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EX VIVO STUDIES ON HOST RANGE AND TROPISM OF INFLUENZA A VIRUSES

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Dedicated to my father,
and his bicycle motto

Summary

Influenza A viruses (IAVs) are single stranded RNA viruses belonging to the *Orthomyxoviridae* family. These viruses exhibit high evolutionary rates and are present in a wide range of animal species. Host switching of IAVs is unpredictable and poses a great threat to human health. Therefore, understanding the underlying mechanisms driving such events is one of the current goals in influenza research. Among the different subtypes, H3N8 IAVs have shown a remarkable tendency to host species jumps. H3N8 equine influenza virus (EIV) is an avian-origin virus that circulated in horses for nearly 40 years before emerging in dogs in early 2000s. In the last decade H3N8 EIV has also been isolated from pigs, key hosts in influenza ecology, and other mammals. To understand the nature of host range determinants it is fundamental to study the dynamics of influenza pathogenesis at the site of infection. Respiratory explants constitute a suitable model to investigate virus replication in the target tissues of the host. This thesis aimed at thoroughly investigating the infection dynamics of host adapted and non-host adapted IAV infections in organ explants of pigs and horses.

We first described the infection phenotype of host adapted viruses such as H3N2 swine influenza (SIV) and H3N8 equine influenza in tissues of their natural hosts to build a solid background knowledge on disease pathogenesis. Swine and equine explants were infected with SIV and EIV, respectively, and accurately monitored for viral replication and structural changes at the site of infection (chapters two and three). This extensive examination has added substantial information to previous literature and confirmed that swine and equine *ex vivo* systems support influenza replication and are suitable to study virus pathogenesis and at the same time observe the 3Rs ethos (Reduction, Replacement and Refinement).

Next, we addressed our research interests on non-host adapted infections focusing on the H3N8 subtype. Enhancing the current knowledge on the role of evolution in the complex phenomenon of viral emergence is of utmost importance. To this end, in chapter four we compared the replication potential of phylogenetically distinct EIVs, each representing an evolutionary different period, in swine cell lines and respiratory explants. All tested EIVs replicated in cell lines whereas only the earliest EIV isolate (Uruguay/63) was able to infect swine explants with a marked tropism for the lower respiratory tract, demonstrating the presence of tissue-specific host barriers at the site of infection. The distinct phenotypes observed *ex vivo* support the view that evolutionary processes play important roles on host range and tropism of EIV. Nonetheless, when compared to SIV H3N2, EIV Uruguay/63 productively infected only a limited number of explants.

H3N8 EIV has originated from the avian gene pool maintained in aquatic waterfowl. To further look into host range shifts of H3N8 IAVs, in chapter five we investigated the replication dynamics of H3N8 avian influenza viruses (AIVs) in equine tracheal explants.

To give an ecologically plausible context to our experiments, we tested AIVs isolated from wild birds in Mongolia, a region densely populated with wild birds and horses. H3N8 AIVs infected tracheal explants, albeit to significantly lower levels and with a different infection phenotype compared to an EIV. This difference was evidenced as lower viral titres, absence of epithelial damage and difficult viral nucleoprotein detection in the infected tissue. These findings, coupled with serological evidence of AIV infections in horses in the field, suggest that introduction of viruses from the avian reservoir can occur and that further adaptive changes may be required for a successful establishment.

Overall, by investigating the infection dynamics of IAVs in *ex vivo* cultures of the respiratory tract we have provided new insights into the different phenotypes displayed by host adapted and non-host adapted viruses at the site of infection. Our results support the hypothesis that viral evolution during long-term transmission of IAVs in host populations could result in dynamic changes in their host range. Such changes must be in line with ecological and epidemiological factors in order to allow the establishment of novel lineages in susceptible hosts. Finally, we have confirmed that organ explants can bridge important gaps between *in vitro* and *in vivo* experiments.

Riassunto

I virus dell'Influenza A sono virus ad RNA con genoma segmentato appartenenti alla famiglia *Orthomyxoviridae* la cui ecologia si distingue per l'ampio spettro di ospiti ed elevato tasso evolutivo. Le frequenti trasmissioni inter-specie ad oggi risultano impredevibili e rappresentano un elevato rischio per la salute umana. Per questo motivo il fronte della ricerca sui virus influenzali è concentrato sullo studio della trasmissione dell'influenza da un ospite all'altro. Tra i diversi sottotipi esistenti, il sottotipo H3N8 ha dimostrato una notevole tendenza al salto di specie. L'influenza equina H3N8 (EIV) è un virus di origine aviare che ha ampiamente circolato nei cavalli per circa 40 anni prima di saltare la barriera di specie e stabilirsi nella popolazione canina dando origine all'influenza canina all'inizio degli anni 2000. Recentemente l'influenza equina è stata occasionalmente isolata anche da maiali, ospiti molto importanti nell'ecologia dell'influenza in quanto suscettibili a numerosi virus di diversa origine animale, e da altri mammiferi. Per studiare a fondo il tropismo dei virus influenzali nei diversi ospiti è fondamentale osservarne il comportamento nei tessuti target. Gli espianti d'organo rappresentano un'alternativa alle sperimentazioni *in vivo*, permettendo di caratterizzare il potenziale replicativo dei virus e riducendo il numero di animali utilizzati a fini sperimentali. Lo scopo di questa tesi è stato caratterizzare infezioni specie-specifiche e non specie-specifiche causate da virus influenzali di tipo A in espianti d'organo di maiale e cavallo.

In primo luogo, con la finalità di acquisire un'importante conoscenza di base sulla patogenesi della malattia, abbiamo caratterizzato due infezioni specie-specifiche nei tessuti target delle rispettive specie ospite: l'influenza suina H3N2 e l'influenza equina H3N8 (capitoli secondo e terzo, rispettivamente). In seguito all'infezione, per ciascun virus è stato descritto un fenotipo d'infezione basato sulla crescita virale e sui cambi strutturali nei tessuti interessati. I risultati ottenuti hanno aggiunto importanti informazioni alla letteratura preesistente ed hanno confermato la sensibilità di questa metodica *ex vivo* per lo studio della patogenesi di virus influenzali nel pieno rispetto del principio delle 3R (Reduction, Replacement and Refinement).

Successivamente abbiamo utilizzato gli espianti d'organo per approfondire lo studio delle infezioni non specie-specifiche causate dal sottotipo H3N8. Lo scopo di questo lavoro è stato valutare l'impatto dell'evoluzione naturale di un virus (dovuta all'ampia circolazione) sul suo spettro d'ospite. Virus dell'influenza equina, filogeneticamente distinti e rappresentanti periodi evolutivi diversi, sono stati testati su linee cellulari primarie di maiale ed espianti d'organo suini (capitolo quarto). Tutti i virus inclusi nello studio sono stati in grado di replicare nelle linee cellulari mentre in espianti d'organo soltanto il primo isolato di influenza equina (Uruguay/63) è stato in grado di infettare diversi tessuti, mostrando un particolare tropismo per le vie respiratorie profonde. Questi dati dimostrano in primis la presenza di barriere specifiche a livello dei tessuti

dell'ospite. Inoltre, le differenze osservate tra virus appartenenti a periodi evolutivi diversi suggeriscono che l'evoluzione ha un ruolo chiave sul tropismo e sullo spettro d'ospite dei virus dell'influenza equina. Tuttavia, pur replicando in tessuti suini, il virus Uruguay/63 ha infettato un numero minore di espianti rispetto ad un virus specie-specifico utilizzato come controllo positivo.

Il virus dell'influenza equina H3N8 è originato da un virus aviare mantenuto nel serbatoio rappresentato dai volatili selvatici. Con la finalità di approfondire ulteriormente le nostre conoscenze sul sottotipo H3N8 ed il suo spettro d'ospite, nel quinto capitolo abbiamo valutato il potenziale replicativo di virus dell'influenza aviare in espianti di trachea di cavallo. Per dare un contesto verosimile ai nostri esperimenti, abbiamo utilizzato virus isolati da volatili selvatici in Mongolia, regione densamente popolata da volatili selvatici e cavalli. I virus testati sono stati in grado di replicare negli espianti di trachea di cavallo mostrando però titoli significativamente più bassi rispetto ad un virus specie-specifico utilizzato come controllo positivo. Inoltre, al contrario di quanto osservato con il virus equino, i virus aviari non hanno causato nessun danno epiteliale e la nucleoproteina virale è stata difficilmente identificata nei tessuti colpiti. L'evidenza sierologica di cavalli infetti con virus aviari in campo supporta i nostri risultati e la teoria per cui contatti sporadici con questi virus avvengono ma affinché siano in grado di stabilirsi nella popolazione equina è necessario un ulteriore adattamento.

In conclusione, infettando espianti d'organo con virus specie-specifici e non, abbiamo fornito nuova evidenza di importanti differenze fenotipiche nei tessuti target. Inoltre, abbiamo dimostrato come l'evoluzione di un virus ne possa influenzare il tessuto tropismo e di conseguenza lo spettro d'ospite. In fine, abbiamo sottolineato come l'utilizzo di una metodica *ex vivo* possa colmare differenze fondamentali tra metodiche *in vitro* ed *in vivo*.

List of abbreviations

AIV	avian influenza virus
ALI	air-liquid interface
BSA	bovine serum albumin
cRNA	complementary RNA
DMEM	dulbecco's modified eagle's medium
EIV	equine influenza virus
ELISA	enzyme-linked immunosorbent assay
HA	hemagglutinin
HI	hemagglutination inhibition
H&E	hematoxylin and eosin
HPAI	highly pathogenic avian influenza
Hpi	hours post infection
IAV	influenza A virus
IHC	immunohistochemistry
LPAI	low pathogenicity avian influenza
LRT	lower respiratory tract
M	matrix protein
MDCK	madin-darby canine kidney
MEM	minimum essential medium
µl	microliter
ml	milliliter
mRNA	messenger RNA
NeuAc	N-acetyl neuraminic acid
NeuGc	N-glycolyl neuraminic acid
NP	nucleoprotein
NPTr	newborn pig trachea
NS	non structural protein
NSK	newborn swine kidney
P/S	penicillin-streptomycin
RNA	ribonucleic acid
RNPs	ribonucleoproteins
PA	polymerase acid
PB	polymerase basic
PBS	phosphate buffer saline
pfu	plaque forming unit
p.i.	post infection
P/S	penicillin streptomycin
SAs	sialic acids
SEM	standard error of the mean
SIV	swine influenza virus
$TCID_{50}$	tissue culture infectious dose with a 50% end point
URT	upper respiratory tract
vRNA	viral RNA

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Chapter 1

General introduction

1.1 Classification

Influenza A viruses (IAVs) are enveloped negative-sense RNA viruses belonging to the *Orthomyxoviridae* family. The other genera included in this family are Influenza B and C viruses, Isavirus (Infectious salmon anemia) and Thogotovirus.

The three influenza virus genera have been classified according to their internal proteins nucleoprotein (NP) and matrix (M) and differ in host range and pathogenicity. Evolutionary studies indicate that these viruses probably diverged from a common ancestor several thousand years ago. Influenza A and B maintain a similar structure whereas the C has a more divergent one (Hay *et al.*, 2001).

The shape of influenza A virions is pleomorphic as they can be found both in a spherical (ranging from 80 to 120 nm) and in a filamentous form (up to 300 nm of diameter). The biological reasons and consequences of this difference are still under investigation. Laboratory-adapted strains are predominantly spherical whereas clinical specimens can harbour several morphologies (Elleman & Barclay, 2004; Harris *et al.*, 2006). Recently, among the filamentous particles found in a human influenza strain, particularly long filamentous virions lacking internal gene segments have been identified (Vijayakrishnan *et al.*, 2013). Interestingly, the authors suggested that these could act as "decoy" for the immune system helping infectious virions to evade immunity. Moreover, due to their length, it has been proposed that they could have a physical action in disrupting the mucociliary barrier present in the respiratory epithelium.

The IAV genome is composed of 8 negative-sense single stranded RNA segments encoding for at least 16 proteins by either alternative splicing or partially overlapping open reading frames (Stubbs & Te Velthuis, 2014; Szewczyk *et al.*, 2014). The ability of these viruses to adapt to new hosts is favoured by their genome structure that confers a high flexibility. Having a segmented genome allows segment exchange leading to reassortment in the likelihood of co-infection of two viruses in the same cell. Also, due

to the error prone polymerase and the lack of proof reading the probabilities of mutations occurring during replication are high.

According to the main surface glycoproteins, the hemagglutinin (HA) and the neuramindase (NA) IAVs have been classified into subtypes. In the avian reservoir 16 HAs and 9 NAs have been identified. Recently, two new subtypes of influenza have been discovered in bats, namely the H17N10 in the little yellow-shouldered bat in Guatemala and the H18N11 in the flat-faced fruit-eating bat in Peru (reviewed in Mehle, 2014). Interestingly, these viruses are evolutionarily distinct from the already known subtypes and at the same time show a high degree of divergence between them therefore opening new horizons to influenza research.

Upon isolation influenza viruses are named following the genus, the host of origin (except for human isolates for which it is omitted), the place of isolation, a strain reference number, the year of isolation and the subtype in parenthesis such as A/swine/Italy/8088/2006 (H3N2).

1.2 Viral structure, protein function and replication cycle

Influenza virions are surrounded by an envelope acquired from the host cell bi-layered lipid membrane during budding. The two major surface spike glycoproteins HA and NA are embedded in the envelope along with the matrix ion channels (M2 proteins). Below the envelope lies a layer formed by the matrix proteins (M1) that interact with both the cytoplasmic portion of the envelope embedded proteins and the 8 ribonucleproteins (RNPs). Each RNP complex is composed by the viral RNA (vRNA) and the nucleocapsid protein (NP). Attached to one end of each RNP there is an RNA-dependant RNA polymerase complex organised in three independent proteins: the polymerase basic protein 1 (PB1) involved in initiating transcription, the polymerase basic protein 2 (PB2) involved in nuclear import and the polymerase acid protein (PA). Each virion also contains a non-structural protein (NS1), a nuclear export protein (NEP, also called NS2) and a PB1-F2 accessory protein identified in early 2000's whose functions are still under investigation.

There are several additional proteins that have been identified in recent years and whose functions are currently unclear (reviewed in Szewczyk *et al.*, 2014), namely the PB1-N40, the PA-X, the PA-N155 and the PA-N182, the M42, the eNP and the NS3.

Several functions have been attributed to the NS1 such as being an interferon antagonist and mediating nuclear import and export. The NEP (or NS2) mediates together with the M2 the nuclear export of RNPs. The M1 protein has the aforementioned role of connecting surface and internal proteins but it also seems to take part to the nuclear transport signalling.

The HA is a homotrimer syntesized as HA0 precursor and cleaved at later stages by host proteases in HA1 and HA2 (Wang *et al.*, 2012). The HA1 subunit represents the globular head of the protein where the receptor binding site and major antigenic sites are allocated whereas HA2 is the stalk region of the protein. Infection of a cell by influenza virus begins with the interaction of the receptor binding pocket of the globular head of the HA and the terminal sialic acids (SAs) found on the surface of the cell (Bouvier & Palese, 2008). Following the attachment, the virion is internalised by endocytosis either by clathrin mediated or independent pathway (Rust *et al.*, 2004) and migrates from the cell surface towards the nucleus through an early and late endosome, respectively. The pH of the endosome is progressively lowered by proton pumps and such acidity causes conformational changes in the HA2 subunit that are crucial for mediating the fusion between the viral envelope and the endosomal membrane. At this stage the M2 proton channels, activated by the acid pH, allow the H^+ ions to penetrate the virion causing the separation of the M1 proteins from the RNPs by weakening protein interaction with subsequent release of the latter into the cytoplasm (Pinto & Lamb, 2006; Wang *et al.*, 2011).

Internalization of RNPs into the nucleus is initialized by nuclear localization signals in the PA, PB1, PB2 and NP that interact with karyopherins such as importins. These proteins have been recognised as important host-specific factors influencing viral replication and host range (Gabriel *et al.*, 2011; Resa-Infante & Gabriel, 2013). Once inside the nucleus, RNA transcription and replication begin. As the vRNA lacks the 5’ methylated cap that initiates transcription, transcription of vRNA into messenger RNA (mRNA) is initiated by

a cap snatching process from the host mRNA by the viral endonuclease PA (Dias *et al.*, 2009). Through the synthesis of a complementary positive-sense RNA (cRNA) that acts as a template, production of new vRNA takes place in the nucleus whereas mRNA, once capped, is translated in the cytoplasm.

The newly synthesised proteins migrate from the cytoplasm to the nucleus where they are assembled with vRNA. Subsequently, through interaction of M1 and NS2 with cellular nucleoporins the RNPs are transported into the cytoplasm. Upon synthesis the HA and NA undergo post-translational changes such as being assembled in oligomers, folded and glycosylated. The final step of viral packaging occurs near the lipid rafts of the inner side of the cellular membrane (Zhang *et al.*, 2000) in which the HA, NA and M2 are already incorporated thanks to apical sorting signals. All gene segments seem to contain small packaging signals to ensure the formation of virions with a full genome (Bouvier & Palese, 2008 and references therein). At this level the M1 and RNPs are assembled followed by budding of the viral particle most likely initiated by an accumulation of M1 at the cytoplasmic side of the cellular membrane. For progeny virions to be ultimately released from the cell they must be detached from the SAs on the cellular surface to which they are bond. This takes place through the activity of the NA, a mushroom-shaped tetramer projecting from the viral envelope. As a sialidase it has a proteolytic function: it cleaves sialic acid terminations from the host cell but also from the virions allowing final release from the cell and preventing aggregation between viral particles. Moreover it seems to have a function in disrupting the mucins contained in the mucus that covers the respiratory epithelium, thus allowing the virus to reach the cellular receptors (Matrosovich *et al.*, 2004). Therefore a balance between HA and NA is essential for optimal viral activity.

1.3 Influenza in avian species: circulation in reservoir hosts and land based poultry

With the exception of the newly identified influenza viruses in bats, it is generally accepted that all subtypes of IAVs are perpetuated in wild birds, especially waterfowl. In particular, birds belonging to the orders *Anseriformes* (e.g. ducks and geese) and *Charadriiformes* (e.g. gulls and shorebirds) are recognised as the main reservoirs. In such hosts a coevolution process has likely occurred leading to asymptomatic infections.

Initially, avian influenza viruses (AIVs) circulating in the wild bird reservoir were believed to be in an evolutionary stasis. More recent evidence has though proved that actually these show rapid evolutionary dynamics (Chen & Holmes, 2006). Different subtypes can be isolated according to the species, the year and the geographical area. The H13 and H16 subtypes are typically isolated from gulls and rarely from other species (Webster & Bean, 1992). The reasons why ducks and shorebirds harbour different influenza subtypes are still to be elucidated.

IAVs replicate in the cells lining the intestinal tract of wild birds. Thus, the major route of transmission in reservoir hosts is the fecal-oral. Viruses are excreted from the cloaca in high concentrations and if the environmental conditions are appropriate (e.g. low temperatures) they can survive for several months on water surfaces.

