTP and 0.5 µl of Dream Taq (Fermentas, St. Leon-Rot, Germany). PCR amplification conditions were 5 min at 94 °C for the initial denaturation, followed by 40 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 40 s and a final extension at 72 °C for 5 min. Aliquot of 10 µl of the amplification products were run by electrophoresis on a 2% agarose gel stained with SYBR® Safe DNA (Invitrogen) at 90 V for 30 min, then photographed with Gel Doc 2000 system (BioRad, USA). Selected samples of positive CQ11 PCR amplifications were purified and sent for sequencing to GATC Biotech (Germany) (www.gatc-biotech.com). The sequences results were compared with data available in NCBI (www.ncbi.nlm.nih.gov) and then deposit in GenBank.

Diversity and Statistical analysis

The results obtained on the survey of Culicidae in Belgian equestrian farms are treated with ecological indices of species diversity and similarity between different study sites. We expressed the species richness (S), relative abundance ($p_i = ni / N$ where ni = number of the i-th species and N is the total number of individuals in the studied station) and frequency of occurrence (Constancy) of species in each study sites. Constancy (C) was calculated with the index $C = P \times 100 / N$, where P is the number of surveys (larval samples) containing the analyzed species and N the total number of surveys. Then it was categorized into five classes using Surge's rule (Scherrer, 1984): Number of classes (N.C.) = 1 + (3.3 log 10 P'), where P' is the total number of inventoried mosquitoes in the study sites. In this study, the following groupings for species categories were proposed: $0 < C \leq 26\%$ accidental (A); $26\% < C \leq 54\%$ infrequent (I); $54\% < C \leq 80\%$ constant (C) and $80\% < C \leq 100\%$ for omnipresent (O) species. In parallel, larval abundance per station and per year was classified into four categories as low (<10 larvae present), intermediate (11-100 larvae present), high (101-500 larvae present), or very high abundance (>500 larvae present). In each study site, Shannon-Weavers's diversity index (H') and Evenness (J') were calculated. The first, $H' = - \Sigma p_i \log_2 p_i$ (where pi the relative frequency of species i-th in the studied habitat) was used to measure and evaluate heterogeneity of mosquito species diversity for each of the six equestrian farms. Evenness (J') or equitability = H' / H_{max} (where $H_{max} = \log_2 S$) was calculated to measure the distribution of individuals within species irrespective of species richness. It varies from 0, if only one species dominates, to 1, if all species show similar abundance (Silver, 2008). The degree of similarity/dissimilarity between study site compared to mosquito species was expressed by index (Qi) and distance (Qi_δ) of Jaccard [$Qi = a / (a + b + c)$ where a is number of species commons to both sites; b and c are number of species absents in one of the two sites] [$Qi_\delta = 1 - Qi$] (Silver, 2008). Jaccard's distance and dendrogram were carried out in R, version 3.1.0 (R Core Team 2014). The mean abundance per species was treated with an analysis of variance (ANOVA-1) with equestrian farm stations (q = 6) and months (n = 4). When assumptions of the one-way ANOVA (normality of populations and homoscedasticity) were not met, Kruskall-Wallis's test was applied. All statistical analyses were performed using Minitab® software version 16. In all the cases, results were considered as statistically significant when the p-value was lower than 5% ($p < 0.05$).

Table 1. Numbers and percentages of sampling of larval abundances per station in 2011 and 2012.

Station	Very high abundance		High abundance		Intermediate abundance		Low abundance		No. Samples	
	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012
Gembloux	4 (16.0%)	3 (10.0%)	8 (32.0%)	5 (16.5%)	8 (32.0%)	10 (33.3%)	5 (20.0%)	12 (40.0%)	25	30
Malonne	0 (0.0%)	2 (7.7%)	2 (10.0%)	8 (30.8%)	16 (80.0%)	12 (46.2%)	2 (10%)	4 (15.4%)	20	26
Chênée	4 (19.0%)	2 (6.5%)	11 (52.4%)	2 (6.5%)	5 (23.8%)	17 (54.8%)	1 (4.8%)	10 (32.3%)	21	31
Warsage	0 (0.0%)	1 (6.7%)	7 (50.0%)	4 (26.7%)	7 (50.0%)	7 (46.7%)	0 (0.0%)	3 (20.0%)	14	15
Sprimont	0 (0.0%)	3 (14.3%)	10 (50.0%)	8 (38.1%)	9 (45.0%)	6 (28.6%)	1 (5.0%)	4 (19.0%)	20	21
La Reid	4 (17.4%)	4 (15.4%)	7 (30.4%)	4 (15.4%)	8 (34.8%)	14 (53.8%)	4 (17.4%)	4 (15.4%)	23	26
Totals	12 (9.8%)	15 (10.1)	45 (36.6%)	31 (20.8)	53 (43.1%)	66 (44.3)	13 (10.6%)	37 (24.8)	123	149