The ecology of waterfowl probably favours the high prevalence of infection in these species (Webby & Webster, 2001). Population size and social behaviour allow frequent contacts and intermingling of different water bird species representing chances for virus transmission to occur. Moreover, aquatic habitats can provide an ideal setting for fecal-oral transmission of pathogens such as influenza. Feeding behaviour, geographical localization and migratory routes can all influence the ecology of the viruses. Also, the presence of different and separate flyways has contributed to the formation of distinct gene pools such as the Eurasian and the American lineages. Within large continents, common breeding areas shared between birds moving along different flyways can represent an opportunity of viral transmission to new populations that subsequently may introduce pathogens into new regions (Olsen *et al.*, 2006a).

Beyond the natural reservoir, IAVs circulate in other hosts in a species-specific manner. Several subtypes of AIVs have been isolated from domestic poultry (e.g. chickens, turkeys and quail) as a result of introductions from the wild reservoir. According to species susceptibility, viral subtype and pathotype, two clinical forms can be encountered. The majority of AIV subtypes fall into the low pathogenicity (LPAI) pathotype, causing mild respiratory symptoms. H5 and H7 subtypes can also be found as highly pathogenic (HPAI) strains causing a systemic infection with high mortality rate.

Poultry in general are regarded as important sources of zoonotic infections and species such as turkeys and quail have gained much attention due to their role as disease amplifiers of AIVs introduced from the wild bird reservoir towards other poultry or mammals. Turkeys are particularly susceptible to AIVs and experimental evidence suggests that replication in this host may enhance their pathogenicity in other poultry such as chickens (Cilloni *et al.*, 2010). Furthermore, there is increasing evidence of their susceptibility to swine influenza viruses. Transmission of influenza from mammals to birds has not been common but outbreaks of swine-adapted influenza in turkeys has been reported (Choi *et al.*, 2004; Nfon *et al.*, 2011).

Quail have been regarded as potential mixing vessels of avian and mammalian influenza viruses. The mixing vessel expression is used to indicate a host species with the capacity to be co-infected with multiple genetically distinct viruses of different animal origins that are able to reassort to generate genetically novel viruses (Nelson & Vincent, 2014). The latter was justified in quail because they harbour receptors that are recognized by both avian and human or swine viruses and because experimental studies showed that they can support virus reassortment (Hossain *et al.*, 2008; Makarova *et al.*, 2003; Thontiravong *et al.*, 2012).

Despite the necessity of further investigating the role of poultry, subtypes relevant to human health such as the HPAI H5N1 and LPAI H9N2 are currently endemic, and occasionally co-circulating, in poultry populations in some areas of Asia and of the Arabic peninsula raising constant alerts (Fusaro *et al.*, 2011; Monne *et al.*, 2013).

1.4 Influenza in mammals: a closer look into swine and horses as important hosts in influenza ecology

IAVs have been isolated from several mammal species including humans, pigs, horses, dogs, minks and marine mammals (Olsen *et al.*, 2006b). Nevertheless, sustained transmission and well-established lineages have been recognized only in few of them (Parrish *et al.*, 2014). Among these, pigs and horses have shown to be important hosts for multiple reasons, as described in detail below.

1.4.1 A brief overview on swine influenza

Swine influenza is a respiratory disease leading to the onset of symptoms such as shortness of breath, cough, fever and lethargy (Janke, 2014). It can also predispose to secondary bacterial infections leading to a more complicated syndrome.

Pigs have been identified as hosts of IAVs since the beginning of the 20th century and ever since the epidemiology of influenza in this host has become quite intricate. Following the emergence of the 2009 H1N1 pandemic virus, they have conquered an unprecedented spotlight. Currently, three subtypes of swine influenza viruses (SIV) are endemic in pigs worldwide: H1N1, H1N2 and H3N2. Within each subtype different lineages exist and their origin and geographical circulation can be strikingly different (reviewed in Vincent *et al.*, 2014). In North America, the classical H1N1 SIV has been circulating after its first detection concomitant with the 1918 human pandemic. This lineage is thought to be derived or be the progenitor of the 1918 pandemic. Therefore, its gene constellation is similar to the latter. Following the introduction of the triple-reassortant H3N2 subtype (TR H3N2) in 1998 which contains genes of human (HA, NA and PB1), avian (PB2 and PA) and swine (NP, M and NS) origin, its spread and gene constellation drastically changed. The North American classical H1N1 lineage was first detected in European swine in 1976. In 1979 this lineage was replaced by the duck-derived avian-like H1N1 virus. H3N2 human IAVs and H1N2 human-avian reassortants are currently circulating in European pigs as well. In Asia besides the classical swine H1N1 and the European avian-like H1N1, an avian-like H1N1 different from that circulating in Europe emerged in the 90's representing another independent introduction of an avian virus into pigs. Further, European H3N2 and the North American TR H3N2 have also been detected in this region (Vincent *et al.*, 2014 and references therein).

This worldwide puzzled picture is further entangled by frequent reassortment events between co-circulating subtypes.

1.4.2 The current debate on the role of pigs

The role of pigs as potential mixing vessels and generators of pandemic strains is currently a topic of debate as scientific evidence has not yet fully explained this theory. The frequent occurrence of reassortment events as well as the similarity between human and swine HAs have long been suggesting their role as intermediate hosts where viruses of different animal origin can replicate simultaneously. This theory was initially supported by the presence of both α2-3 (recognised by avian and equine viruses) and α2-6 (recognised by swine and human viruses) sialated glycans, the main influenza receptors known and studied up to date. The current understanding of influenza receptor distribution has been relying mainly on lectin staining that has occasionally yielded contrasting results. Initially, both receptor types were found in the upper respiratory tract (URT) of pigs giving evidence of a possible site for viruses of different animal origin to reassort (Ito *et al.*, 1998; Suzuki *et al.*, 2000). This analysis was later conducted by several others revealing a paucity of α2-3 and a predominance of α2-6 receptors in the URT and a presence of both receptor types in the lower respiratory tract (LRT) therefore resembling the distribution seen in human and some poultry species tissues (Löndt *et al.*, 2012; Van Poucke *et al.*, 2010). Binding assays on swine tissues using labelled viruses confirmed the preference of AIVs for the LRT. Also, infections of *ex vivo* cultures of the swine respiratory tract showed a greater replication in lung explants compared to nasal mucosa or tracheal explants (Van Poucke *et al.*, 2010; van Riel *et al.*, 2007). Lectin staining not only produces contrasting results, but is also limited to detecting the presence of silated glycans without identifying the actual glycan.

According to mass spectrometry, swine trachea expresses mainly α2-6 receptors whereas in the lungs both α2-6 and α2-3 are abundantly expressed (Chan *et al.*, 2013a). Further, mass spectrometry provided additional information on sialic acid species other than the type of linkage. The N-acetylneuraminc (NeuAc) acid, also present in avian tissues, was abundantly detected whereas the N-glycolylneuraminc (NeuGc) acid, which is dominant in horses, was less present.

Improvement of carbohydrate study technologies has shown that other factors such as glycan length, topology, and additional sulphation, syalation or fucosylation influence

virus binding. Using a novel approach such as shotgun glycan microarray, Byrd-Leotis and colleagues have described the glycome present in swine lung tissue through an extensive overview of the multitude of natural N-glycans in this important site (Byrd-Leotis *et al.*, 2014). Additionally, the authors investigated the binding avidity of several IAVs for the isolated glycans reporting three main important findings: first, avidity changes within a subtype; second, some human and avian strains have a similar affinity for either α2-3 or α2-6 glycans; third, some strains bound to glycans that are not identified by lectins stressing the need for innovative approaches to receptor investigations.

In light of such findings, and in line with previous studies supporting the replication of AIVs independently from α2-3 expression (Oshansky *et al.*, 2011), they stated that virus receptor recognition is far more complicated than an α2-3/α2-6 preference.

As mentioned above, presence of both receptor types has also been found in humans and poultry species (Costa *et al.*, 2012; Kimble *et al.*, 2010; Shinya *et al.*, 2006) therefore suggesting that the mixing vessel role might not be unique to this host.

To further challenge the theory, De Vleeschauwer *et al.* argued that natural infections of swine with AIVs normally result in sporadic dead-end cases with the great exception of the well-established European H1N1 avian-like swine lineage that appeared in the pig population in late 70's. Moreover they stated that AIVs replicate in pigs to a lower extent than SIVs and are not efficiently transmitted to other pigs (De Vleeschauwer *et al.*, 2009a, b).

Despite the gaps concerning scientific explanations, it is undeniable that pigs are unique hosts as regards to influenza ecology. The evidence provided by the 2009 pandemic has been leading the research front towards increased surveillance in swine populations worldwide. Since then, repeated sporadic isolations of human and avian influenza viruses as well as occasional detection of equine influenza in pigs have confirmed their peculiar susceptibility to IAVs of different origin. It is also important to acknowledge that zoonotic infections are bi-directional and cases of human influenza in swine have been

well documented highlighting the high likelihood of novel reassortants arising from pigs. In this context, swine can be considered as mixing vessels in the sense that pigs are more capable than humans of harbouring a large number of co-circulating IAV lineages that can generate diverse combinations of segments via reassortment (Nelson & Vincent, 2014).

Pigs' susceptibility to IAVs, either in the form of sporadic or well established infections and frequency of exposure to sources of infection (poultry, humans, other mammals) resulting in often isolations of reassortants worldwide are unique to this mammal so far.

In conclusion, the mechanisms underlying why and how pigs are important hosts deserve to be fully understood.

1.4.3 Influenza in horses: a historically well-known disease recently capable of surprising

Episodes of respiratory disease in horses have been reported for centuries, often in association with similar disease in humans or other animal species such as poultry. A panzootic that was initially reported in horses in Canada in 1872, followed by an epizootic of in poultry in the US, is thought to represent the first documented case of HPAI (Morens & Taubenberger, 2010). In the 1956 the first subtype of equine influenza was identified (Sovinova *et al.*, 1958). Interestingly, it was an H7N7, the same subtype of HPAI circulating at that time in birds. To further strengthen the hypothesis of relatedness, this EIV had an expanded cleavage site, a typical trait of HPAI viruses (Morens & Taubenberger, 2010).

The H7N7 subtype is now believed to be extinct as it has never been isolated after 1979 and serological positivity thereafter was attributed to vaccination (Webster, 1993). In 1963 a second subtype of EIV was identified in Florida in horses imported from Argentina (Waddell *et al.*, 1963). The H3N8 EIV has thereafter spread worldwide outcompeting the H7N7 and is still a cause of important disease and economic losses in the equine industry (Daly *et al.*, 2011). Reassortment between the two subtypes has occurred resulting in H7N7 viruses bearing H3N8 internal genes, but not vice versa (Murcia *et al.*, 2011). Several reasons have been proposed for the extinction of this

subtype, such as the introduction of vaccination, a lower fitness compared to H3N8 proved by the gene flow during reassortment and most interestingly, a lower adaptation to mammals. The latter was proposed following whole genome analyses and phylodynamics highlighting the reassortment of H7N7 with AIVs, whereas this was not identified for the H3N8, perhaps indicating a major adaptation to mammals.

A recent study has confirmed that both EIVs originated from the avian gene pool (Worobey *et al.*, 2014). In addition, a second independent introduction of an avian H3N8 occurred in 1989, further supporting the susceptibility of horses to AIVs (Guo *et al.*, 1992). Influenza receptor distribution in the respiratory tract of horses has been studied through lectin staining. Nonetheless, it has not been thoroughly described as in other species yet. The dominant sialic acid linkage is the α2-3 that though bears some differences compared to linkage found in birds. α2-3 NeuGc acid bound to galactose represents the 90% of the SAs present in the horse trachea (Suzuki *et al.*, 2000), whereas the remaining 10% is the α2-3 NeuAc acid, which in turn is predominant in other species such as pigs and birds. As mentioned for pigs, there are some discrepancies on the results provided by this technique. According to Muranaka and colleagues there is a predominance of α2-3 SAs from the epithelium of the nasal mucosa down to the bronchus, whereas α2-6 SAs are only present on cilia, goblet cells and accessory glands (Muranaka *et al.*, 2011). Interestingly, equine lungs seem to lack both receptor types. These results are in contrast with previously reported studies that did not detect α2-6 SA in equine trachea (Daly *et al.*, 2008; Suzuki *et al.*, 2000). The study conducted by Suzuki *et al*. also compared receptor affinity of several EIVs for different species of SAs such as NeuAc acid and NeuGc acid either α2-6 or α2-3 bond to galactose (NeuAcα2-6Gal, NeuAcα2-3Gal and NeuGcα2-3Gal). In first place they showed that none of them had affinity for the NeuAcα2-6Gal moiety. Secondly, among the tested viruses, EIV A/equine/Miami/1963 (the first H3N8 isolate) was the only isolate possessing a strong affinity for NeuAcα2-3Gal and poor for NeuGcα2-3Gal whereas later isolates from 1979 to 1991 had either double avidity or major affinity for the latter.

The avian origin of EIV H3N8 and the subsequent adaptation to the horse resulting in an increased avidity for the NeuGcα2-3Gal could potentially explain the different receptor preference. Nevertheless, extensive replication of EIVs in embryonated chicken eggs has

been shown to shift the receptor avidity towards a NeuAcα2-3Gal. This leads to the need of carefully interpreting the results of binding assays, as it is difficult to trace back the passage history of old isolates.

Horses have proven in recent years that further to being a source of viruses capable of establishing in other mammals, as it occurred with dogs, they are also increasingly causing sporadic spillovers in other species. Both arguments are described in detail below.

1.5 Inter-species transmission of IAVs: the interesting story of the H3N8 subtype

Transmission of IAVs between animal species is one of the most unpredictable features of these pathogens, making them object of constant surveillance and intense study. Because of the relevance to human health and historical background, many of these efforts are focused on understanding the transmission triangle represented by humans, birds and pigs. Nevertheless, as mentioned above, IAVs infect several other hosts and episodes of inter species transmission have and do occur among them.

When it comes to host switching among animal populations the H3N8 subtype stands out as a remarkable example. This subtype is among the most frequently isolated viruses in the wild reservoir in different parts of the world (Gilbert *et al.*, 2012; Hénaux *et al.*, 2012). Avian H3N8 was efficiently introduced into horse populations leading to well-established infections twice. The first example is the aforementioned emergence of EIV H3N8 in 1963. Secondly, in 1989 an independent introduction of an avian H3N8 occurred in China which then disappeared after two years (Guo *et al.*, 1992, 1995).

Recently, an avian H3N8 caused an outbreak of influenza among seals with a high mortality rate (Anthony *et al.*, 2012). Genomic characterization of this virus showed that it originated from wild birds without any evidence of reassortment with mammalian viruses. Moreover, avian H3N8 isolates belonging to the same clade as the seal isolate showed enhanced infectivity in mammalian cells and relevant animal models such as ferrets and mice and also had an increased binding avidity towards α2-6 receptors (Karlsson *et al.*, 2014). Overall, the evidence provided by both natural infections and experimental data suggests that H3N8 AIVs strains with enhanced mammalian tropism

and transmissibility amongst them circulate in the wild reservoir. Therefore, their replication potential needs to be further investigated.

Further looking into this subtype, H3N8 equine influenza has conquered much attention. Following the establishment of H3N8 in horses, EIV has circulated in horse populations worldwide with low evolutionary rates, suggesting a high degree of pathogen-host adaptation. In fact, the lack of exchange of gene segments with other influenza A viruses suggested that the horse may be a dead-end host.

However, in 2004 an EIV was identified as the cause of a severe outbreak of respiratory disease in dogs (Crawford *et al.*, 2005). Phylogenetic analyses determined that the canine isolates were closely related to contemporary EIVs.

This episode was just the first testimony of the emergence of H3N8 canine influenza virus (CIV) from EIV. After its first appearance, transmission of EIV to dogs was shown not to be unique to the American continent and was reported also in the UK and Australia (Crispe *et al.*, 2011; Daly *et al.*, 2008).

Up to date, the emergence of CIV represents the only well-established transfer of EIV into another species. Albeit, increasing spillover infections into other mammals reported in recent years are raising major concerns. During an outbreak that affected horses in China in 2007, EIV was detected in donkeys for the first time (Qi *et al.*, 2010). This may be not very surprising given the phylogenetic close-relatedness between horses and donkeys but it still represents a novel transmission episode. More interestingly, two H3N8 EIVs have been isolated from pigs showing respiratory symptoms in China (Tu *et al.*, 2009). These viruses were closely related to European EIVs and possessed mutations at the cleavage site and near the receptor binding domain of the HA protein that had been previously detected in CIV. Ultimately, at the beginning of 2014 EIV was isolated from a Bactrian camel in Mongolia (Yondon *et al.*, 2014), a region densely populated with horses showing high seroprevalence of H3N8 EIV infection (Pablo R. Murcia, personal communication) and where H3N8 AIVs circulate in wild birds (Gilbert *et al.*, 2012).

The reasons for which sustained transmission occurred only in dogs remain unclear. Preliminary evidence of receptor distribution has partially explained the susceptibility of dogs (Muranaka *et al.*, 2011). Nevertheless, virological, epidemiological and other host specific factors are currently under investigation to explain why sustained transmission in different parts of the world occurred in that specific time frame.

All the aforementioned natural episodes of inter species transmission of H3N8 IAVs, summarized in figure 1, highlight the necessity of further understanding how the viruses circulating in the avian reservoir replicate in mammals and whether extensive circulation and evolution in these hosts over time can increase the likelihood of infection in other mammals.

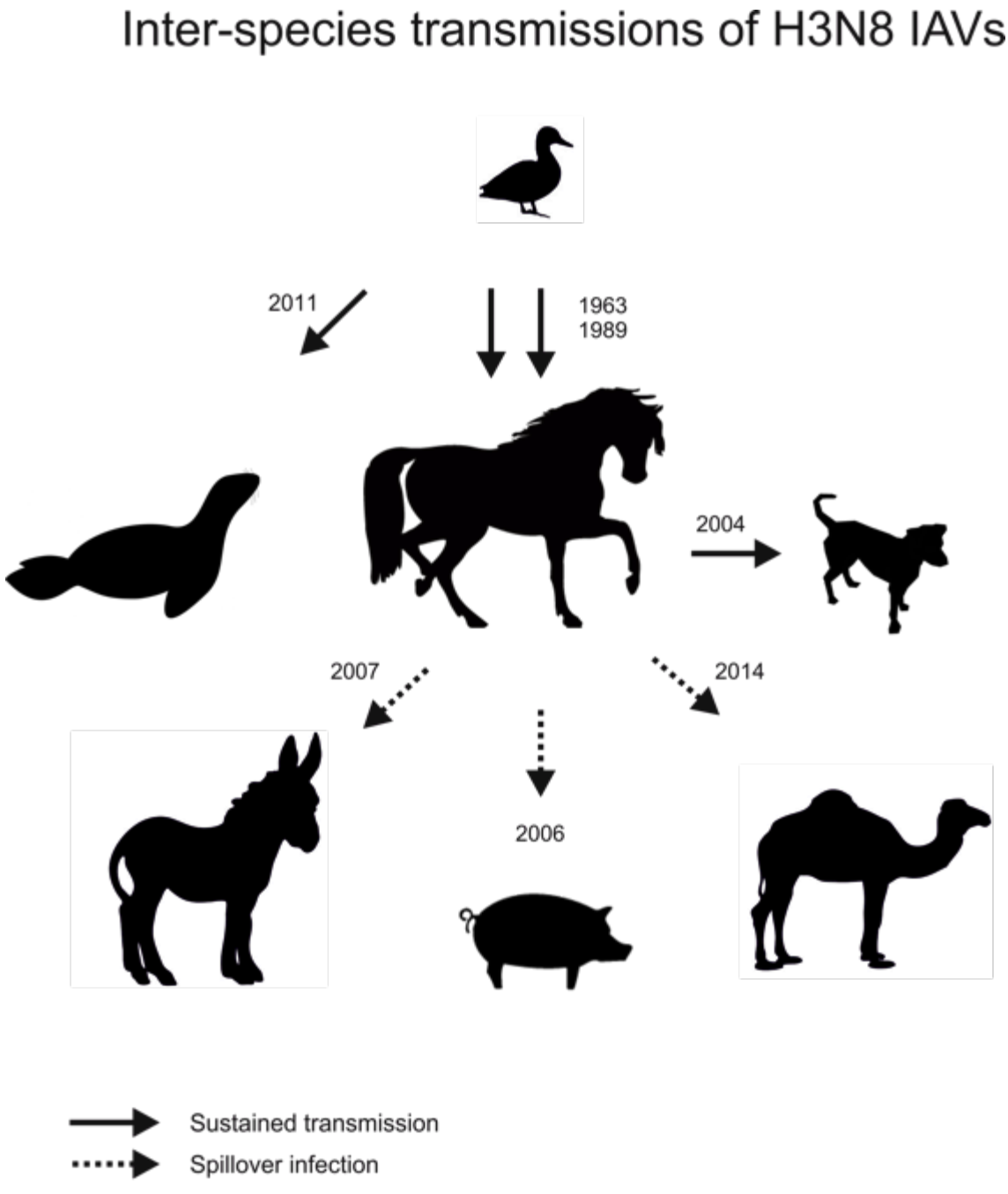

Figure 1. Schematic representation of inter-species transmission events reported for H3N8 IAVs. References are included in the main text.