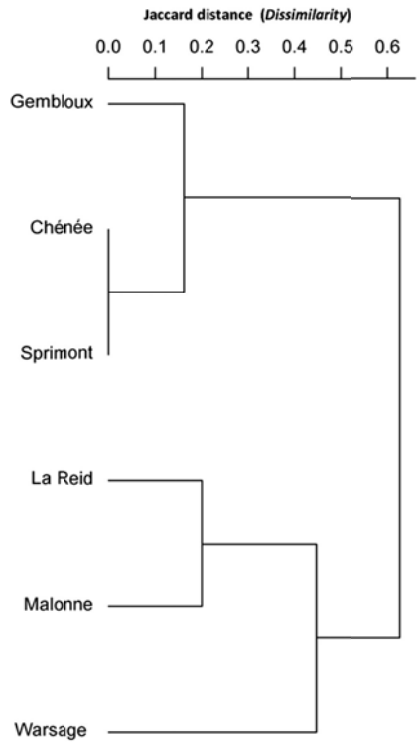

Figure 4. Cluster analysis of mosquito fauna, by Jaccard's distance between equestrian farms studied in 2011 and 2012.

Results

In total, 123 and 149 larval samples were collected from the six equestrian farms in 2011 and 2012 respectively. Samples with intermediate larval abundance presented 43.1% (2011) and 44.3% (2012), followed by high abundance with 36.6% (2011) and 20.8% (2012) of all samplings per year (Table 1).

A total of 51493 larvae and 319 adults were collected, morphological identifications showing the presence of 11 species: 7 obtained by larval sampling and 10 by adult trapping (Table 2 and Table 3). Among the larval species identified in all equestrian farms, *Cx. pipiens s.l.* (68.0% in 2011; 79.1% in 2012) and *Cx. torrentium* (22.4% in 2011; 15.5% in 2012) were the most abundant species. In parallel, the two most abundant adult species were *Cq. richiardii* (54.2%) and *Cx. pipiens* s.l./*Cx. torrentium* (35.7%). Morphological differentiation between adult females of *Cx. pipiens* s.l. and *Cx. torrentium* was not possible and these two species were referred to as *Cx. pipiens* s.l./*Cx. Torrentium*.

All specimens belonging to the *An. maculipennis* species complex were not identified to the species level. However, molecular identification of *Cx. pipiens* species complex using multiplex PCR based on the microsatellite locus CQ11 allowed to detect the presence of three molecular form, *Cx. pipiens* form *pipiens*, *Cx. pipiens* f. *molestus* and *Cx. pipiens* f. hybrid (heterozygotous). Among the 126 specimens selected for molecular study, only 65 PCR gave positive results. Sixty individuals belonged to *Cx. pipiens* f. *pipiens* (92.3% of positive PCR) only 3 to *Cx. pipiens* f. *molestus* (4.6%), and 2 to *Cx. pipiens* f. hybrid (3.1%). Nucleotide sequences of 10 *Cx. pipiens* f. *pipiens* and 2 *Cx. pipiens* f. *molestus* are deposited in GenBank under accession numbers KP666140, KP675968-KP675972, KP675976, and KP675978-KP675980 for Pipiens form and KP675973 and KP675982 for Molestus form.

Taxonomic diversity differed among the six equestrian farms and years (2011 and 2012) (Table 2). Richness and Shannon-Wiener diversity index were both higher in Sprimont (2011) and Chênée (2012) where S = 7, H' = 1.53 and S = 7, H' = 1.6, respectively. These two stations were very close (≈ 8.3 km) and similar (Qi = 100%; Qi_{δ} = 0.0%). During larval sampling for both years, lowest species richness was observed in Warsage (only 3 species were identified). The lowest similarities of Jaccard were observed between all pairs of Warsage and each of others stations (Qi < 60%) (Figure 4). Even so, highest evenness were highlighted that in this station, the three present species were in equilibrium between them and there was not a clear dominance in 2011 (J = 0.66) and 2012 (J = 0.86). The evenness value in 2011 was also high in Malonne (0.71) and Chênée (0.68).