1.6 An overview on the use of *ex vivo* organ cultures as complementary models of influenza infection

In recent years the use of *ex vivo* organ cultures of the respiratory tract has increased significantly to investigate several aspects of influenza infection dynamics. Within a laboratory setting organ explants are the closest culture system to the *in vivo* condition achievable, providing evidence of host-pathogen interactions while reducing the number of animals used for experimental purposes, in accordance with the 3 R's principle (reduction, replacement and refinement).

Along with explants, primary cell cultures have long been regarded as a good method. Nevertheless, these have limitations that explants can overcome. Primary cell cultures consist of bi-dimensional monolayers of a single cell type. Following removal from their natural environment as well as serial passaging, some fundamental properties such as glycan profile may vary making the substrate less reliable (Gawlitzek *et al.*, 1995). On the other hand, explants maintain the architecture of tissues as in the host from which they were taken presenting an important cellular complexity to viral infections. Moreover, relevant features in virus invasion and spread may be optimally studied in this system as cell-to-cell contacts are preserved (Vandekerckhove *et al.*, 2009). An additional important advantage is the fact that many explants can be collected from the same individual, therefore the experimental variability associated with inter animal variation can partially be overcome by comparing different viruses under identical conditions.

Ex vivo cultures to study influenza were first reported in 1974 (Schmidt *et al.*, 1974). This first well documented report of respiratory tissue cultures in influenza research showed that these were viable for at least 48 hours without major changes, indicating their suitability for studying viral replication. Over the following years*, ex vivo* organ cultures deriving from different poultry species, pigs, horses, dogs, ferrets and humans have been developed (Chan *et al.*, 2013b; Lin *et al.*, 2001; Petersen *et al.*, 2012; Van Poucke *et al.*, 2010; Siegers *et al.*, 2014). According to the animal species, distinct tracts are cultured. Such choice may depend on animal size, tissue availability (human explants) and origin (abattoir or animals used for experimental purposes). A summary of *ex vivo* tissue cultures of the respiratory tract used in influenza research is presented in table 1.

As receptor distribution changes along the respiratory tract, offering different conditions for viral replication, it would be advisable, where implementable, to obtain explants from as many respiratory tissues as possible.

Animal species	Tissue	Reference
Dog	Trachea	Gonzalez et al., 2014
Ferret	Nasal mucosa, trachea and lung	Roberts et al., 2011; Siegers et al., 2014
Horse	Nasal mucosa and trachea	Lin et al., 2001; Vandekerckhove et al., 2009
Pig	Nasal mucosa, trachea, bronchus and lung	Van Poucke et al., 2010 Nunes et al., 2010
Poultry	Trachea	Petersen et al., 2012
Human	Nasopharynx, bronchus, lung and conjunctiva	Chan et al., 2013b

Table 1. Summary of *ex vivo* organ cultures of the respiratory tract (and human conjunctiva) for the study of influenza infection in different animal species. Reported is the most recent complete reference literature.

By infecting explants with a known quantity of virus and collecting samples at different time points many aspects related to infection and host response can be studied such as: viral growth, target cells, mechanisms of cellular response to infection (e.g. apoptosis and regeneration), alterations in organ primary functions (e.g. ciliary clearance) and innate immune responses.

Since this method was first reported, several steps forward have been achieved to improve its culture methodology towards mimicking the conditions found in the respiratory tract of the living host. For example, the culture method changed from fully or semi submerged cultures to air-liquid interface systems that most closely resemble the physiology of respiratory tissues. Reagent composition has also been modified to avoid influencing cellular activity.

Regarding the infection technique, different approaches have been described. Some research groups have conducted infections by fully submerging explants in a volume

containing high viral loads (e.g. 10^5 or 10^6 plaque forming units [pfu]) for 1h of incubation and then removing the inoculum and thoroughly washing the explants to remove unbound particles. Others have adopted lower viral loads in small volumes (10^2 pfu in 5-20 µl) inoculated on the explant surface without further removing the inoculum or washing steps. Several options in sample collection have also been proposed, such as quantifying viral presence in the culture medium, recovering the infectious virus present at the level of the epithelial surface by serial washes or directly collecting individual explants and quantifying the viral load released by submerging the tissue in a liquid medium. The choice of a method over the other in both infection technique and sample collection may depend on experimental purposes (e.g. testing the susceptibility of a host to viruses of different origin or looking for the target cells).

Explants have been exploited in the context of inter species transmission of IAVs, a field of great interest and uncertainty in influenza research. Multiple insights in the replication of avian influenza viruses have been shown by working on swine and human explants, primarily their tropism for the lower respiratory tract. Also, Gonzalez and colleagues have recently described the use of canine tracheal explants to study the infection phenotypes of canine, equine and human influenza (Gonzalez *et al.*, 2014). By testing several equine influenza viruses belonging to phylogenetically distinct clades, they were able to provide evidence of a different ability of replicating according to the isolate, stating that viral evolution may have an important impact on host range and tropism of EIV.

Cultures of human nasopharynx, bronchus, lung and conjunctiva have been described and used in assessing the tissue tropism of emerging respiratory viruses such the Middle Eastern Respiratory Syndrome (MERS) coronavirus and the latest H7N9 zoonotic influenza virus emerged in China (Chan *et al.*, 2013a, 2014). As *in vivo* experiments may not be implementable promptly in animals, since they require careful experimental design, deeper ethical considerations (compared to *ex vivo* experiments), specific permits and proper facilities, *ex vivo* cultures are a rapid alternative to gain preliminary insights into an unknown pathogen. Therefore, the great advantage offered hereby by explants is to further characterize a new pathogen isolated from a clinical specimen.

Novel possible applications of culturing respiratory explants could be looking into tissue-associated viral quasispecies to describe population dynamics in the different parts of the respiratory tract. Mucosal immunity is increasingly gaining attention in vaccine development because of its important role as a primary line of defense. Therefore, tissue associated immune response at the mucosal level following infection could also be further explored by means of *ex vivo* studies.

Nevertheless, the *ex vivo* technique has some limitations in the type of data it can provide. Thus, *in vivo* experiments might be requested to further ascertain disease pathogenesis. When testing the replication potential of avian, human and swine influenza viruses in swine explants, Van Poucke and colleagues confirmed that the observed data were consistent with previous *in vivo* studies (Van Poucke *et al.*, 2010). On the other hand, a study aiming at evaluating the replication of avian influenza viruses in equine tracheal explants and ponies highlighted incongruent results as none of the viruses that replicated *ex vivo* was able to productively infect ponies (Chambers *et al.*, 2013). This provides evidence that organ explants are useful tools to obtain preliminary data on virus behaviour but that the barriers present in the living host might be far more complicated to overcome because of physiological and immunological conditions. Furthermore, the nasal mucosa and nasopharynx may be of paramount importance in preventing infection of respiratory pathogens.

Several issues representing the limits of using these cultures, such as variability among explants from the same animal and partial divergence between virological and histological data, are presented and discussed in this thesis.

1.7 Aims of the thesis

Host switching of influenza A viruses is unpredictable and understanding the underlying mechanisms driving such events is one of the current goals in influenza research. For this purpose, looking at viral tropism at the site of infection is fundamental and respiratory explants allow a closer and detailed insight into a pathogen's phenotype while reducing the number of animals used for experimental purposes.

H3N8 IAVs have been isolated from a relevant number of animal species and have shown a unique tendency to host species jumps. Among the hosts involved in their intricate spectrum, pigs and horses stand out as important species in influenza ecology in which disease dynamics need to be further elucidated.

Therefore this thesis was aimed at:

- Characterizing the infection phenotype of host adapted influenza viruses in *ex vivo* cultures in order to build solid background knowledge on swine and equine influenza in a relevant biological system. To this end, swine and equine explants were infected with SIV and EIV, respectively, and accurately monitored for viral replication and structural changes at the site of infection (chapters two and three). These data were generated as reference phenotype to compare to non-host adapted infections, adding information to the pre-existing literature.
- Given the preliminary evidence on the impact of evolution on host range and tropism of H3N8 EIV and the isolation of EIV from pigs, chapter four aimed at comparing the replication potential of phylogenetically distinct EIVs in swine cell lines and respiratory explants. Further to providing a comparison between *in vitro* and *ex vivo* data we also wanted to evaluate the tissue tropism of EIVs for the different parts of the swine respiratory tract.
- Based on the strong genetic evidence of the avian origin of EIV, chapter five aimed at investigating how avian influenza viruses isolated from wild birds replicate in equine tracheal explants. For this purpose H3N8 AIVs isolated in Mongolia, a region densely populated with wild birds and horses, and belonging to different clades were tested and compared to the infection phenotype displayed by an EIV.

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Chapter 2

Characterization of H3N2 Swine Influenza in an *ex vivo* culture system of the swine respiratory tract

2.1 Introduction

Influenza in pigs is a worldwide spread respiratory disease. Typical symptoms include coughing, fever, depression and abdominal breathing (Richt *et al.*, 2003). First documented cases in symptomatic animals were reported following the 1918 human cases of Spanish flu (Shope, 1931). The current enzootic subtypes in the pig population are H1N1, H1N2 and H3N2. Many of these circulating viruses are reassortants containing genes of swine, human and avian origin in different combinations according to the geographical area (Vincent *et al.*, 2014). Since this disease was first recognized, pigs have been increasingly pointed out as important hosts in influenza ecology due to their susceptibility to influenza viruses originating from different animal species. Historical and epidemiological data support the vision that the pig can contribute to the polyhedric viral gene pool with unknown potential for humans.

The mixing vessel theory for which pigs are considered recipient hosts where influenza viruses of different origin can reassort was first proposed in 1985 (Ma *et al.*, 2008). Indeed, all the zoonotic infections that have originated from pigs confirm the key role of this host. This role for swine was in first place attributed due to the receptor distribution in their respiratory tract. Both human-like alpha 2-6 and avian-like alpha 2-3 sialated glycans, which are globally recognized as key receptors for influenza virus binding and entry (Matrosovich *et al.*, 2013), are present in the pig with different abundance according to the respiratory tissue. Studies on the detection of such receptors through lectin staining have yielded controversial results concerning their distribution (Van Poucke *et al.*, 2010). Recent evidence based on mass spectrometry has confirmed the presence of both types in the swine trachea and lung (Chan *et al.*, 2013) and thus the likelihood of co-infection with viruses of different origin. However, enhanced knowledge has demonstrated that the presence of both receptor types is not unique to this host, as these are also found in land based poultry such as quail (Thontiravong *et al.*, 2012) and

human lungs (Imai *et al.*, 2012). Moreover there is consistent evidence that sialated glycans are only in part determinants of influenza virus tropism as other co-receptors may be involved. Despite the huge scientific efforts put in place to understand the biological reasons behind the role of pigs in influenza ecology further evidence is required for a complete understanding.

For all the aforementioned reasons updated reliable methods to study influenza infection in this host are highly required. To investigate disease dynamics under controlled conditions *in vivo* experiments are the ideal condition. Nonetheless, a high number of animals is required to have statistical power. This aspect has an important impact from both an ethical and economical side and it is not always feasible. Primary cell cultures have been implemented for the isolation of SIV (Ferrari *et al.*, 2003). Nevertheless cell lines have some limitations. In first instance they do not represent the cellular complexity of a tissue that is usually composed of different cell types. Despite recent advance on suspended tri-dimensional cell culturing, usually cell cultures are bi-dimensional monolayers. Moreover, after serial passages primary cell cultures can undergo modifications and thus become less reliable.

In the recent years the research efforts to develop swine *ex vivo* organ cultures have offered a valid alternative to *in vivo* experiments in the influenza field. Development of *ex vivo* tissue cultures of the respiratory tract has been a fundamental tool to look at some aspects of host-pathogen interaction at the site of infection. Culturing respiratory tissues allows the maintenance to a great extent of the complex architecture and physiology of the epithelium as it is found in the living host with the advantage of working in a laboratory setting.

The first report of the use of swine tracheal organ cultures in influenza research dates back to 1974 (Schmidt *et al*., 1974). Given the viability of these organ cultures for more than three days they resulted suitable for studying infection dynamics over short time periods. Over the years the technique has been abundantly improved and extensively used to describe the infection dynamics of swine, avian and human influenza viruses. The direct comparison between *in vivo* and *ex vivo* data on virus replication has proved that this technique is reliable and that the results yielded are comparable (Van Poucke *et*

al., 2010). As for the possibility of looking at other aspects of infection Nunes *et al.* (2010) have described the cytokine expression following influenza infection in swine tracheal explants. This system cannot be implemented to study the intervention of the immune system upon infection, as it lacks blood supply. Albeit, the cytokine profile at the site of infection can still be studied. As a further application, swine explants have been used to evaluate the effect of serial adaptation of an avian H5N1 virus on swine cell lines on its tropism and pathogenicity in pig tissues (Londt *et al.*, 2013).

Only few studies have looked at the tropism of viruses throughout the respiratory tract, from the nasal mucosa down to the lungs. Most studies were focused on specific aspects of infection in one or two anatomical locations of the respiratory tract. Currently, despite some incoherency with earlier studies, there is consistent information available on the different receptor distribution along the respiratory tract of pigs and its relation to influenza virus tropism. Therefore, when possible, the use of the entire respiratory tract should be implemented when assessing viral replication dynamics in this particular host.

The aim of the present chapter is to thoroughly describe the phenotype of infection of an H3N2 swine influenza virus in swine nasal mucosa, trachea and lung explants throughout a 96 hours time course. The purpose of this work is to comprehensively characterize a host adapted influenza infection (SIV in swine tissues), to compare this with other species-specific influenza infections (equine influenza in horse tracheal explants, chapter three) and to have a standard reference when testing other influenza viruses in this system (equine influenza, chapter four). To import the technique into the Division of Comparative Biomedical Sciences at the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe), a two-weeks training in the virology laboratory of the faculty of Veterinary Medicine of the University of Ghent, where the protocol was initially developed, was arranged under the supervision of Prof. Kristien Van Reeth and Dr. Sjouke Van Poucke. This exchange period was conducted within the context of the EU FP7 Project FLUPIG (Project Nr° 258084).

To describe the infection dynamics of swine influenza in the *ex vivo* system, we infected swine nasal mucosa, tracheal and lung explants with A/swine/Italy/8088/2006 (H3N2). A

detailed phenotype of infection was described through virus quantification, evaluation of the ciliary activity, histological examination and immunohistochemical detection of viral nucleoprotein (NP).

2.2 Materials and Methods

2.2.1 Virus

To describe the infection dynamics of H3N2 swine influenza in a swine *ex vivo* system a Eurasian H3N2 isolated in Italy in 2006 was used. A stock of A/swine/Italy/8088/2006 (SIV H3N2) was obtained by inoculating 9 to 11 day old SPF embryonated chicken eggs. After 72 hours allantoic fluids were harvested, clarified by centrifugation at 3000 rpm for 10 minutes, aliquoted and stored at -80°C. Viral titres were determined by standard plaque assay on MDCK cells (Matrosovich *et al.*, 2006) followed by immunostaining as described below.

2.2.2 Animals

Six to 8-week old commercial hybrid female piglets were purchased from a high health status farm. To assess seronegativity of the herd to Influenza A virus, blood samples were collected at the farm before purchase and at the time of euthanasia. Sera were tested in duplicate with an ELISA kit targeting anti viral NP antibodies (IDScreen®, IDvet). Animals were housed in HEPA-filtered BSL-3 facilities with unlimited access to water and feed. Environmental enrichments such as chains and balls were also provided. Upon arrival animals were treated by IM injection of Ceftiofur (Naxcel® Pfizer, 1ml/20 kg pv) to clear the airways from the major bacterial pathogens such as *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Haemophilus parasuis* and *Steptococcus suis*. Euthanasia was performed by IV administration of 12.5 mg/kg of Pentothal sodium (Intervet) and subsequent exsanguination. Upon euthanasia the head and whole respiratory tract, from the larynx to the lungs, were collected and aseptically transported to the laboratory for explant preparation. All experiments were conducted after the approval of the IZSVe ethical committee (Approval nr. CE IZSVe_11/2012).

2.2.3 Explant preparation

Nasal, tracheal and lung explants were prepared as previously described (Nunes *et al.*, 2010; Van Poucke *et al.*, 2010), with slight modifications.

2.2.3.1 Air-liquid interface culture system (ALI)

All explants described below were cultured according to the air-liquid interface principle. This culture technique consists of resembling the physiological condition of respiratory tract tissues that receive nutrients from the blood and are exposed to air. To obtain such conditions *in vitro* we used the technique described by Nunes *et al*. (2010). Explants were placed on sterile filter paper strips with the epithelium facing upwards. The strips were placed on top of sterile agarose plugs in six-well plates (fig. 1A) that allowed an elevation of the explant in the well. Each well contained five ml of culture medium in order to soak the paper strip and have a thin film of medium covering the basal part of the explant (fig. 1B). The upper part of the explant was in turn exposed to air.

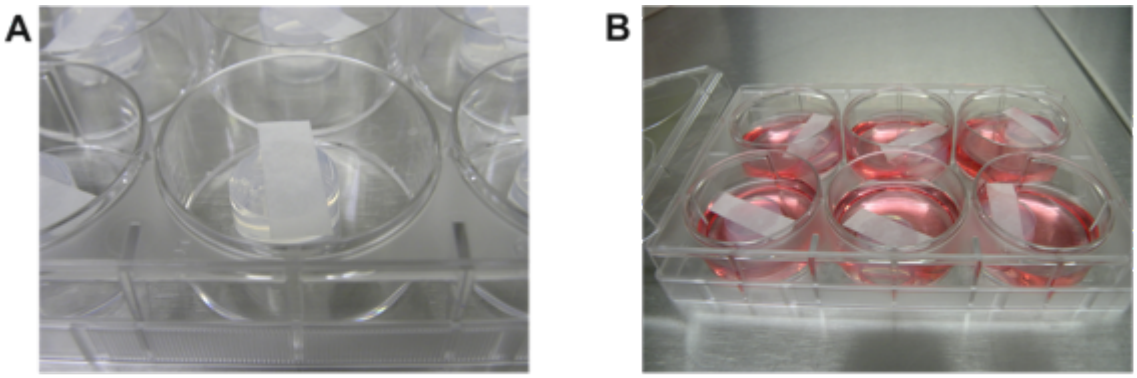

Figure 1. Air-liquid interface culture system. A) Agarose plug with filter paper on top. B) Six-well plate containg 5 ml of medium as used for all explant cultures.

2.2.3.2 Nasal mucosa explants

Upon euthanasia the head was separated from the rest of the body, thoroughly washed with disinfectant soap and rinsed with ethanol. The skin and muscles around the nose were removed and the nose was sawed off the skull. The nose was opened lengthways and the nasal mucosa was carefully stripped from the nasal septum (fig. 2A) and the medial part of the ventral turbinates (fig. 2B). The tissue was placed in a sterile Petri dish and abundantly washed with phosphate buffered saline (PBS) containing 10% penicillin-streptomycin (P/S, Gibco), 5% gentamycin (Gibco) and 1% fungizone (Gibco). Next it was cut into squares of approximately 0.5 x 0.5 cm (fig. 2C) and ALI cultured in a humidified incubator at 37°C with 5% CO_2. Each well contained five ml of 50:50 of Dulbecco's Modified Eagle's Medium (DMEM, Gibco) and Roswell Park Memorial Institute (RPMI, Gibco) supplemented with 1% P/S and 1% gentamycin. From each animal approximately 50 nasal explants, of exclusively respiratory mucosa, were obtained.

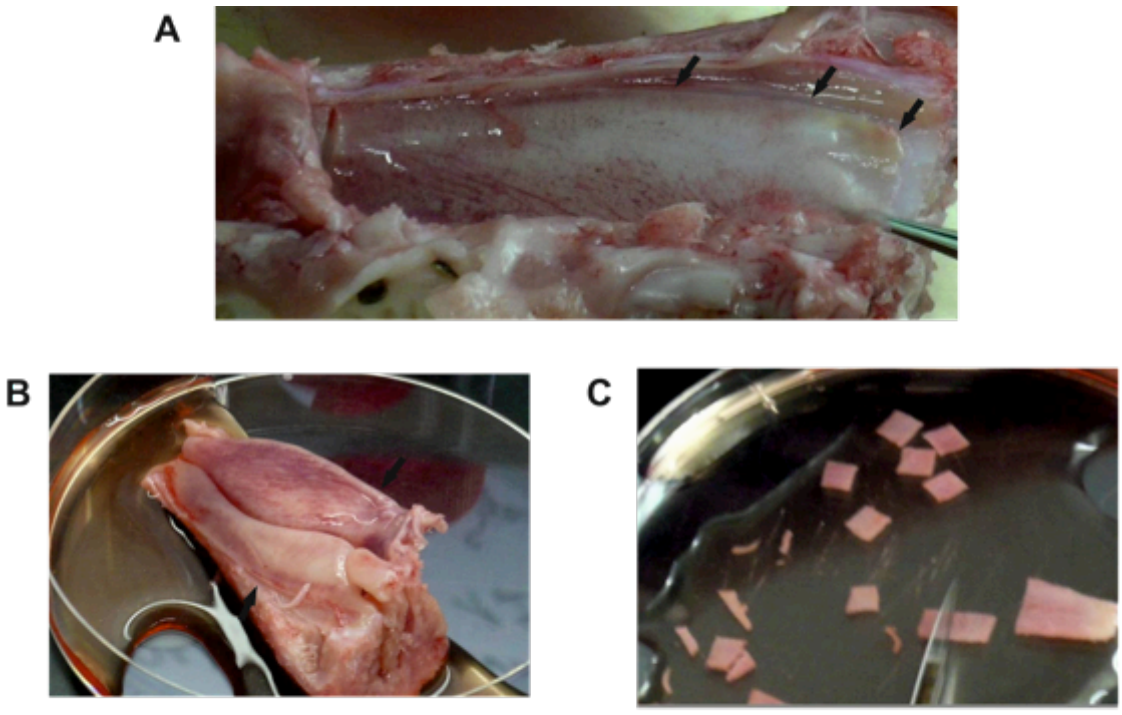

Figure 2. Preparation of nasal mucosa explants. A) Mucosa of the nasal septum indicated by black arrows. B) Mucosa of the nasal turbinates indicated by black arrows. C) Upon collection of the nasal mucosa from the aforementioned sites the tissue was cut into squares in a petri dish.

2.2.3.3 Tracheal explants

The trachea was excised from the larynx, separated from the lungs just above the level of the bifurcation (fig 3A), placed in warm medium (50:50 DMEM-RPMI) supplemented with 1% P/S, 1% fungizone and kept in a humidified incubator at 37°C with 5% CO_2. The medium was replaced five times every 40 minutes to reduce bacterial presence. After the last wash the connective tissue surrounding the cartilage was removed. The trachea was opened lengthways, pinned onto a sterile board with needles (fig. 3B) and cut into squares of approximately 0.5 x 0.5 cm that were ALI cultured in five ml of the same medium used for the washing step (fig. 3C) in a humidified incubator at 37°C with 5% CO_2. From each animal 70 to 90 tracheal explants were obtained.

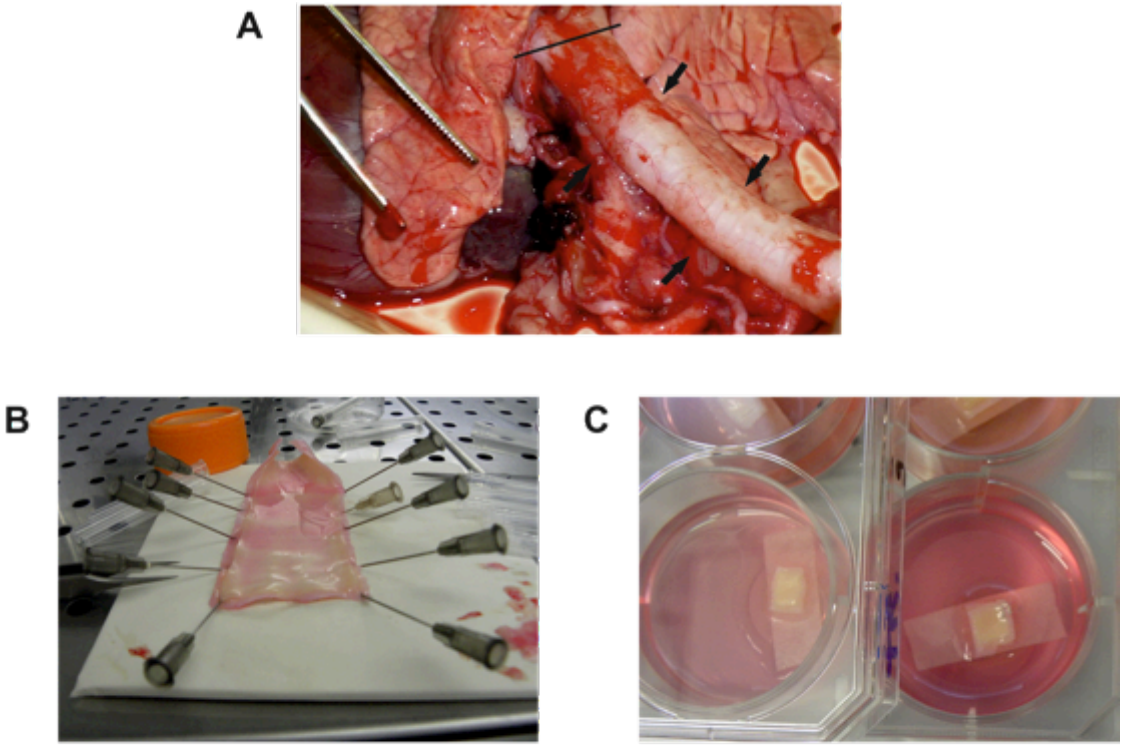

Figure 3. Preparation of tracheal explants. A) The trachea (indicated by black arrows) was separated from the lungs just above te bifurcation (indicated by a black line). B) Trachea opened legnthways and pinned onto a sterile board.C) Tracheal explant cultured at the air-liquid interface in a 6-well plate.

2.2.3.4 Lung explants

Lung explants were obtained by filling the right apical lobe with 1% agarose (Type VII A, Sigma Aldrich) through an 18G catheter introduced down the tracheal bronchus (fig. 4A). The lung was then placed at 4°C for 20 min to allow the agarose to polymerize. The expanded lobe was then cut into pieces that were placed in a 20 ml syringe filled with 4% agarose (fig. 4B). After an incubation of 20 min at 4°C the closed part of the syringe was cut off and the agarose cylinder with the lung pieces inside was pushed forward. Thin slices were then cut with a razor blade (fig. 4C). The agarose surrounding the lung was carefully removed and the tissue was cut to obtain explants of approximately 0.5 x 0.5 cm. Lung explants were abundantly washed in PBS containing 10% P/S, 5% gentamycin and 1% fungizone and ALI cultured with 5 ml of DMEM, bovine insulin (2.5 µg/ml, Sigma Aldrich), hydrocortisone (0.5 µg/ml, Sigma Aldrich), vitamin A (0.5 µg/ml, Sigma Aldrich) and gentamycin (0.1 mg/ml, Gibco) in a humidified incubator at 37°C with 5% CO_2. After 24 hours of culture and prior to infection, lung explants were thoroughly washed with warm PBS to remove any remaining agarose. From each animal approximately 100 lung explants were obtained.

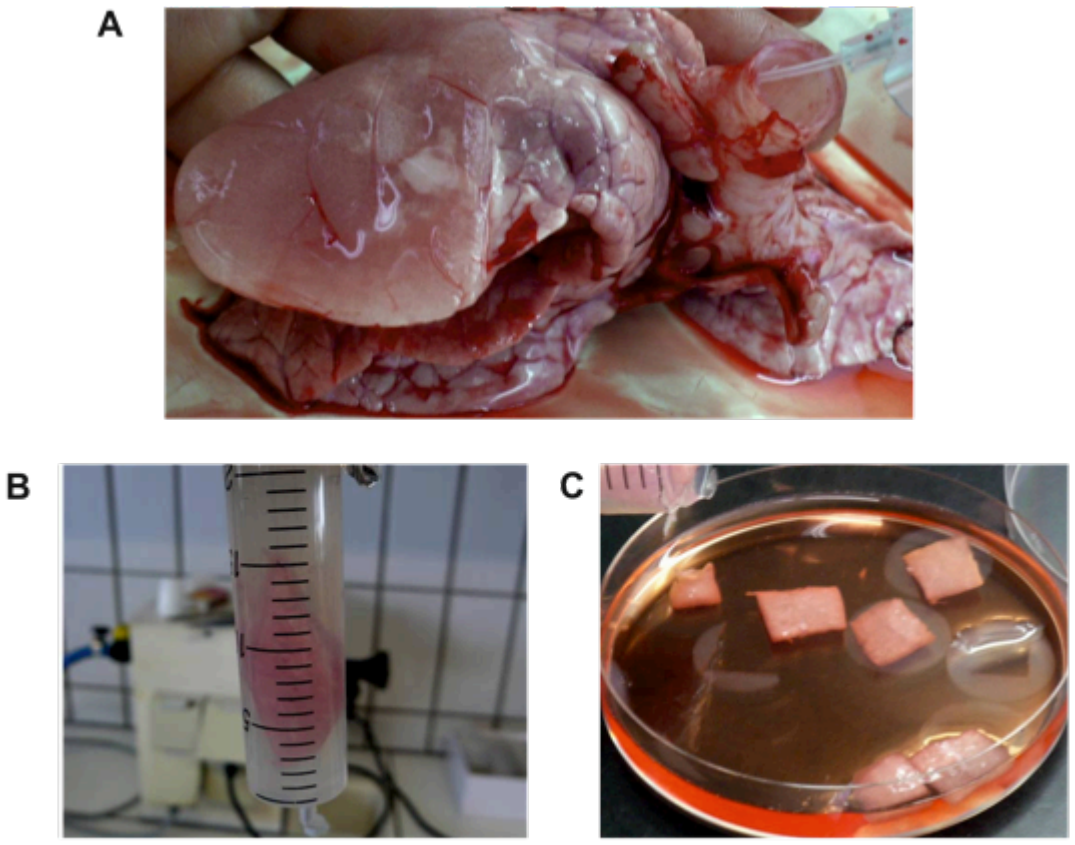

Figure 4. Preparation of lung explants. A) The right apical lobe was filled with 1% agarose through a sterile catheter introduced down the tracheal bronchus. B) The inflated lobe was cut into pieces that were introduced into a syringe filled with 4% agarose. C) The agarose cilinder containing the lung pieces was pushed forward and thin slices of tissue were cut in a petri dish.

2.2.4 Vitality of the explants

Vitality of the explants was checked daily through examination of the ciliary activity under a light microscope and changes in media color. Additionally, in tracheal explants a colored bead assay was performed to assess the reduction of ciliary activity as a consequence of viral infection as described previously (Nunes *et al.*, 2010). Five microliters (µl) of blue-dyed polystyrene microspheres (Polybead®, Polysciences Inc) were pipetted on top of tracheal explants (fig. 5, time 0) that were then placed back in the incubator. After five minutes explants were checked for bead clearance over a 50 minutes time course. The assay was considered positive when the beads were completely cleared to one side of the explant as shown in figure 5. A bead assay was attempted on nasal explants but the results observed were highly variable in both mock and SIV infected explants. Thus, these were not included as a feature of the phenotype of infection. As lung tissue does not have a clear orientation and cilia are present only in bronchial and bronchiolar structures, the colored bead assay is not applicable.

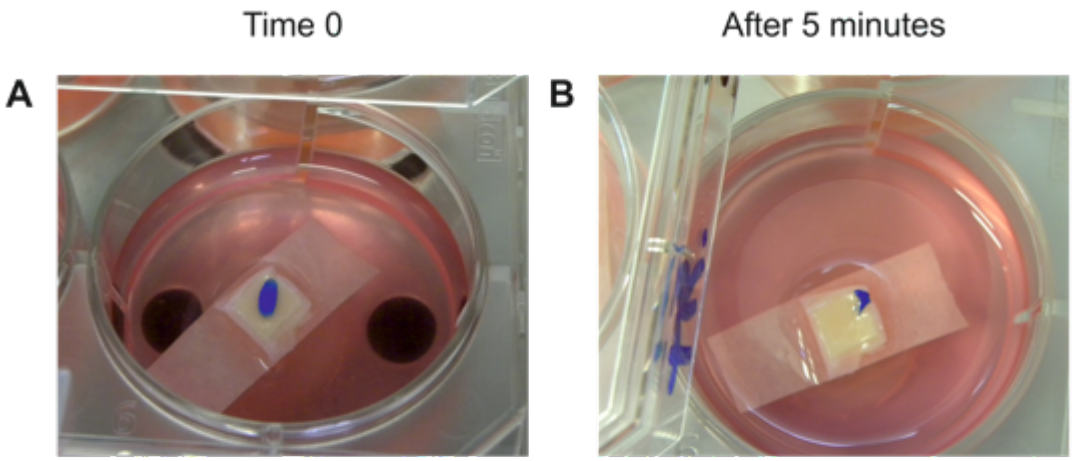

Figure 5. Colored bead assay. A) Five microliters of blue dyed microsphere beads were placed in the middle of a tracheal explant. B) Positive bead clearance at 5' expressed as lateralization of the beads to one side of the explants caused by a coordinated activity of the cilia present in the tracheal epithelium.

2.2.5 Explant infection

After 24h of culture explants were infected with five µl of viral suspension containing 200 plaque forming units (pfu). Stock virus was diluted in MEM supplemented with 1% P/S, 1% L-glutammine and 1 µg/ml of TPCK treated trypsin. Mock-infected explants (inoculated with five µl of medium used for virus dilutions) were included in all experiments. Replicate samples were collected at 6, 24, 48, 72 and 96 hours post infection (hpi) for virus quantification and histological examination. Nasal explants were kept only until 72 hpi because of a high incidence of bacterial contamination observed after this time point. Data reported herein results from at least three independent experiments.

2.2.6 Virus quantification in infected explants

Explants were immersed in 500 µl of cold sterile PBS, shaken in a tissue lyser (Qiagen) at 20 Hz for ten minutes and centrifuged at 16000xg for 10 min at 4°C. The collected supernatant was used for virus titration using the Tissue Culture Infectious Dose ($TCID_{50}$) assay on MDCK cells according to the Reed and Muench formula. To accurately determine viral titres at 72 hpi, plates were checked for presence of cytopathogenic effect (CPE), haemagglutinating activity of the supernatant and immunostained. Viral titres were expressed as $\log_{10}$ $TCID_{50}$/ml.

2.2.7 50% Tissue Culture Infectious Dose Assay ($TCID_{50}$)

This assay was performed to determine the endpoint dilution of viral presence. MDCK cells were seeded in 96-well plates in order to obtain a confluent monolayer in 24 hours. Samples were serially diluted (1:2 or 1:5) in MEM supplemented with 1% P/S, 1% L-glutammine and 1 µg/ml of TPCK treated trypsin (infection medium). Plates were washed twice with warm PBS and 50 µl of each dilution were inoculated onto the monolayer. After 2 hours of incubation at 37 °C, 100 µl of infection medium were added to each well and plates were placed back in the incubator for 72 hours. Samples were tested in quadruplicate wells. Plates were checked for presence of virus induced cytopathogenic effect such as rounding of the cells and destruction of the monolayer.

2.2.8 Hemagglutination Test in Microtitre Plates

To obtain a more precise evaluation of viral titres we looked for the presence of haemagglutinating activity in the supernatants of the 96-well plates were the $TCID_{50}$ assay was conducted. This method is based on the tendency of the viral HA protein to bind receptors that are present on the surface of red blood cells causing them to agglutinate. The haemagglutinating activity of the supernatants was visualized by placing 50 µl of supernatant in a well of a 96-well plate and subsequently adding 50 µl of 0.5% chicken red blood cells (RBCs). After 40 minutes of incubation plates were checked for the presence of RBCs aggregates (positive) or presence of a button of RBCs at the bottom of the well (negative).

2.2.9 Immunostaining of plates

To finally confirm viral titres, plates were washed twice with PBS and fixed in 80% acetone. A 10 minutes permeabilisation step using 0.5% Triton X in PBS was performed followed by a 30 minutes blocking step using PBS-0.05% Tween supplemented with 10% Bovine serum albumnin (BSA). Immunostaining was conducted using a mix of two primary commercial mouse monoclonal anti Influenza A NP antibodies (MAB 8257 and MAB 8258 Millipore, 1:4000) and a secondary Peroxidase labeled goat anti-mouse IgG secondary antibody (KPL, 1:2000). True Blue™ (KPL) peroxidase substrate was used to visualize infected cells.