Table 2. Taxonomic diversity; Specific richness, S; Relative abundance, RA%; Frequency occurrence (constancy), Co; and diversity index (Shannon-Wiener diversity, H'; Evenness, J) of mosquito larvae per sampling station in 2011 and 2012.

2011	Gembloux RA% (Co)	Malonne RA% (Co)	Chênée RA% (Co)	Warsage RA% (Co)	Sprimont RA% (Co)	La Reid RA% (Co)
Cx. torrentium[a]	10.60 (O)	18.70 (O)	21.30 (C)	63.60 (C)	62.60 (O)	8.40 (C)
Cx. territans	-	-	-	-	1.30 (I)	-
Cx. pipiens s.l.	74.00 (O)	71.60 (O)	65.80 (C)	34.70 (C)	20.30 (O)	90.90 (O)
Cx. hortensis	-	-	3.90 (I)	-	0.10 (A)	-
Cs. annulata	6.50 (A)	9.60 (O)	-	-	3.80 (C)	0.60 (I)
An. claviger	3.50 (I)	-	-	-	0.04 (A)	0.02 (A)
An. maculipennis s.l.	5.50 (C)	-	9.00 (C)	1.70 (A)	11.80 (I)	-
Total of specimens collected	6412	1379	5766	1847	2644	6845
Species Richness (S)	5	3	4	3	7	4
Shannon-Wiener diversity (H')	1.32	1.12	1.37	1.05	1.53	0.48
Evenness (J)	0.57	0.71	0.68	0.66	0.55	0.24
2012						
Cx. torrentium[a]	9.60 (C)	8.50 (O)	16.50 (C)	32.50 (I)	26.00 (O)	12.80 (O)
Cx. territans	0.02 (A)	-	0.10 (A)	-	0.10 (A)	-
Cx. pipiens s.l.	88.50 (O)	86.40 (O)	62.50 (O)	55.60 (O)	71.50 (O)	86.50 (O)
Cx. hortensis	-	-	9.60 (C)	-	0.90 (I)	-
Cs. annulata	0.40 (A)	4.70 (O)*	0.70 (I)	-	0.80 (I)	0.50 (C)
An. claviger	0.50 (C)	0.40 (I)	0.40 (A)	-	-	0.04 (A)
An. maculipennis s.l.	1.00 (C)	-	10.10 (O)	11.90 (A)	0.60 (A)	0.20 (A)
Total of specimens collected	4397	6299	2903	2054	4758	6182
Species Richness (S)	6	4	7	3	6	5
Shannon-Wiener diversity (H')	0.62	0.73	1.60	1.36	1.03	0.62
Evenness (J)	0.24	0.36	0.57	0.86	0.40	0.27

[a] Identification of this species especially based on male genitalia after emerging in laboratory; (A) Accidental; (I) Infrequent; (C) Constant; (O) Omnipresent; * Value of density of this species differ significantly between the stations ($H_{(5)}$= 11.76; P = 0.038)

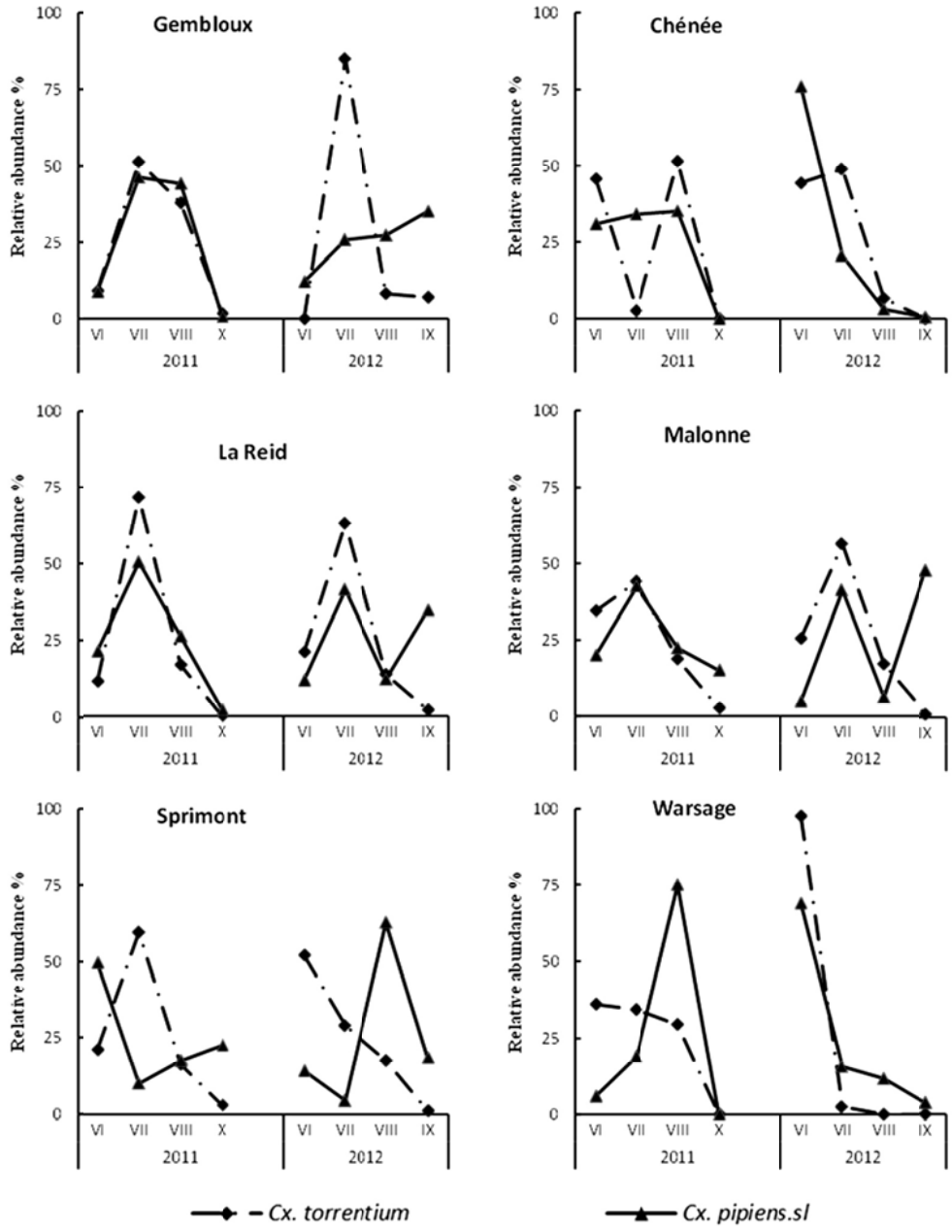

Figure 5. Seasonal abundance (Relative abundance %) of *Cx. pipiens s.l.* and *Cx. torrentium* larvae in equestrian farms in 2011 (June, July, August & October) and 2012 (June, July, August & September).

However, in 2012, *Cx. pipiens s.l.* was strongly dominant (up to 88.5% in Gembloux) and evenness values were low in Gembloux (0.24), La Reid (0.27), Malonne (0.36), and Sprimont (0.40) (Table 2).

Table 3 shows that some species have a high ecological plasticity and their larvae can develop and encounter a wide range of artificial or natural/semi-natural breeding sites. There were in particular, the larvae of *Cx. pipiens s.l.*, *Cx. torrentium,* and *Cs. annulata*. However, other species are more related to characteristic ecological features of some natural breeding sites (such as *An. claviger* and *Cx. territans*). At the level of six equestrian farms studied, water trough, ponds, and purin (liquid manure) were the most favorable habitats in development of mosquito larvae. The highest mosquito diversity was observed in ponds (all species) and water trough (6 species). Of all the breeding sites (111) sampled in studied stations, 100% contained larvae of *Cx. pipiens s.l.*, *Cx. torrentium*. The highest larval densities for these two species for artificial breeding sites occurred in water troughs, puddles, and abandoned buckets. For natural/semi-natural breeding sites, the highest larval densities occurred in ponds and liquid of manure (slurry) (Table 4).