2.2.10 Histological examination

Samples of the selected organs were collected, immediately fixed in 10% neutral buffered formalin, routinely processed and paraffin embedded for histological examination. Three micrometer sections were cut, stained with hematoxylin and eosin (H&E) and examined under light microscope.

Immunohistochemical staining for virus nucleoprotein was performed using BenchMark ULTRA (Roche) autostainer with ultraView Universal DAB Detection Kit (Roche). Antigen retrieval was performed with Protease 2 (Roche) for 12 minutes and Normal Horse Serum (VECTOR Laboratories) was used for 20 minutes as a reagent for blocking non-specific binding. The sections were incubated for one hour and 20 minutes at room

temperature with a rabbit polyclonal antiserum targeting equine influenza NP protein (provided by Pablo Murcia, University of Glasgow) diluted 1:2000 with Ventana Antibody Diluent with Casein (Roche). A second incubation was done using Ventana Antibody Diluent with Casein (Roche) for 12 minutes to reduce the background staining. To ascertain structural details, sections were counterstained with the Hematoxylin II (Roche) for 8 minutes. Positive and negative control slides were present in each immunohistochemistry run. Samples were evaluated for the presence and distribution of immune staining.

2.2.11 Statistical analyses

Results were analysed using GraphPad PRISM® Version 6 software for statistical analysis performing a one-way Anova. Differences were considered significant when $P<0.05$.

2.3 Results

2.3.1 Phenotype of infection of SIV H3N2 in nasal explants

To validate our *ex vivo* system we infected the different organ cultures with an H3N2 SIV. First we wanted to determine whether the virus was able to replicate in cultured tissues by quantifying the viral load contained in the explants at different time points. Increasing viral titres were recorded in nasal mucosa explants from 24 to 72 hpi as shown in figure 6A. Observed titres ranged from 3 to 7 $\log_{10}$ $TCID_{50}$/ml.

Next, we wanted to determine whether there was any evident virus-induced damage in the tissue. Histological examination of infected and control explants is shown in figure 6B. Mock-infected explants maintained their structure throughout the study without major changes. Presence of typical features of influenza infection such as epithelial disruption and vacuolization were observed at 48 and 72 hpi in SIV infected explants. Some samples also displayed a thinning of the epithelium and loss of cilia at the apical surface.

To confirm that the lesions observed were virus induced and to determine virus localization in the tissue we conducted IHC. Viral NP was present in the epithelium from 24 hpi (few cells), increased at 48 and by 72 hpi it was scattered in vacuolised areas. As shown by the two panels in figure 6B the histological damage and IHC positivity matched. Both the damage observed through H&E and viral presence by IHC were detected in localized areas of the explants suggesting that the infection was focal.

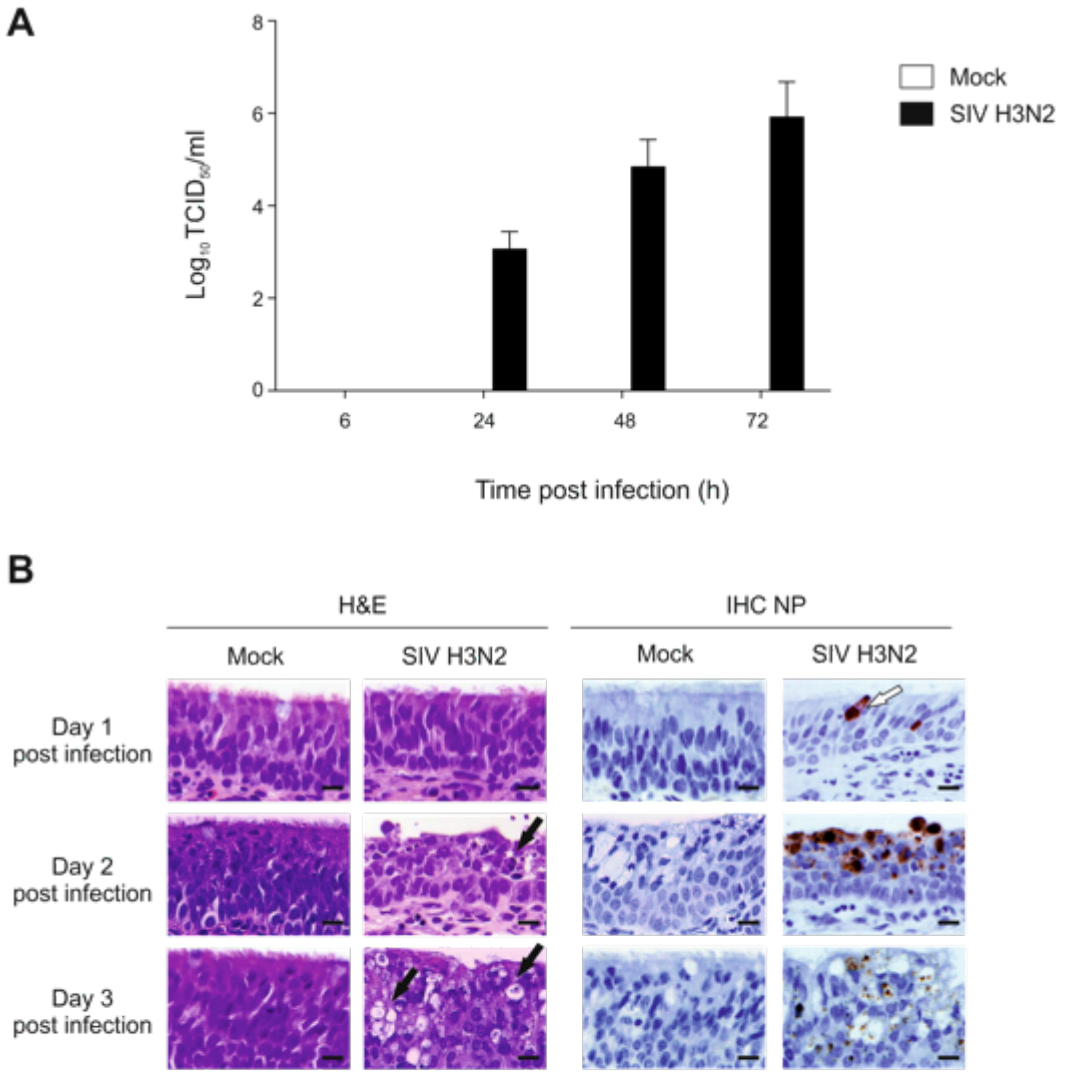

Figure 6. SIV replication in nasal mucosa explants. A) Virus quantification in infected explants at different time points. Bars represtent the mean and SEM of three independent experiments. B) Histological examination. Hematoxylin and eosin (H&E) panel on the left showing histological damage in SIV infected explants compared to mock-infected. Black arrows show epithelial disruption on day 2 p.i. and vacuolisation on day 3 p.i. The right panel displays viral nucleoprotein detection (IHC NP). Positive cells are stained in brown and indicated by white arrows. Black horizontal bars represent 50 µm.

2.3.2 Phenotype of infection of SIV H3N2 in tracheal explants

SIV H3N2 replicated in tracheal explants as a 10^3 fold increase in viral load (compared to the inoculum) was detected at 24 hpi. Replication dynamics presented a first peak at 48 and a second peak at 96 hpi (fig. 7A). Viral titres reached a $\log_{10}$ $TCID_{50}$/ml titre of seven.

In samples collected for histological examination presence of virus induced damage was evident from 48 hpi onwards (fig. 7B). Such feature included loss of cilia at the apical surface, thinning of the epithelium and presence of mucus on the surface of the explant. In some explants the damage was already evident at 24 hpi. Mock-infected explants maintained their structure throughout the study without major changes.

To ascertain the cellular target of infection we conducted IHC for viral NP. Positivity was detected in epithelial cells from 24 hpi onwards with a maximum distribution across the epithelium at 48 hpi. Furthermore positive signal was detected in the mucus and cellular debris on the surface of the explant derived from epithelial disruption (day 3 pi).

To investigate whether viral infection affected ciliary activity we conducted a colored bead assay. Mock-infected explants maintained their clearance ability unaltered until 96 hpi. This test revealed a reduced clearance in SIV infected explants from 48 hpi onwards (fig. 8A). Compared to mock-infected, the difference was significant at 48, 72 and 96 hpi ($P<0.001$). Figure 8B shows a bead assay conducted at 96 hpi. A mock-infected explant is still able to lateralize the beads to one side of the explant whereas in a SIV infected the beads remain in the center of the explant. An overall trend in clearance decrease along time was observed but the recorded delay ranged from 15 minutes to complete absence of clearance depending on the explant, on the day of sampling and on the animal. Such difference might be due to a varying degree of virus-induced damage in each explant.

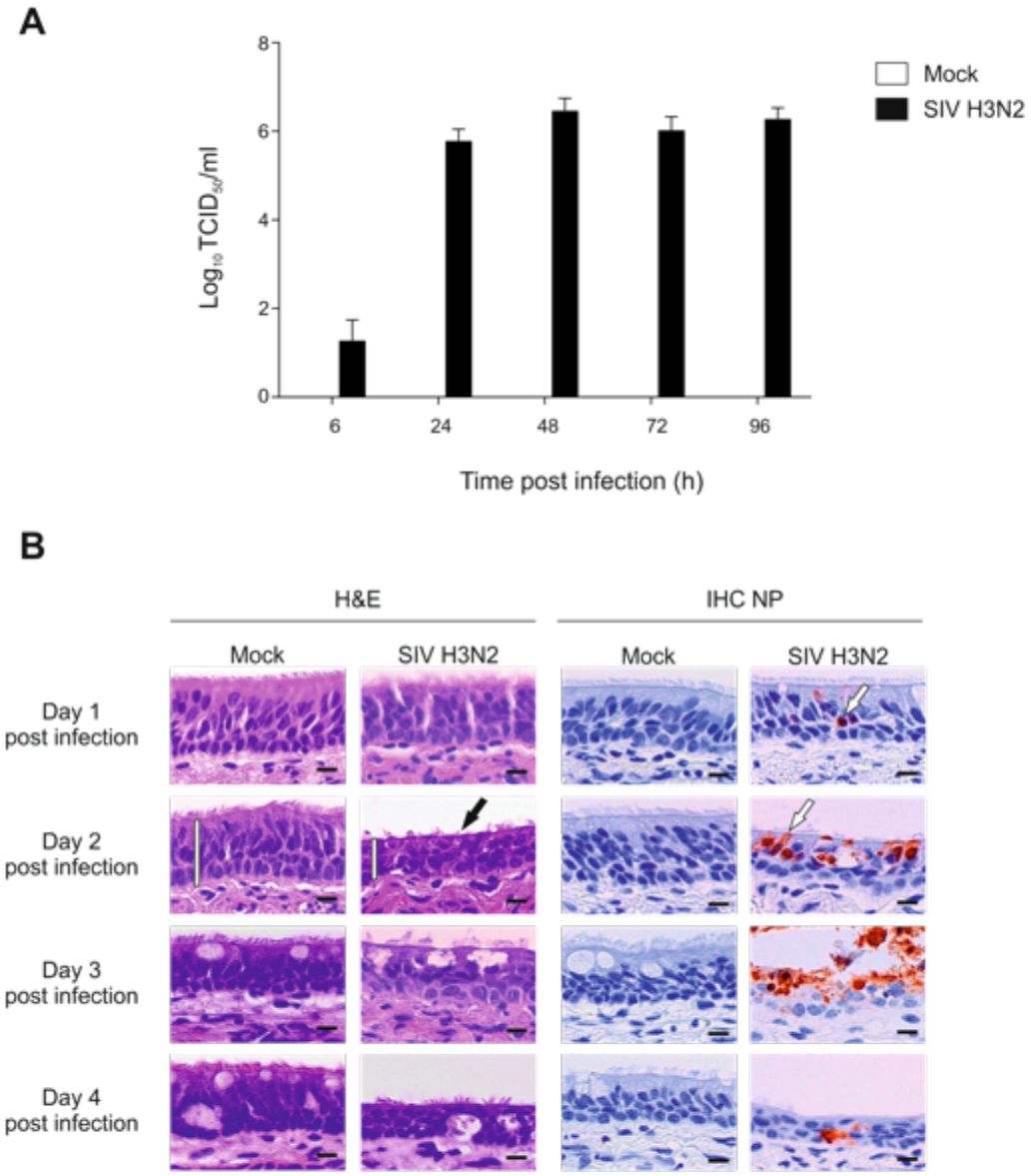

Figure 7. SIV replication in tracheal explants. A) Virus quantification in infected explants. Bars represent the mean and SEM of three independent experiments. B) Histological examination. Hematoxylin and eosin (H&E) panel on the left showing histological damage in SIV infected explants and mock-infected. White vertical lines show the difference in epithelial thickness. Black arrows show the loss of cilia on the epithelial surface. The right panel displays viral nucleoprotein detection (IHC NP). Positive cells are stained in brown and indicated by white arrows. Black horizontal bars represent 50 µm.

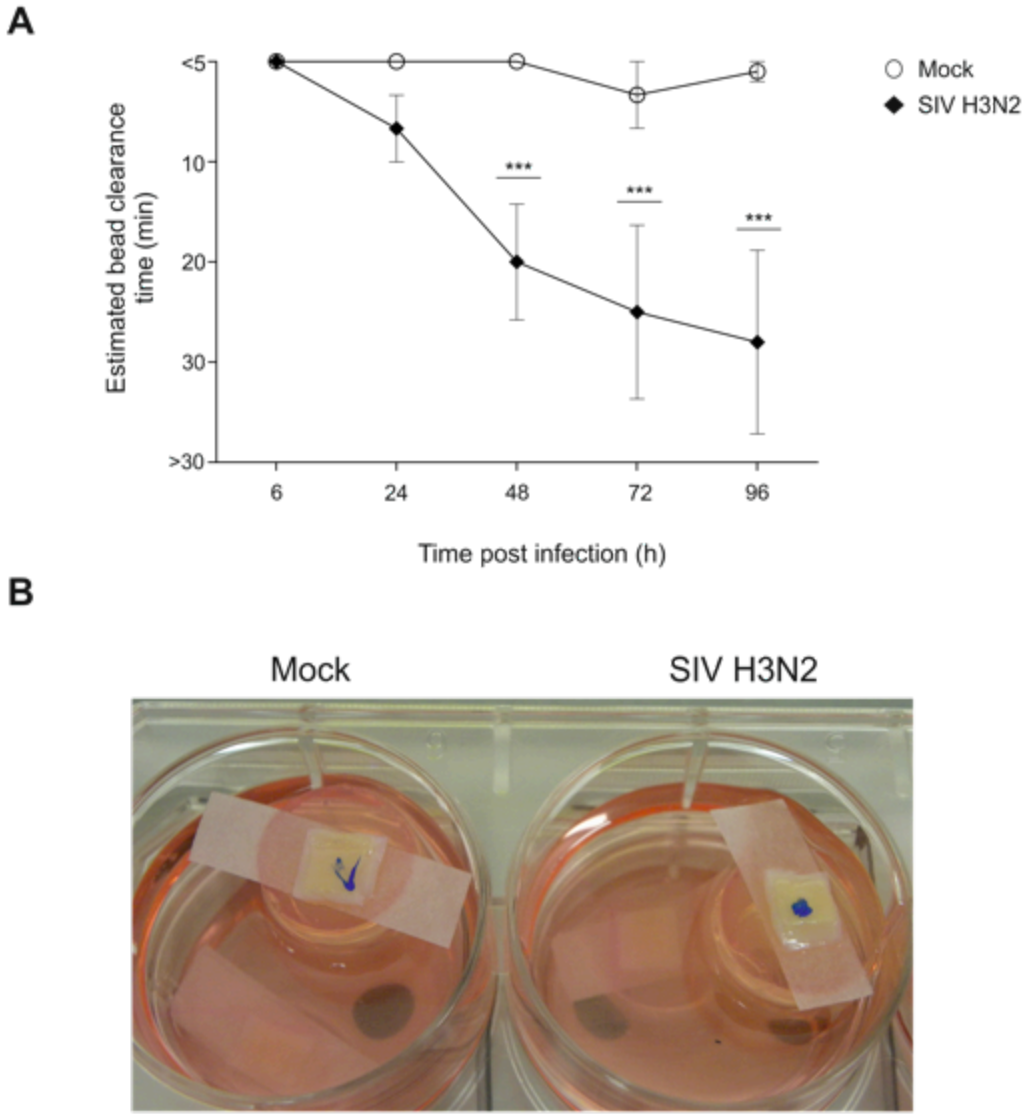

Figure 8. Bead assay in SIV infected tracheal explants. A) Graph shows estimated bead clearance time in mock and SIV infected explants over a 96 hours time course. Lines represent the mean and SEM of three independent experiments. *** P<0.001. B) Image of a 96 hpi bead assay in mock and SIV infected explants.

2.3.3 Phenotype of infection of SIV H3N2 in lung explants

Lung explants yielded increasing viral loads from 24 to 96 hpi as shown in fig. 9. Titres reached in this explant system varied from 4 to 6 $\log_{10}$ $TCID_{50}$/ml.

At histological examination, (fig. 9B) infected samples displayed damage at the level of the bronchiolar epithelium similar to that observed in nasal and tracheal epithelium. The damage observed in bronchioli was restricted to a limited area of each bronchiolar structure suggesting once more a focal infection. Mock-infected samples also displayed areas of epithelial damage due to the insult represented by the presence of the remaining agarose and the manipulation of the organ during explant preparation. Therefore IHC was key to confirm that the lesions observed were due to virus replication. Given these findings, histological examination of lung explants was considered with caution and the phenotype of infection mainly relied on IHC and virological findings.

As for IHC, NP staining confirmed viral localization mainly in the bronchiolar epithelium and only few alveolar cells were found positive. The presence of the first positive cells was detected at 24 hpi and the number of positive bronchiolar structures increased until 96 hpi. The peak of viral presence detected virologically and by IHC was recorded between 72 and 96 hpi. Compared to the other two explants types, infection dynamics in the lungs seemed slightly delayed.

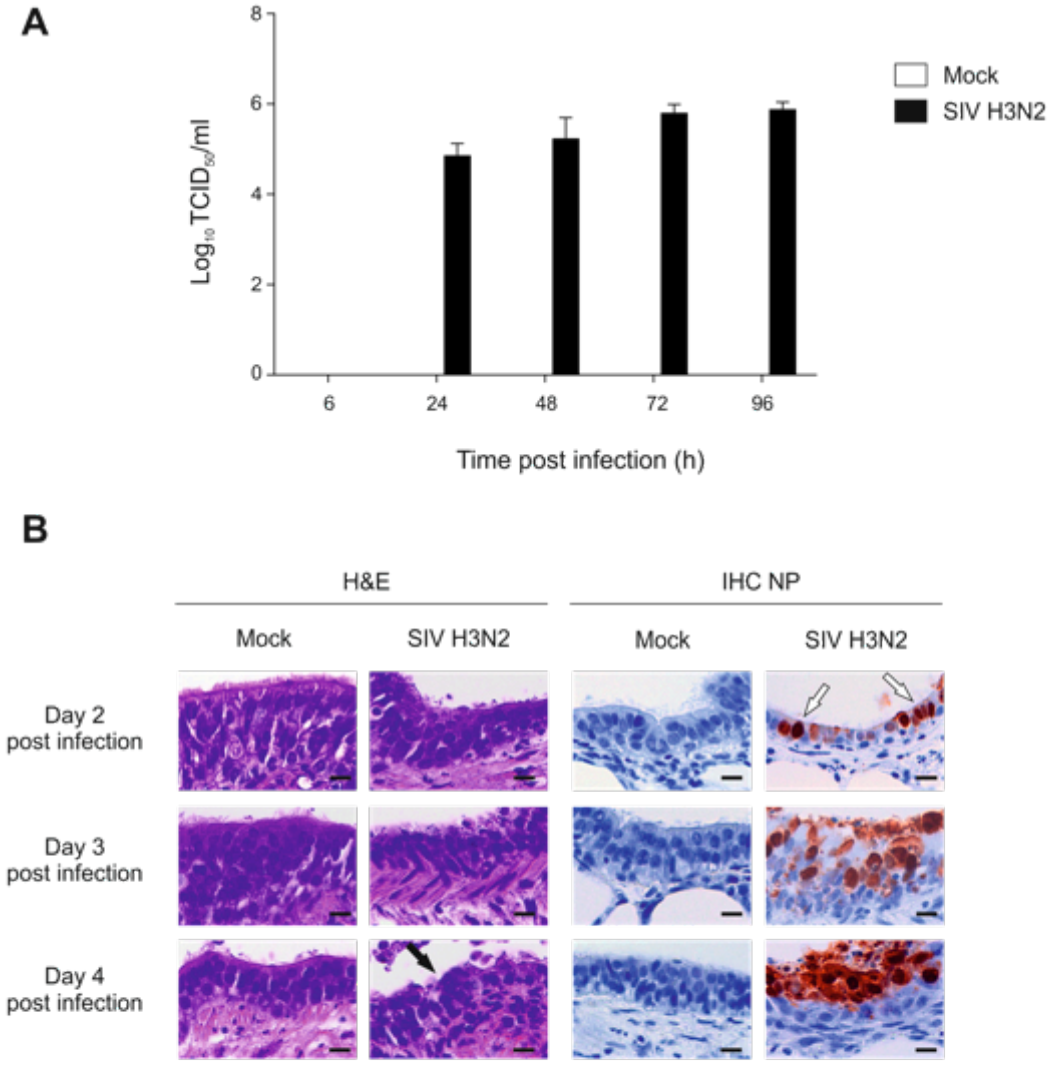

Figure 9. SIV replication in lung explants. A) Virus quantification in infected explants at different time points. Bars represent the mean and SEM of three independent experiments. B) Histological examination. Hematoxylin and eosin (H&E) panel on the left showing histological damage in SIV infected explants, as indicated by black arrows, compared to mock-infected. The right panel displays viral nucleoprotein detection (IHC NP). Positive cells are stained in brown and indicated by white arrows. Black horizontal bars represent 50 µm.

2.4 Discussion

Swine influenza virus affects the respiratory tract of pigs causing the onset of symptoms such as cough, nasal discharge and dyspnea. In the present chapter, we described a detailed phenotype of infection of an H3N2 SIV in a swine explant system that covered the whole respiratory tract (e.g. from the nose to the lungs) in order to look at the dynamics of disease at the site of infection.

Previous studies have investigated the replication of SIV in swine explants, however to a limited extent and with some differences compared to this work. Van Poucke *et al*. (2010) first described the establishment of this complete explant system and used three different swine influenza viruses to validate it. In their study they followed virus replication only up to 48 hpi and they did not quantify the virus in the explants but in the culture medium. Moreover histological findings associated with infection were not described and viral detection in infected cells was performed only at one time point (48 hpi). Nunes and colleagues used swine tracheal explants to investigate the cytokine expression following SIV H1N1 infection (Nunes *et al.*, 2010). This work described a similar phenotype of infection over a five day time course and the results obtained on virus quantification, histological damage and IHC clearly resemble those described herein with a virus of a different subtype (H3N2). Nevertheless Nunes *et al*. only used tracheal explants. An *ex vivo* study on the infectivity of the pandemic H1N1 and H5N1 AIV in tracheal, bronchial and lung explants was conducted but the results related to the positive SIV H1N1 control were again limited to 48 hpi and only based on virus quantification and IHC (Löndt *et al.*, 2012).

We have compared the replication potential in the different parts of the respiratory tract over a 96 hour time course. To mimic a natural contact between the virus and the target tissue we have chosen to administer a low viral dose through droplet infusion on the surface of the explants. This infection technique combined with the air-interface system avoids re-infection by contact of the epithelial surface with the culture medium, as in semi-submerged systems. To have a precise measurement of the virus yields at the level of the tissue we decided to collect the individual replicate explants and quantify the virus released from the explant following immersion in PBS and tissue rupture. SIV

H3N2 abundantly replicated throughout the respiratory tract with a similar trend. In all tissues we recorded a marked increase in viral titres beginning at 24 hpi with slight differences in the following time points depending on the anatomical region. In nasal mucosa and lung explants viral titres increased daily until the last time point at which samples were collected (72 and 96 hpi, respectively) whereas in tracheal explants a double peak of replication at 48 and 96 hpi was observed. Such differences are most likely due to tissue specific innate immune responses that develop following infection (Nunes *et. al*., 2010).

Through histological and immunohistochemical examination we were able to identify the epithelium of all three systems (nasal, tracheal and bronchiolar) as the major target of infection. The thickness of the epithelium decreased as the infection progressed. By examining samples every 24 hours we were able to follow epithelial changes associated with the ongoing infection. These changes included epithelial disruption, vacuolization of epithelial cells, progressive loss of cilia and presence of cellular debris at the apical surface and thinning of the epithelium. The extent of histological damage varied among individual explants. This difference was also reflected in the bead assay conducted on tracheal explants, which is essentially an indirect measure of virus-induced damage. The delay in bead clearance varied from 15 minutes to complete absence of clearance. Similar results in variations were reported by Meng *et al.* (2013) when observing under a light microscope the ciliary activity of bronchiolar structures present in precision cut lung slices infected with SIV.

Staining for viral nucleoprotein confirmed these observations: in some explants viral NP was spread across the epithelium whereas in others it was only present in one or more focal areas. As the exact number of susceptible cells in the explants cannot be controlled we can hypothesize that such factor can influence the outcome of infection. Furthermore the innate immunity triggered as a consequence of the infection of epithelial cells may also play a role as the magnitude of its effect also depends on the number of cells in each explant. Taken together, these results show that the extent of infection in explants can vary from focal to widespread.

Overall the results provided in this chapter offer an extensive description of multiple aspects of SIV infection along the respiratory tract of naïve pigs. Such findings are consistent with previous studies and could be used as a reference when studying the infection dynamics of any other influenza virus in this system. As previously reported, we have confirmed that this system is susceptible to swine influenza infection and that lesions observed in the respiratory epithelium closely resemble those of naturally and experimentally infected animals (Janke, 2014). Finally we have also highlighted a different degree of infection as an aspect to consider when using this technique.

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Chapter 3

Development and validation of an *ex vivo* culture system of the equine trachea to study influenza infection

The present work is the result of a collaboration that was conducted at the Centre for Virus research, University of Glasgow, together with Dr. Gaëlle Gonzalez and under the supervision of Dr. John Marshall and Dr. Pablo R. Murcia.

3.1 Introduction

Equine influenza is a respiratory disease of horses representing one of the most common causes of respiratory distress. Up to date two subtypes of equine influenza virus (EIV) have been identified. The H7N7 was the first EIV discovered in association with respiratory disease in horses in Czechoslovakia in 1956 (Sovinova *et al*., 1958). In 1963 a second subtype was identified in Florida in race horses imported from Argentina (Waddel *et al*., 1963) and the genetic features of this virus revealed that it had originated from the avian gene pool (Worobey *et al.*, 2014). Both subtypes circulated worldwide until the 1980's after which there was an increased detection of uniquely the H3N8. Given the missed detection of H7N7 in the past 30 years it is believed that this subtype is no longer present. Epizootics of equine influenza have been associated with important economic losses in the equine field due to the high morbidity of this disease. Therefore investigating disease pathogenesis and assessing the efficacy of prevention tools is of utmost importance.

The horse has been increasingly recognized as an important host in influenza ecology. It is susceptible to avian influenza viruses, as demonstrated by the aforementioned origins of the 1960's H3N8. To further support this statement, a second independent introduction of an avian H3N8 occurred in horses in 1989 in Jilin, China (Guo *et al*., 1991). This virus circulated in horses in China for a couple of years before disappearing. The fact that the H3N8 has been quite a stable lineage in horses for many decades together with the lack of gene exchange with other influenza viruses led to the initial impression that the horse could have been a dead-end host (Gorman *et al*., 1991). Instead, it is from an H3N8 EIV that canine influenza first emerged in dogs in the early

2000s (Crawford *et al.*, 2005). After its first appearance in the dog population, repeated introductions were subsequently detected. EIV has been detected also in pigs (Tu *et al.*, 2009) and more recently in Bactrian camels (Yondon *et al.*, 2014). These inter-species transmission events are a huge potential source of information concerning host switching of IAVs. Nevertheless, the experimental study of disease in horses is rather difficult. Beyond an economical and practical reason, there are several ethical issues in using horses in research as they are seen by many as companion animals. Furthermore animals suitable for influenza experiments are hard to find as this disease is commonly found in horses and the majority of animals are routinely vaccinated.

As an alternative to *in vivo* experiments, *ex vivo* cultures of the equine respiratory tract have been previously described (Hamilton *et al.*, 2006; Lin *et al.*, 2001; Vandekerckhove *et al.*, 2009). These studies have implemented the use of equine nasal mucosa, nasopharynx, guttural pouch and trachea as *in vitro* models to investigate infectious diseases and more in general tissue physiology and pathology. The advantages of using such a technique have been extensively described in chapter one and two.

As described for the pig in chapter two, in order to have a reliable technique to study influenza infection in the horse host under controlled conditions we developed an equine tracheal explant system. Before using this system to study the replication dynamics of IAVs of different origin in the horse, it is fundamental to have a solid species-specific disease pattern as a reference control. Therefore, we first validated this system by infecting it with an EIV and described the infection dynamics over five days. The infection dynamics were determined by assessing viral replication, changes in the ciliary function, presence of histological damage, immunohistochemical examination for viral NP and induction of apoptosis.

3.2 Materials and methods

3.2.1 Explant preparation

Horse tracheas were collected from healthy Welsh ponies obtained from the Animal Health Trust and euthanized for a congenital heart disease. Blood was collected from each animal at the time of euthanasia to investigate the presence of anti equine influenza virus antibodies by means of hemagglutination inhibition (HI) test. Upon euthanasia, tracheas were aseptically collected, divided into proximal, middle and distal part and transported in pre-warmed medium consisting of a 1:1 mixture of DMEM and RPMI supplemented with penicillin (100U/ml, Gibco, Life Technologies), streptomycin (100 µg/ml, Gibco, Life technologies) and fungizone (2.5 µg/ml, Gibco, Life technologies). Tracheas were then placed in an incubator at 37°C, 5% CO_2 and 95% humidity. The culture medium was replaced six times every 50 minutes in order to reduce bacterial presence (fig. 1A). After the washing steps, the connective tissue outside the cartilage was removed and tracheas were opened lengthways (fig. 1B and 1C). Each third of trachea (proximal, middle and distal) was further divided into two parts lengthways (fig. 1D). Each part consisted of approximately 14 (half) tracheal rings and each (half) ring was divided into 6 explants of approximately 1 x 1 cm (fig. 1E). The mucosa was carefully stripped off the cartilage as previous experiments conducted in our laboratory had shown that the explants were not vital if left intact because of the thickness of the cartilage. Explants were placed with the epithelium facing upwards onto a sterile section of filter paper that was in turn placed onto agarose plugs in six well plates containing five ml of the same medium used for the washing step (fig. 1F). Only a thin film of medium covered the filter paper and the basal portion of the explants as described previously (Gonzalez *et al.*, 2014) thereby mimicking the air-interface found in the respiratory tract of the living animal. Explants were maintained at 37°C, 5% CO_2 and 95% humidity for up to five days. From each animal approximately 500 tracheal explants were obtained. Experiments were conducted at least in triplicate.

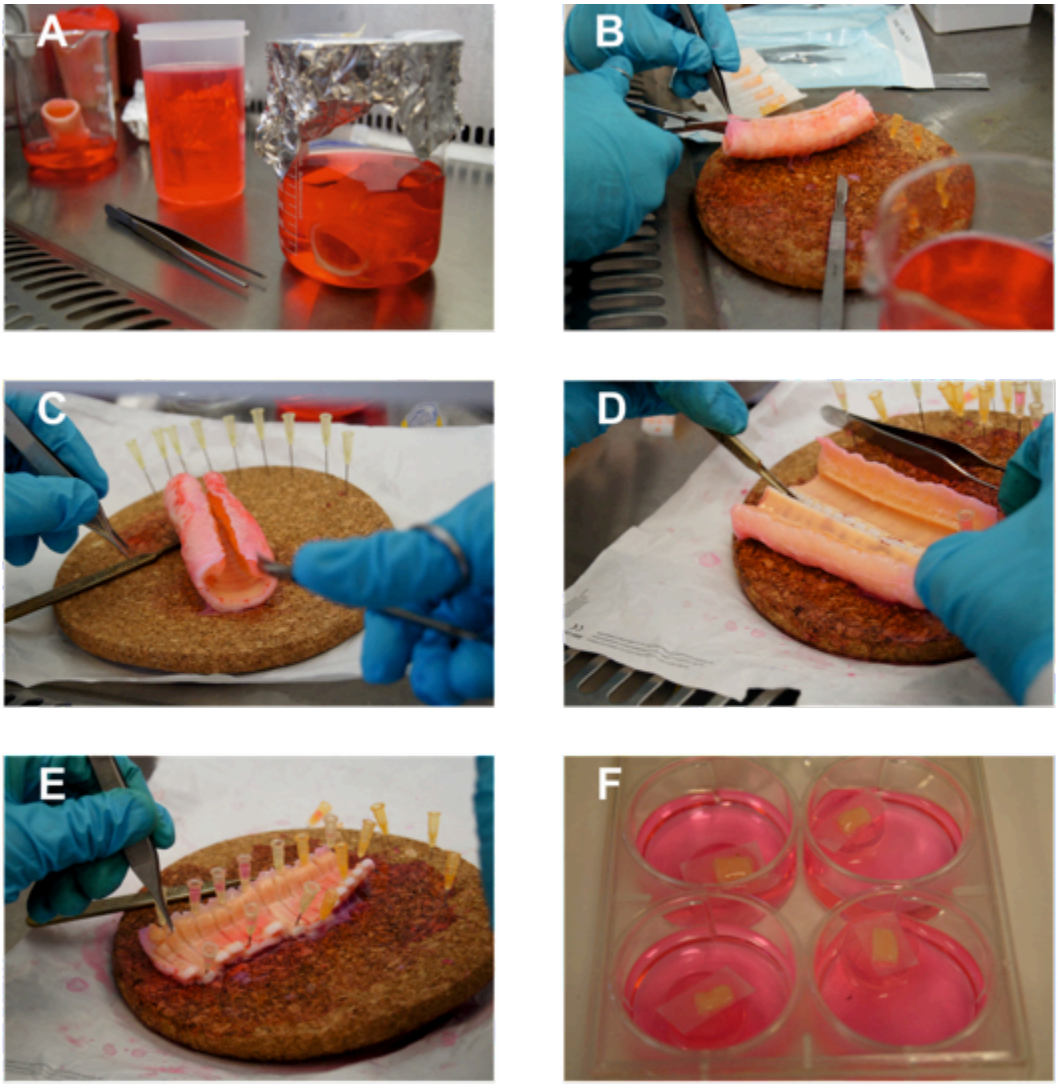

Figure 1. Equine tracheal explants technique. A) Washing steps. B and C) The trachea was opened lenghtways. D) The trachea was divided into two halfs leghtways. E) Each half was cut into strips along tracheal rings and each strip was further cut into six explants. F) Explants were cultured at the air-liquid interface.

3.2.2 Virus and cells

We infected explants with the isolate A/equine/South Africa/2003 (South Africa/03). Virus stocks were produced by inoculating 9 to 11 day old embryonated chicken eggs. Each experiment was performed at least in triplicate using tracheas from different animals. Viral titres were determined by plaque assays on MDCK cells (Matrosovich *et al.*, 2006) followed by an immunostaining of plaques (see below).

3.2.3 Experimental infections

Explants were infected with a dose of 200 pfu in five µl 24 hours after explant preparation. Stock virus was diluted in MEM supplemented with 1% P/S, 1% L-glutammine and 1 µg/ml of TPCK treated trypsin. Mock-infected explants (inoculated with five µl of the same medium used for virus dilutions) were included in all experiments. Inoculated explants were sampled for bead clearance, histology and viral replication at 6 hours and every 24 hours post-infection for five days.

3.2.4 Assessment of viability of organ culture by ciliary beating

Ciliary beating of the tracheal explants was checked on every 24 hours in order to assess presence of virus-induced damage in the epithelium. Five microliters of polystyrene microsphere beads (Polysciences, Northampton, UK) were placed onto the apical surface of the explants and plates were placed back in the incubator at 37°C, 5% CO_2, 95% humidity. Bead clearance was evaluated every five minutes over a 50 minutes period. Tissue pieces were considered viable when the beads were completely cleared to one side of the explants by co-ordinated cilia movement.

3.2.5 Virus quantification

Infected explants were immersed in 500 µl of sterile PBS and vortexed for at least 5 minutes. The supernatant was used in plaque assays in MDCK cells followed by an immunostaining of plaques as described below. Results were expressed as an arithmetic mean in pfu/ml of at least three independent experiments.

3.2.6 Immunostaining of plaques

Viral titres were determined by plaques assays on MDCK cells followed by an immunostaining of plaques. Briefly, after the incubation time, cells were fixed in 80% acetone solution for 20 minutes and then permeabilised with 0.5 % triton X100 + PBS 1X for 15 minutes at room temperature. A mouse monoclonal anti influenza A NP antibody (European Veterinary Laboratory, clone HB65, dilution: 1:500) and Horseradish Peroxidase-conjugated rabbit anti-mouse IgG antibody (AbD serotec, Oxford, UK) were used as primary and secondary antibodies, respectively. To visualize the infected cells, True Blue peroxidase substrate (Insight Biotechnology, Wembley, UK) was added, and color development was terminated after 15 minutes of incubation by washing with tap water. Viral titers were calculated by counting blue plaques. Counts were expressed as $\log_{10}$ pfu per milliliter.

3.2.7 Histological analysis

To investigate the presence of virus induced epithelial changes we conducted histological examination of explants at different time points. After collection, the explants were fixed in 10% (v/v) buffered formalin and paraffin embedded. Subsequently, 4 µm paraffin sections were cut and subject to Hematoxylin and Eosin (H&E) staining. Histological images were captured using cell^D software (Olympus).

3.2.8 Immunohistochemistry

To confirm viral presence in the tissue we conducted two types of immunohistochemical analyses. Fist we looked directly for viral nucleoprotein. Next we conducted a staining for apoptosis, a feature of viral infection. Tissue sections were deparaffinised and hydrated using standard procedures. For the apopstosis staining antigen an retrieval step was performed by pressure cooker heating. A permeabilization step was performed using 0.5 % triton X100 + PBS 1X for 15 minutes at room temperature. Next, in order to quench endogenous peroxidase, sections were incubated in a peroxidase blocking buffer for ten minutes. Sections were incubated overnight at 4°C with the following primary antibodies diluted in 10% normal goat serum: a rabbit polyclonal antiserum targeting equine influenza NP protein (designed by Pablo Murcia, University of Glasgow, dilution

1:500) and monoclonal rabbit antiserum tareting cleaved caspase 3 (Cell signaling, dilution: 1:800). Immunohistochemistry was peformed with DAKO supevision system (DAKO) and slides were counterstained wit Mayer's hematoxylin. Histological images were captured using cell^D software (Olympus).