Figure 5 presents seasonal dynamics (relative abundance) of *Cx. pipiens s.l.* and *Cx. torrentium* larvae in the six equestrian farms in 2011 (VI, VII, VIII and X) and 2012 (VI, VII, VIII and IX). The relative abundance of these mosquito larvae was expressed as the mean number of larvae in each month by the total number of larvae of the species in each station. Seasonal dynamics of these two species showed different variations depending on stations and months. In most stations, the relative abundance of *Cx. pipiens s.l.* and *Cx. torrentium* follows a classic thermophilic trend. However, variations and heterogeneity of the dynamics were recorded mainly in 2012. The calculation of Pearson correlation coefficients (PCC) shows that there is a significant relationship between the relative abundance of *Cx. pipiens s.l.* larvae and the mean temperature in Chênée in 2011. The correlation coefficient of 0.972 ($p = 0.028$) reflects indeed a strong positive linear relationship between the sampled larvae and the mean temperature measured in this station. Strong correlations were also obtained in 2011 [Chênée: *Cx. torrentium* (PCC = 0.914; P = 0.086) and Warsage: *Cx. torrentium* (PCC = 0.937; P = 0.063)] and 2012 between larval abundance of both species and mean temperatures. In parallel, the PCC shows a significant correlation between the relative abundance of *Cx. torrentium* and rainfall in 2012 [Chênée: PCC = 0,988; P = 0.012 and La Reid: PCC = 0,976; P = 0.024]. There were no significant relationships between relative abundance of both species and rainfall in 2011 and they also were remained low for most stations in 2012.

Table 3. Relative abundance (%) of adult mosquitoes collected using Mosquito Magnet

Species	Gembloux	Malonne	Chênée	Warsage	Sprimont	La Reid
*Cx. pipiens s.l./Cx. torrentium**	84.0	76.9	76.2	63.6	38.9	3.4
Cx. hortensis	-	-	4.8	-	-	-
Cs. annulata	4.9	15.4	9.5	18.2	-	0.6
An. claviger	4.9	7.7	4.8	-	-	3.4
An. maculipennis s.l.	-	-	4.8	9.1	-	-
An. plumbeus	-	-	-	9.1	-	-
Oc. cantens	-	-	-	-	-	1.1
Oc. geniculatus	3.7	-	-	-	-	-
Cq. richiardii	2.5	-	-	-	61.1	91.4
Number of specimens collected	81	13	21	11	18	175

Liberty Plus® in equestrian farms studied in 2012**.**

*Morphological identification between females (majority of specimens) of *Cx. pipiens s.l.* and *Cx. torrentium* was not possible, because characters that are used to separate these species are damaged or not visible.

Table 4. Mosquito larvae densities sampled according to breeding sites in all studied equestrian farms.

Breeding sites	*Cx. torrentium*	*Cx. territans*	*Cx. pipiens s.l.*	*Cx. hortensis*	*Cs. annulata*	*An. claviger*	*An. maculipennis s.l.*
Artificial							
Water trough (20 nbc)	2877	0	7340	458	462	135	697
Tarpaulin cover (1 nbc)	76	0	44	0	5	0	0
Abandoned Bucket (17 nbc)	2305	0	2395	0	32	0	49
Tank of rainwater collection (1 nbc)	211	0	166	0	0	0	264
Used tires (10 nbc)	848	0	749	0	179	20	3
Fossa (washing horses) (2 nbc)	4	0	71	0	1	0	0
Abandoned pool (2 nbc)	131	0	368	0	63	0	0
Puddle (2 nbc)	699	0	3382	1	0	0	331
Natural and Semi-natural							
Drain (11 nbc)	309	0	98	0	31	0	0
Watercourse (6 nbc)	159	1	723	0	54	111	32
Pond (25 nbc)	1218	42	13154	94	226	1	345
Slurry (liquid manure) (12 nbc)	860	0	9359	0	48	16	116
Horse hoofprint (2 nbc)	5	0	118	0	0	5	0
***Total* (111 nbc)**	9703	43	37968	553	1101	288	1838

nbc: Number of breeding sites collected

Discussion

Compared with 2011, the number of collected mosquitoes in the six equestrian farms studied (Gembloux, Malonne, Chênée, Warsage, Sprimont, and La Reid) was nearly identical that in 2012. In both years, most samples were characterized with intermediate and high abundance of larvae. Eleven species were encountered during the present study on Belgian equestrian farms, whereas 30 confirmed species have been recorded on the entire country (Dekoninck et al., 2011a and Versteirt et al., 2013). Only seven taxa were identified by larval morphotaxonomic. Adult morphotaxonomic and genitalia analysis have allowed to identify four others species which are *An. plumbeus*, *Oc. cantens*, *Oc. geniculatus,* and *Cq. richiardii*. Romi et al. (2004) also recorded eleven species in a study on potential vectors of WNV following an equine disease outbreak in Toscana region (Italy).