3.2.9 Statistical analyses

Results were analysed using GraphPad PRISM® Version 6 software for statistical analysis performing a one-way Anova. Differences were considered significant when $P<0.05$.

3.3 Results

3.3.1 Replication of EIV South Africa/03 in equine tracheal explants

To validate our equine tracheal explant system we first wanted to determine whether it could support the replication of an equine influenza virus. Tracheal explants were infected with the EIV isolate South Africa/03 and maintained in culture for 5 days. Quantification of extracellular infectious virus recovered from explants at different time points was assessed through standard plaque assay followed by immunostaining. The virus productively infected tracheal explants as increasing viral presence was detected starting from 24 hours post infection (hpi). A peak of viral growth was detected at day 2 reaching titres of 7 log_{10} pfu/ml. From day 3 to 5 post infection viral titres progressively decreased (fig. 2). The overall titres recorded ranged from 4 to 7 log_{10} pfu/ml, showing that this system can support equine influenza virus replication.

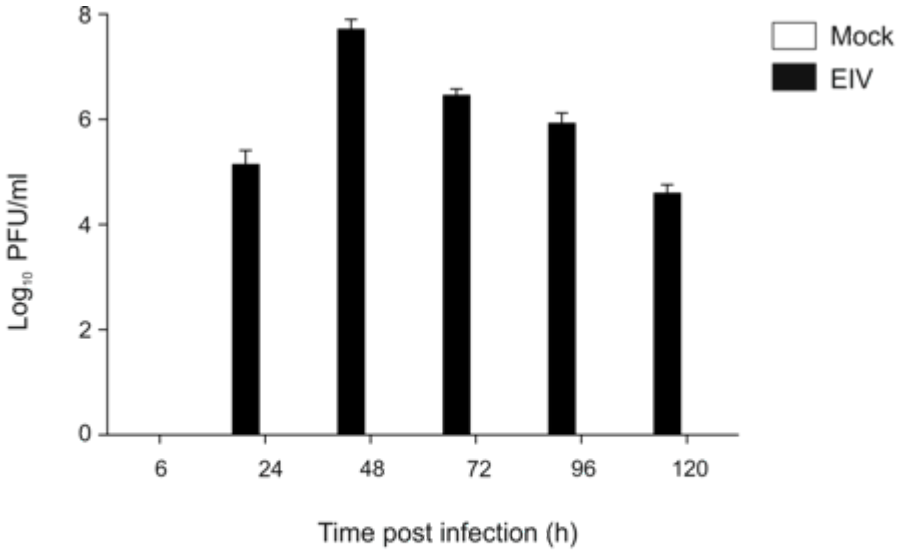

Figure 2. Equine influenza replication dynamics in tracheal explants. Bars represent the mean and SEM of three independent experiments.

3.3.2 Assessment of the ciliary function in EIV South Africa/03 infected explants

To investigate presence of virus-induced damage in the epithelium we assessed the function of the ciliary apparatus by conducting a colored bead assay on the surface of the explants every 24 hours. As shown in figure 3, bead clearance in mock-infected samples was maintained within 5 minutes throughout the duration of the experiment (5 days). EIV infected samples started to show a decrease in bead clearance efficiency (and increase in bead clearance time) from 48 to 120 hpi. Compared to mock-infected, the difference was significant from 72 hpi onwards ($P<0.001$). The observed delay in clearance ranged from 10 minutes to complete absence of bead clearance depending on the explant and on the day of examination.

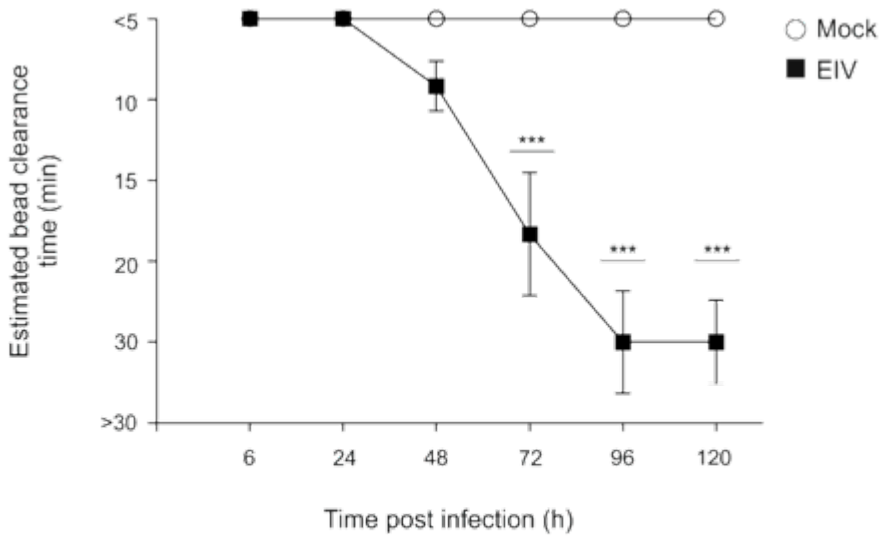

Figure 3. Bead assay in EIV infected tracheal explants. The graph shows estimated bead clearance time in mock and EIV infected explants over a 5 days time course. Lines represent the mean and SEM of three independent experiments. *** P<0.001

3.3.3 Histological findings following infection with EIV South Africa/03

To look for signs of EIV replication we collected samples for histological examination following H&E staining. Mock-infected samples preserved their structure without major changes throughout the study period. Signs of influenza virus infection such as loss of cilia at the apical surface and cellular disruption began to be evident by day 2 post-infection in EIV infected samples. As the infection progressed a marked thinning of the epithelium was also observed (fig. 4, left panel). These lesions were observed in EIV infected explants up to day 5 post infection.

3.3.4 Immunohistochemistry

To confirm that the damage observed was virus-induced an immunohistochemical staining for viral NP was conducted. Positivity spreading from few cells to the entire epithelial lining was recorded from day 1 to day 5 post-infection. Infected cells were found only in the epithelium (basal to the apical part) and the positive signal was detected mainly in the nucleus and occasionally in the cytoplasm (fig.4, middle panel). No staining was detected in the lamina propria nor submucosal glands.

To further characterize the infection phenotype the presence of virus induced apoptosis was assessed. Consistent with the previous findings of viral infection, apoptotic cells were detected in EIV infected samples from day 1 to 5 post-infection (fig.4, right panel). Apoptotic cells were not detected in mock-infected samples.

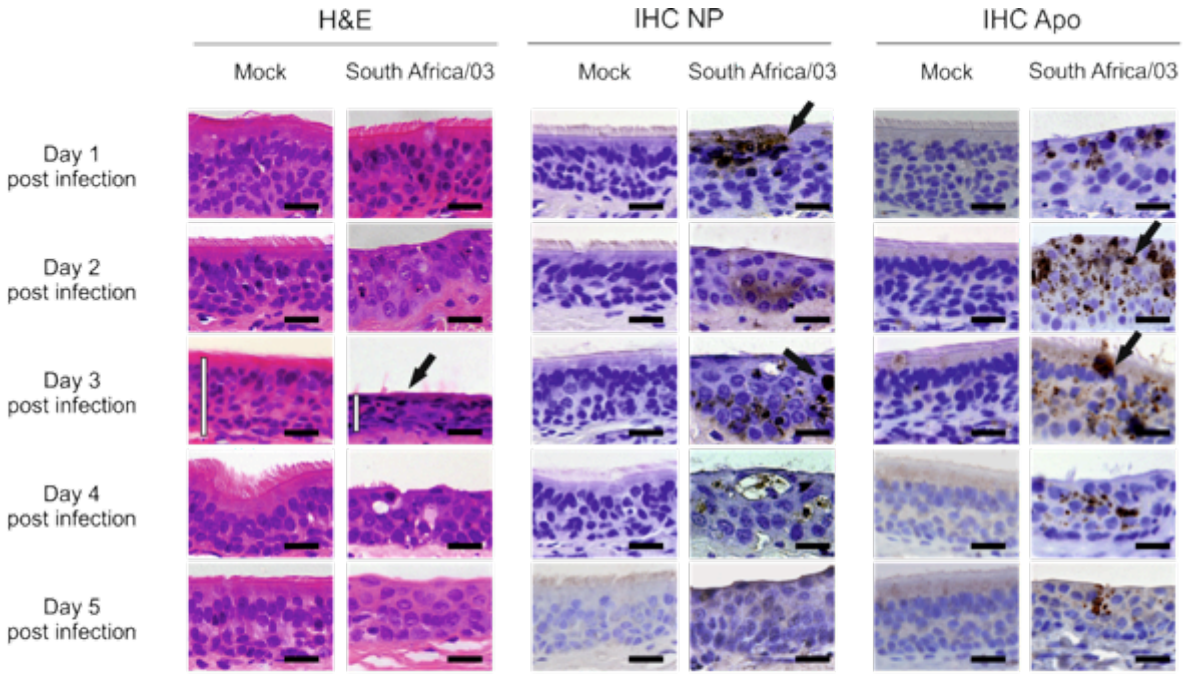

Figure 4. Histological and immunohistochemical examination of equine tracheal explants infected with EIV A/equine/South Africa/2003. Shown in the left panel are EIV and mock-infected explants stained with hematoxylin and eosin. White vertical lines show the difference in epithelial thickness between mock and EIV infected samples. Black arrows indicate loss of cilia at the apical surface of EIV infected explants. In the middle panel immunohistochemistry for viral nucleoprotein (NP) and in the right panel immunohistochemistry for apoptosis (Apo). Positive cells are stained in brown and indicated by black arrows. Black horizontal bars represent 50 µm.

3.4 Discussion

Equine influenza is an infectious disease of horses affecting the respiratory apparatus. Several *ex vivo* cultures have been described previously with the aim of developing study systems of respiratory disease resembling as possible the living host. There are few examples of the application of such systems in the equine infectious diseases field. Vandekerckhove and colleagues (Vandekerckhove *et al.*, 2011) have described the use of nasal mucosa explants to compare the mechaninsms of pathogenesis of equine herpesvirus 1 and 4. Among the advantages reported, the preservation of a three-dimensional structure allowing the maintainance of inter-cellular contacts provided by this technique resulted fundamental to properly study disease dynamics. Lin et al (Lin *et al.*, 2001) have described the establishment of an equine tracheal explant system. In their study they validated a semi submerged culture system of entire tracheal explants (cartilage to mucosa) over 96 hours to study viral infections. Equine explants have been used in bacteriology to study the virulence of the *Streptococcus equi* infection (Hamilton *et al.*, 2006). These examples have provided evidence that the *ex vivo* culture system is reliable. Thus, when feasible, it should be implemented to study diseases dynamics in the target tissues of a host.

Here, through several approaches, we have characterized the replication of an EIV in equine tracheal explants over five days. We decided to focus on influenza replication in the tracheal epithelium because this site is recognized as one of the targets of influenza infection in the horse (Sutton & Viel, 1997). We opted for culturing only the mucosa at the air liquid interface to prevent reinfection from the culture medium and because previous attempts of culturing the entire trachea revealed less vitality. As several infection methods had been published, we chose to adopt a technique described previoulsy in canine and swine explants (Gonzalez *et al.*, 2014; Nunes *et al.*, 2010). A low infectious dose (200 pfu of virus contained in one drop of approximately five µl) was used and the inoculum was left on the epithelial surface. As an alternative, incubation with a higher dose followed by several washes to remove unbound virus has been described for equine, swine and human explants (Chan *et al.*, 2011; Lin *et al.*, 2001; Van Poucke *et al.*, 2010). In our opinion, the protocol we adopted mimicks more closely a

natural infection and thus, is more suitable for our purpose of establishing an *ex vivo* system resembling the *in vivo* dynamics between virus and host.

In first instance, we followed viral replication by quantifying the extracellular viable virus contained in the surface of the explants. To have a more precise measurement of the real viral production we collected indivudual explants and evaluated the viral load released by each piece following vortexing. Alternative methods consist in quantifying the virus released in the culture medium. This has the advantage of allowing the follow up of replication dynamics in the same explant over time but it cannot avoid quantifying eventually residue inoculum. Overall, the variability obsereved among the different explants collected and in tissues coming from different animals was minimal. Therefore, our method can be used as a valid quantification system to look at tissue associated viral load. Already at 24 hpi a marked increase in viral load was observed followed by a further rise reaching maximum titres at 48 hpi. Viral presence was detected until the last time point of collection.

After having demonstrated that EIV could replicate we determined the type of histological changes associated with infection. Infected samples started displaying changes in the epithelial morphology by day 2 post infection. Loss of cilia at the apical surface, cellular shrinkage and epithelial thinning were increasingly observed until day 5 post infection. Immunohistochemical localization of viral NP confirmed that the damage displayed was virus induced. EIV infection affected the whole epithelial layers from the apical to the basal membrane. No staining was detected in the lamina propria nor submucosal glands.

To have a final confirmation of the suitability of this system to study influenza infection we investigated the cellular response to infection. Apoptosis, or programmed cell death, is one of the defense mechaninsms that can be induced in a cell following different types of insults: viral infections are among these. IAVs have been shown to induce apoptosis via both the intrinsic and extrinsic pathway (Herold *et al.*, 2012). As our goal was to detect apoptosis, but not its pathway of activation, we investigated its presence by immunostaining of the cleaved caspase 3, an enzyme that is activated at later stages of the apoptosis cascade and is thus common to both pathways. Positive signal was

detected in EIV infected samples as early as day 1 post-infection and continued to be present until day 5 confirming that apoptosis is a hallmark of influenza virus infection.

In the present chapter we have described the establishment of an equine tracheal explant system and its validation through infection with an EIV. Furthermore, we have discussed some aspects related to the choice of culture method, infection and sample collection. We have shown that this system can support influenza replication providing information on several aspects of infection. Thus, it is suitable for studying the replication potential of other influenza viruses in this host.

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Chapter 4

Phylogenetically distinct H3N8 equine influenza viruses show different tropism for the swine respiratory tract

4.1 Introduction

Influenza A viruses (IAVs) viruses can infect a wide variety of hosts, and IAVs have caused cross-species transmissions that led to either spillover infections or emergence and sustained transmission in new host populations.

Among the different IAV subtypes, H3N8 equine influenza virus (EIV) has shown a remarkable ability to cross species barriers. EIV is thought to have originated from the avian reservoir (Worobey *et al.*, 2014) and has circulated in horses for over 50 years. In early 2000s EIV crossed the species barrier and established in dogs as canine influenza virus (CIV) (Crawford *et al*., 2005). During 2004-2006 influenza surveillance in China two H3N8 viruses closely related to European EIVs have been isolated from pigs showing respiratory signs of illness (Tu *et al.*, 2009). The two isolates possessed mutations near the cleavage site and in the receptor binding domanin that had been previously reported in canine H3N8. Another spillover infection of H3N8 EIV occurred recently in mongolian bactrian camels (Yondon *et al*., 2014).

A second introduction into the equine population of an H3N8 virus from the avian gene pool (Guo *et al.*, 1992) as well as the recent detection of an avian H3N8 in harbour seals (Karlsson *et al.*, 2014) rise concerns on the ability of this subtype of infecting mammals.

Patterns of viral emergence are complex to understand as they can be determined by ecological drivers, such as the efficent transmission of a pathogen as a result of contact between a reservoir host and a new susceptible species, or adaptive drivers which instead require natural selection of adapting mutations for successful transmission to occurr (Holmes & Drummond, 2007). The latter may be achieved natural cycles of replication that occurr upon extensive circulation. Recently it has been suggested that EIV evolution could have played an important role in the emergence of CIV: equine influenza viruses that circulated in the 1960s displayed a highly attenuated phenotype in

dog tracheas, whereas an EIV isolated in 2003 -around the time of emergence of canine influenza virus- exhibited an infection phenotype indistinguishable from that of CIV (Gonzalez *et al*., 2014).

Historical and epidemiological evidence support the vision that pigs play an important role in influenza ecology (Vincent *et al.*, 2014) and might be a source of reassortant viruses with unknown potential. For this reason, it is important to determine if IAVs that are endemic in domestic animals could potentially infect pigs and contribute –via reassortment- to the IAV gene pool that could eventually transfer to humans as it happened during the 2009 pandemic (Smith *et al.*, 2009).

To investigate whether changes in host range along EIV evolutionary history included other animal species besides dogs we focused on pigs as this species can behave as mixing vessel (Ma *et al*., 2009) and because EIV has been isolated from pigs after natural transfers in the past.

As extensively discussed in chapter two, *ex vivo* cultures of swine respiaratory tissues allow the study of several aspects of influenza infection. In the present study we compare the replication potential of a panel of phylogenically distinct H3N8 EIVs in swine cell lines and determine the phenotype of infection of EIV in swine respiratory explants in comparison to a swine influenza virus.

4.2 Materials and Methods

4.2.1 Cells

Newborn Pig Trachea (NPTr), Newborn Swine Kidney (NSK) and Madin-Darby Canine Kidney (MDCK) cells were maintained in Minimum Essential Medium (MEM, Sigma) supplemented with 10% Foetal Calf Serum (Euroclone), 1% 200mM L-glutammine (Sigma) and 1% antibiotic solution of Penicillin-Streptomycin (Gibco) and Nystatin (Sigma). Cell lines were passaged twice weekly and maintained in a humidified incubator at 37°C with 5% CO_2. NPTr and NSK cell lines were kindly provided by the Istituto Zooprofilattico Sperimentale dell'Emilia Romagna e Lombardia.

4.2.2 Viruses

A panel of phylogenetically distinct H3N8 equine influenza viruses and an H3N2 Eurasian swine influenza virus used as positive control were tested in the present study. Virus stocks of A/swine/Italy/8088/06 (SIV H3N2), A/equine/Uruguay/1963 (Uruguay/63), A/equine/Fontainebleau/1979 (Fontainebleau/79), A/equine/Argentina/1995 (Argentina/95) and A/equine/South Africa/2003 (South Africa/2003) were obtained by inoculating 9 to 11 day old SPF embryonated chicken eggs. After 72 hours allantoic fluids were harvested, clarified by centrifugation at 3000rpm for 10 minutes, aliquoted and stored at -80°C. Viral titres were determined by standard plaque assay on MDCK cells followed by immunostaining as described in chapter two.

These viruses represent evolutionary distinct clades of EIV and despite the unavailability of complete passage history they were chosen for specific reasons: Uruguay/63 is one of the first H3N8 isolated from horses, South Africa/2003 represents the clade of viruses circulating at the time of CIV emergence and the other two isolates included represent intermediate clades.