Among the identified larvae in all equestrian farms, *Cx. pipiens s.l.* accounted for more than 73% of the mosquitoes collected during both studied years. Sebastian et al. (2008) found similar results and confirmed that *Cx. pipiens* is the most abundant species found during an entomological investigation of WNV epidemic in a horse farm. Molecular identification of *Cx. pipiens* species, allowed detecting the presence of three molecular forms (Pipiens, Molestus, and Hybrid). Pipiens form constituted itself more than 92% of positive selected specimens. Similar results were found by Osório et al. (2014), where they found that the majority of specimens of *Cx. pipiens s.l.* were identified as *Cx. pipiens* f. *pipiens* and followed with less importance by *Cx. pipiens* f. *molestus*, and hybrid forms. Rudolf et al. (2013) also confirmed that the predominant species in Germany are *Cx. pipiens pipiens* biotype *pipiens* and *Cx. torrentium*. In temperate areas, *Cx. pipiens s.l.* can be subdivided into two distinct biotypes, which are morphologically indistinguishable but differ greatly in physiology and behaviour. The biotype *pipiens* is mainly ornithophilic, anautogenous, and eurygamous, whereas the biotype *molestus* is mammalophilic, autogenous, and stenogamous (Becker et al., 2010 and Osório et al., 2014). Hybridization between the both forms can also be found in sympatric with the two distinct biotypes *pipiens* and *molestus* (Bahnck and Fonseca, 2006). This hybrid form has been observed in different countries in Europe (Reusken et al., 2010 and Osório et al., 2014). The hybrids showed opportunistic feeding behaviour and therefore can bite both birds and mammals. Its behaviour and ecological flexibility to grow in the same habitats of *pipiens* and *molestus* forms may serve to be as important bridge vectors for the transmission of arboviruses from infected birds to mammals, and thus complicating the epidemiologic landscape (Reusken et al., 2010 and Osório et al., 2014).

Many researches demonstrated that *Cx. pipiens* is one of principal vectors of WNV in Europe (Hubalek and Halouzka, 1996 and Becker et al., 2010), including in Italy where it was suspected to play a major role in the transmission of this infectious agent in horse farms (Romi et al., 2004).

Culex torrentium was also largely abundant and omnipresent in the most equestrians farms studied. Morphological differentiation between larvae of *Cx. pipiens s.l.* and *Cx. torrentium* was very delicate and even impossible between females of these both species. For that reason, we based much more on male genitalia examinations, which were considered as a good discriminatory character between these two species (Schaffner et al., 2001 and Becker et al., 2010). Others researches confirmed that this species is widespread and common in Europe where it is dominant in northern areas (Rudolf et al., 2013 and Hesson et al., 2014). Despite the ignorance of a vectorial role of *Cx. torrentium* relative to the transmission of WNV, its abundance and co-dominance alongside of *Cx. pipiens* in central and northern Europe require deepening taxonomic studies to avoid identification mistakes and determine the true vectors. In parallel, it is also advisable to carry out local studies on vector competence and capacity of *Cx. torrentium* for WNV and other arboviruses (Weitzel et al., 2011 and Hesson et al., 2014).

We confirmed the presence and establishment of *Cx. hortensis*, recently identified for the first time in Belgium by the MODIRISK (Versteit et al., 2012). In this study, *Cx. hortensis* was found in two localities (Chênée and Sprimont) with both the larval and adult stages. Larvae of this species were sampled primarily in the water troughs in grasslands and less important at the ponds level. Larvae of *Cx. hortensis* may develop in varied breeding sites with presence or not of plants such as cemented drinking troughs, puddles in a torrent-bed, ponds sides, and drain pipes (Schaffner et al., 2001). *Culex territans* was found only in the larval stage and only in the natural/semi natural breeding sites (ponds and streams). Becker et al. (2010) noted that preferred larval habitats of *Cx. territans* are permanent bodies of water such as ponds, swamps, pools along streams, edges of lakes, or slow streams. Just like the females of *Cx. hortensis*, *Cx. territans* feeds especially on batrachians and reptiles. Both species have never been involved in the transmission of human pathogens (Schaffner et al., 2001). However, recent research suggests that some snakes and amphibian species were found competent hosts for EEEV. In addition, some snakes may represent an overwintering host and can support long-term virus replication, especially at low temperatures (White et al., 2011). Burkett-Cadena et al. (2008) demonstrated that *Cx. territans* can also feed on birds and mammals what

constitutes the possibility that this species may be important in the transfer of arboviruses to and from ectotherms and other host groups. The competency of *Cx. territans* for EEEV is unknown to this day (White et al., 2011), but this mosquito was found to be infected by this arbovirus (Burkett-Cadena et al., 2008). *Culex territans* can therefore be considered as potential and probable vector of EEEV in the case of its overgrowth and accidental biting of horses.

For adult trapping, *Cq. richiardii* was the most abundant species in equestrian farms, but this abundance may be explained by the studied station "La Reid", where a marsh with water plants was present around the farm. These aquatic plants are essential for the biology of *Cq. richiardii* immature stages. Becker et al. (2010) noted that breeding sites of this species may be various permanent water bodies where larvae and pupae live submerged and obtain oxygen from the aerenchyma of aquatic plants. In addition, the females can cause a severe nuisance to humans and domestic animals in the surroundings of fresh waters or slightly saline marshes, lakes, and estuaries (Becker et al., 2010). Few adult specimens of *An. plumbeus* were captured in equestrian farm (only in Warsage). However, a recent data indicated a strong population expansion of this potential vector of *Plasmodium falciparum* (Haemosporida: Plasmodiidae) and WNV all over Belgium and demonstrated its adaptation to man-made artificial breeding sites (Dekoninck et al., 2011b).

In the majority of breeding sites found in the equestrian farms studied, *Cx. pipiens s.l.* and *Cx. torrentium* were the most frequent mosquito larvae collected, where they co-occurred in the majority of artificial and natural/semi-natural breeding sites sampled. Higher number of mosquito species coexisted in water troughs, used tires, ponds, and watercourses compared with other breeding site types. Our results suggest that water troughs and ponds are numerous and more productive habitats for *Cx. pipiens s.l.* and *Cx. torrentium*, along with abandoned buckets, puddles, and liquid manures (slurry). Although slightly less abundant, *Cs. annulata* preferred similar habitats. Very wealthy in organic matter, liquid manure (or slurry) which flow from the dung heaps was one of the most favorable breeding sites for the development of *Cx. pipiens s.l.* larvae. Elimination and cleaning of this breeding site may therefore play an interesting role in the reduction and control of nuisance caused by *Cx. pipiens s.l.* None breeding site was found inside of the studied stations for the four species *An. plumbeus*, *Oc. cantens*, *Oc. geniculatus,* and *Cq. richiardii* that were trapped only in adult stages. Despite the availability of favorable breeding sites for the development of *Aedes*/*Ochlerotatus* larvae, no species of this genus were identified in the larval stage.

In parallel, equestrian farms studied appear to contain various favourable habitats to both *pipiens* and *molestus* forms, and therefore a high-level of hybridization outcome of interbreeding between both forms. Further detailed researches are needed on local ecology (breeding sites, seasonal dynamics, and feeding behaviours) of the three identified forms of *Cx. pipiens* complex for understanding and assessing epidemiological risks of potential arboviruses such as WNV and equine encephalitis viruses.

In this study, seasonal dynamics of *Cx. pipiens s.l.* and *Cx. torrentium* larvae showed different variations based on stations and months. In most stations, larval abundance of *Cx. pipiens s.l.* and *Cx. torrentium* follows a classic thermophilic trend and a strong relationship between this abundance and the mean temperature measured in some equestrian farms was observed. However, variations and heterogeneity of the dynamics were recorded mainly in 2012. While no direct mechanism may be determined from this study, we hypothesize that human intervention is behind the deletion and cleaning of some artificial breeding sites, which were identified as suitable for proliferation of mosquitoes. This was the case of water troughs in grasslands where horse breeders were informed that these breeding sites may be suitable for the development of mosquito larvae. Several studies demonstrated that high temperatures appeared to favor high mosquito larval abundance and shorten their development time, especially for larvae of some *Culex* species (Gardner et al., 2012). This abiotic factor may have significant implications for WNV activity and increases also the ability of *Cx. pipiens* as vector to transmit this arbovirus (Dohm et al., 2002). In parallel, only larval abundance of *Cx. torrentium* was influenced by the precipitation in equestrian farms of Chênée and La Reid. In general, there were no significant relationships between larvae abundance of *Cx. pipiens s.l.* and *Cx. torrentium* and rainfall during the study period in equestrian farms. Several studies of WNV and their vectors have found positive associations between precipitation, human cases and larval abundance of *Culex* species as vectors. Others have indicated that an excess of rainfall may actually limit WNV incidence and vector production (Gardner et al., 2012). Gardner et al. (2012) demonstrated that low rainfall promotes high larval abundance while high rainfall was associated with absence of larvae. These authors assumed that high rainfall flushed *Culex* larvae out of their breeding sites and prevented also adult females from laying egg rafts until water flow slowed.

Although some of our equestrian farms studied had international activities including receiving horses from some countries of Eastern Europe, no exotic mosquito species had been identified in our study.

It is also worth noting that services of the Belgian Federal Agency for the Safety of the Food Chain (FASFC) recorded several cases of equine infectious anemia (EIA) in the Province of Liège including horses in two of our study sites. All these cases of EIA were horses originated mainly from Romania (FASFC, http://www.afsca.be/santeanimale/aie/#SituationBelgique). The more recent record of new exotic species in Belgium, were *Oc. japonicus* (Theobald) in Natoye (Namur) and *Oc. koreicus* (Edwards) in Maasmechelen (Limburg), both collected during the national inventory MODIRISK (Versteirt et al., 2013) and *Ae. albopictus* (Skuse) in a platform of imported used tires located in Vrasene (Oost-Vlaanderen) (Boukraa et al., 2013) and also in Lucky bamboo [*Dracaena braunii* Engl. (Asparagales, Asparagaceae)] shipments originating from the South coast of China (Demeulemeester et al., 2014).

Based upon their behaviour and ecology in equestrian farms, several species are potential vectors of WNV (*Cx. pipiens s.l.*, *Cx. torrentium*, *An. plumbeus*, *Cq. richiardii*, *Oc. geniculatus*), Tahyna virus (*Cs. annulata*, *Oc. cantans*) and Usutu virus (*Cx. torrentium*, *Cx. pipiens s.l.*, *An. maculipennis s.l.*) (Schaffner et al., 2001, Becker et al., 2010 and Calzolari et al., 2013). However, none of the identified species seemed able to transmit EEEV, WEEV and VEEV, besides *Cx. pipiens s.l.* which is considered as a potential vector of EEEV and WEEV (Pfeffer and Dobler, 2010) or *Cx. territans* which can be considered as a probable vector of EEEV. In addition to arboviral diseases, it is to note that equestrian farms seem gave suitable environments and breeding sites for the proliferation of mosquitoes, that can cause a real nuisance problem for horses and neighboring inhabitants. Our results about the diversity of breeding sites and the importance of these ones in the proliferation of potential vectors of arboviruses, can be used by equine farmers to design management strategies to reduce mosquito populations and thus eliminate nuisance sources, especially preventing the potential transmission of arboviruses.

Conclusion

Given the low variation in species composition of potential mosquito vectors and their breeding sites in the studied stations, the transmission dynamics of probable mosquito-borne viruses will likely be similar between Belgian equestrian farms. However, it is important to note that other factors can also be involved in arboviruses incidence such as the local weather variability, the management of equestrian farms and hygiene compliance to reduce potential breeding sites, and also composition and distribution of avian species as reservoirs. Although none autochthonous transmission of the four equine arboviruses (WNV, EEEV, WEEV, and VEEV) was detected in Belgium, this country undergoes important changes just like Europe in terms of intensification of the international trade with endemic countries of the diseases, increasing the risk of arboviruses importation. In this regard, an intensified vigilance of veterinary services, as well as deepened entomological surveys in order to assess the bionomics of potential vectors in relation to potential reservoirs species and other environmental factors are highly recommended.

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