4.2.3 Viral growth in NPTr and NSK cells

EIVs ability to replicate in swine cell lines was monitored over a 72 hours time course. NPTr and NSK cells were seeded in 24-well plates to obtain a confluent monolayer. Prior to infection, two wells per cell line were washed twice with warm PBS and trypsinised for cell count. Plates were washed twice and triplicate wells per time point were

infected with 200 µl of several EIVs at a multiplicity of infection (MOI) of 0.001 pfu/cell. After 1h of incubation at 37°C the inoculum was removed, cells were washed once with warm PBS and 1ml of culture medium supplemented with 1 µg/ml of tosylsufonyl phenylalanyl chloromethyl ketone (TPCK)-treated tripsyn was added. At 6, 24, 48 and 72 hours post infection (hpi) 300 µl of supernatant were collected and stored at -80°C. Mock-infected wells were included in all experiments. Viral titres in the supernatant were calculated using the Tissue Culture Infectious Dose ($TCID_{50}$) assay on MDCK cells according to the Reed and Muench formula. At 96 hpi plates were checked for presence of cytopathic effect (CPE) and haemagglutinating activity of the supernatants.

4.2.4 Animals

Six to 8-week old female piglets (n=3) were purchased from a high health status farm. To assess seronegativity of the herd to Influenza A, blood samples were collected in the farm before purchase and at the time of euthanasia. Sera were tested in duplicate with an ELISA kit targeting anti viral nucleoprotein (NP) antibodies (IDScreen®, ID.vet). Animals were housed in HEPA-filtered BSL-3 facilities with unlimited access to water and feed. Environmental enrichments such as chains and balls were also provided. Upon arrival animals were treated by IM injection of Ceftiofur (Naxcel® Pfizer, 1ml/20 kg pv) to clear the airways from the major bacterial pathogens such as *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Haemophilus parasuis* and *Steptococcus suis*. Euthanasia was performed by IV administration of 12.5 mg/kg of Pentothal sodium (Intervet) and subsequent exsanguination. All experiments were conducted after the approval of the local ethical committee (approval number CE IZSVe 11_2012).

4.2.5 Explant preparation

Swine nasal, tracheal and lung explants were prepared as described in chapter two. Each virus was tested in duplicates in tissues obtained from three different pigs.

4.2.6 Explant infection

After 24h of culture explants were infected with five µl of each viral suspension containing 200 PFU. Mock infected explants were included in all experiments. Replicate samples were collected at 6, 24, 48, 72 and 96 hpi for virus quantification and histological examination. Nasal explants were kept only until 72 hpi. Vitality of the explants was checked daily through examination of the ciliary activity under a light microscope and changes in media color.

4.2.7 Bead clearance assay

To assess the vitality of the explants and to evaluate the presence of tissues damage as a consequence of viral infection, we conducted a colored bead assay on tracheal explants as described in chapter two.

4.2.8 Virus quantification in infected explants

Explants were collected in 500 µl of cold sterile PBS, shaken in a tissue Lyser (Qiagen) at 20 Hz for 10 min and centrifuged at 16000xg for 10 min at 4°C. The collected supernatant was used for virus titration using the Tissue Culture Infectious Dose ($TCID_{50}$) assay on MDCK cells according to the Reed and Muench formula. In addition to CPE reading and hemagglutinating activity of the supernatant plates were also immunostained as described in chapter two.

4.2.9 Histology and Immunohistochemistry

To investigate the presence of histological changes associated with infection and to determine viral tropism, histological and immunoistochemical analyses for viral NP detection were performed as described in chapter two.

4.2.10 Statistical analyses

Results from *in vitro* and *ex vivo* growth curves were analysed using GraphPad PRISM® Version 6 software for statistical analysis. In order to compare all the viruses at each time point a Two-way Anova with Bonferroni's post-test for multiple comparisons was performed. Differences were considered significant when $P<0.05$.

4.3 Results

4.3.1 Equine influenza viruses replicate in swine cell lines to comparable levels to an H3N2 SIV

All tested EIVs were capable of infecting NPTr and NSK cells as shown in figure 1. Although there were slight differences in replication dynamics observed at 6 and 24 hpi, no significant differences were detected between SIV and any of the EIVs tested at later time points (P>0,05 two-way Anova with Bonferroni's post-test for multiple comparisons), showing that EIV can readily infect and replicate in swine cell lines (fig. 1a and 1b).

Presence of CPE was evident from 48 hpi and at 72 hpi the monolayer was completely destroyed in both cell lines with all viruses.

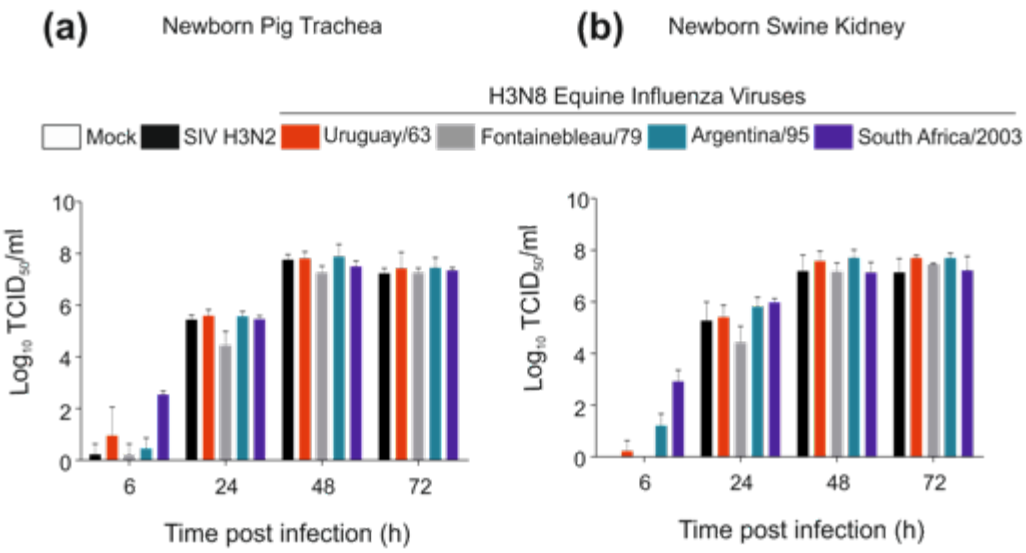

Figure 1. Growth kinetics of phylogenetically distinct H3N8 EIVs in swine cell lines. (a) Newborn pig trachea cells.(b) Newborn swine kidney cells. Vertical bars show the mean and standard deviation of three independent experiments.

4.3.2 H3N8 EIVs do not infect swine nasal mucosa explants (respiratory part)

To further characterize EIV's tropism for swine tissues we first infected nasal mucosa explants. Infection with SIV H3N2 revealed increasing viral titres from 24 hpi onwards (not shown). Typical signs of influenza infection such as epithelial disruption and vacuolization were observed from 48 hpi as shown in figure 2 and viral NP was increasingly detected across the epithelium from 24 to 72 hpi.

None of the EIVs tested replicated in nasal mucosa explants. According with this finding, no histological damage was observed in any of the explants infected with EIVs and immunohistochemistry for viral NP resulted negative (fig. 2).

An additional experiment up to day 4 post infection was conducted to exclude viral presence at a later time point.

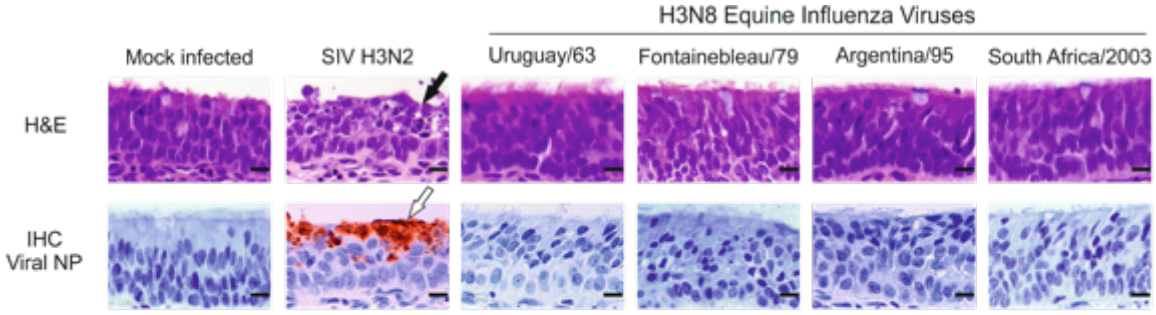

Figure 2. Infection of swine nasal mucosa explants with phylogenetically distinct H3N8 EIVs. Histological features of nasal mucosa explants infected with H3N2 SIV (positive control) and various H3N8 EIVs (Uruguay/63, Fontainebleau/79, Argentina/95 and South Africa/2003). Lesions are shown in sections stained with hematoxylin and eosin and indicated by a black arrow. Infected cells were detected by immunohistochemical staining of the NP viral protein. Positive cells are stained in brown and indicated by white arrows. Black horizontal bars represent 50 µm. Shown is day 2 post infection.

4.3.3 The early isolate Uruguay/63 is the only EIV able to replicate in swine tracheal explants displaying a different infection phenotype from SIV

As expected, replication of SIV H3N2 in tracheal explants was abundant from 24 to 96 hpi (fig. 3a). Consistent with this finding the colored bead assay showed a progressive decrease in ciliary activity up to complete absence of bead clearance (fig. 3b). Histological damage, such as loss of cilia and reduction in epithelial thickness, was evident from 24 to 96 hpi (shown in fig.4a). In accordance, viral NP was detected in the epithelium from 24 hpi onwards. (fig. 4b).

With regards to EIVs, only the early isolate (Uruguay/63) was able to replicate, albeit to a lower level than SIV. A peak of viral replication up to 5 $\log_{10}$ $TCID_{50}$/ml was observed at 72 hpi (4 out of 6 replicas) and to a lower extent and smaller number of samples at 48 and 96 hpi as shown in figure 3a. As for the other EIVs, the virus titres detected were always lower or equal to the initial inoculum leading to the consideration that very low to no replication occurred. The differences recorded between Uruguay/63 and the other EIVs were significant at 72 hpi ($P<0,05$ two-way Anova with Bonferroni's post-test for multiple comparisons).

However, even when infecting, Uruguay/63 did not cause any changes in bead clearance or histological damage (figs. 3b and 4a, respectively). Furthermore, despite having serially sectioned through duplicate samples at all time points within each experiment no viral antigen was detected in the infected tracheas examined (fig. 4b). In agreement with virological findings, none of the other EIVs caused epithelial damage and viral NP was not detected in the tracheal explants examined.

Within each experiment, replicate samples infected with EIVs and collected at the same time point harvested different viral loads whereas minimum variability was observed in H3N2 infected samples as shown in figure 3a.

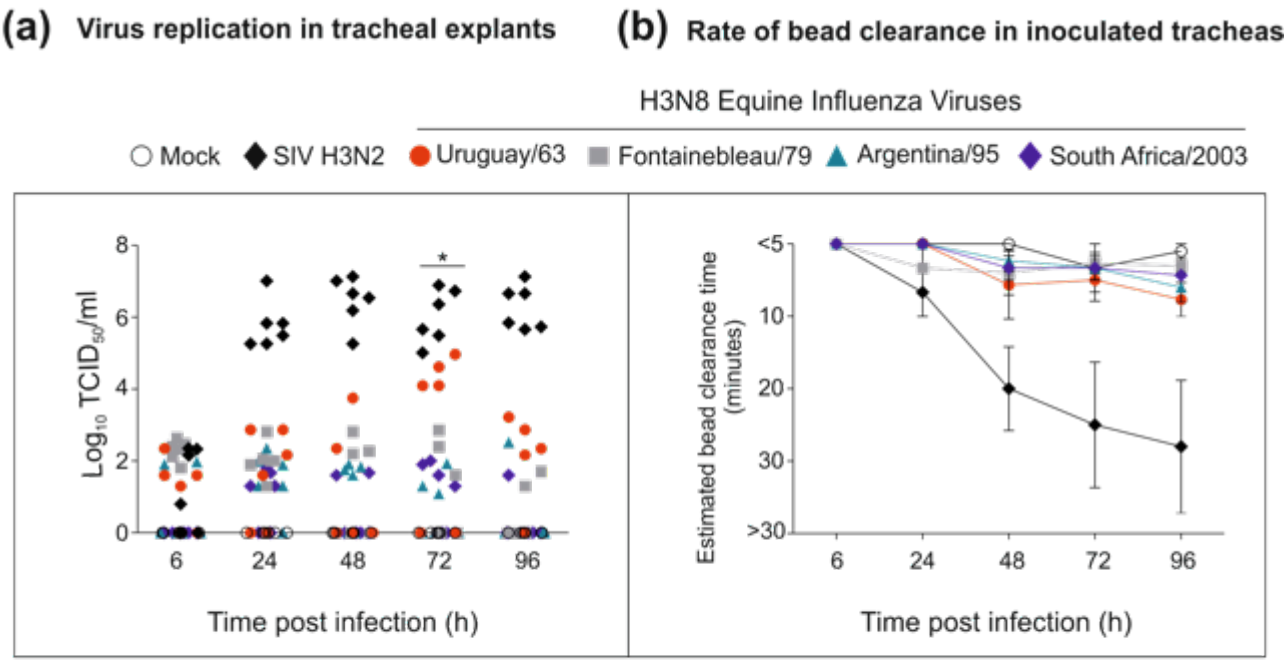

Figure 3. Infection of explants derived from swine nasal mucosa and trachea with phylogenetically distinct H3N8 EIVs. (a) Growth kinetics of SIV H3N2 and a panel of H3N8 EIVs in swine tracheal explants. Dots represent values of individual replicates, * P<0.05. (b) Graphical representation of bead clearance assays in control, SIV-infected and EIV-infected tracheal explants. Lines represent the average time to clear the beads in three independent experiments.

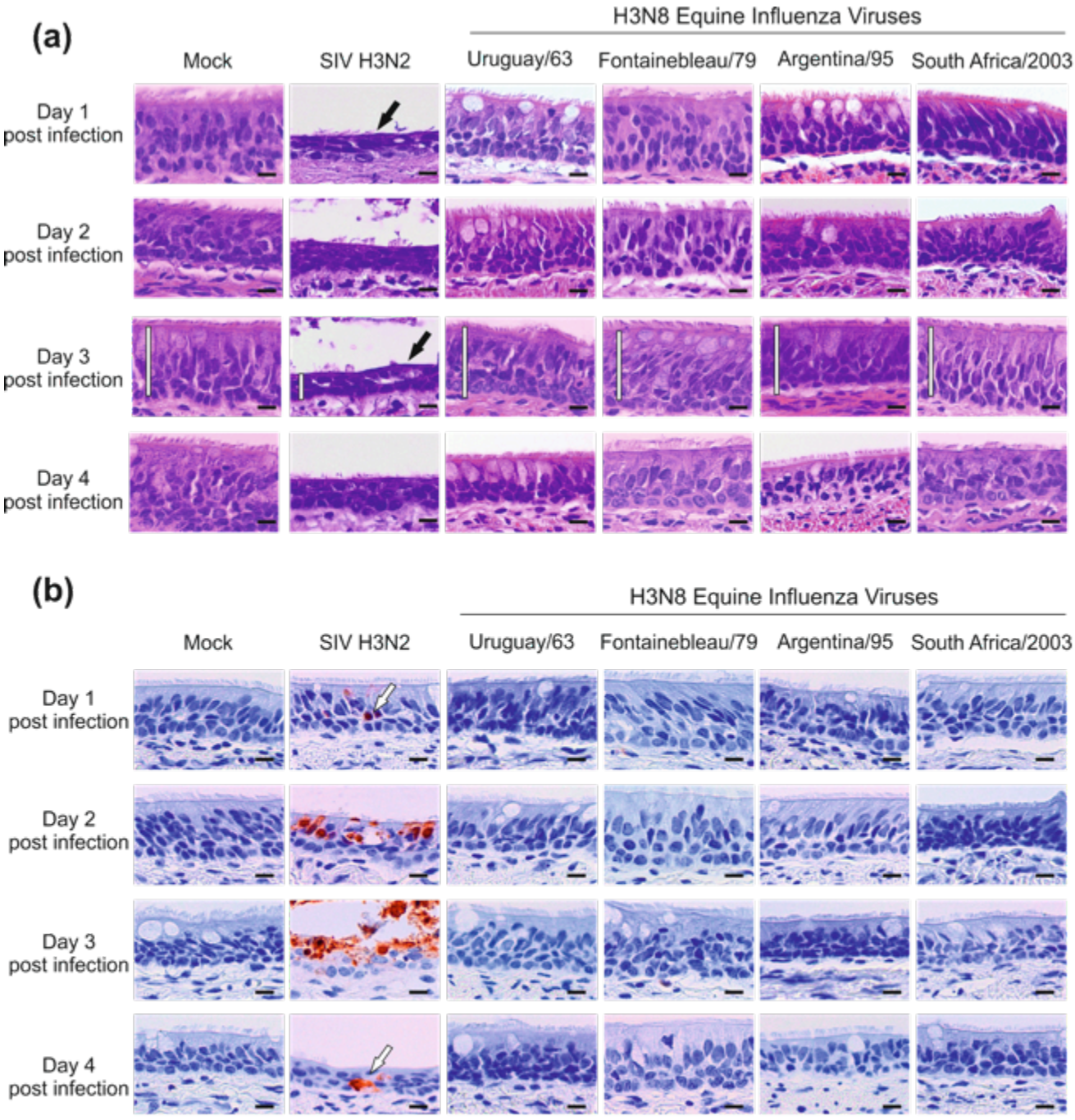

Figure 4. Histological and immunoshistochemical features of swine tracheal explants infected with phylogenetically distinct H3N8 EIVs. (a) Hematoxylin and eosin staining of swine tracheal explants infected with SIV and a panel of phylogenetically distinct EIVs. White vertical bars show the difference in epithelial thickness between mock, SIV and EIV infected explants. Black arrows show loss of cilia at the apical surface. (b) Immunohistochemical detection of viral NP. Positive cells are stained in brown and indicated by white arrows. Black horizontal bars represent 50 µm.

4.3.4 EIV Uruguay/63 replicates to high titres in swine lung explants targeting the bronchiolar epithelium.

As for the other explants systems, SIV H3N2 virus replicated to increasing titres from 24 hpi onwards and in this time frame viral NP was detected in bronchiolar and rare alveolar cells. In this explant system difference in infection phenotypes between EIVs was even more evident: Uruguay/63 displayed high replication efficiency by day two post-inoculation, with up to four log increases and peaking at day four, although some inter-animal differences in replication dynamics were observed (fig. 5). In fact, at 96 hpi titers observed in some explants were similar to those exhibited by SIV H3N2 and significantly higher than those of the other EIVs ($P<0.0001$ two-way Anova with Bonferroni's post-test for multiple comparisons). Moreover, Uruguay/63 increasingly infected the bronchiolar epithelium and rare alveolar cells, at similar levels to those seen for SIV H3N2 (fig. 6). As reported for tracheal explants, some samples infected with the other EIVs contained viable virus but again the titres recorded were inferior or equal to the inoculum and immunohistochemistry yielded negative results.

Variability in virus quantification (as reported for tracheal explants) and IHC analyses of replicate samples infected with Uruguay/63 was observed whereas this was never the case with SIV H3N2 infected samples.

Histological changes were observed in the bronchiolar epithelium of explants infected with SIV H3N2 and EIV Uruguay/63 from 72 and 96 hpi, respectively, but also in mock-infected explants. The latter may be due to organ manipulation during explant preparation and thus histological examination of lung explants was not included as a feature of viral replication.

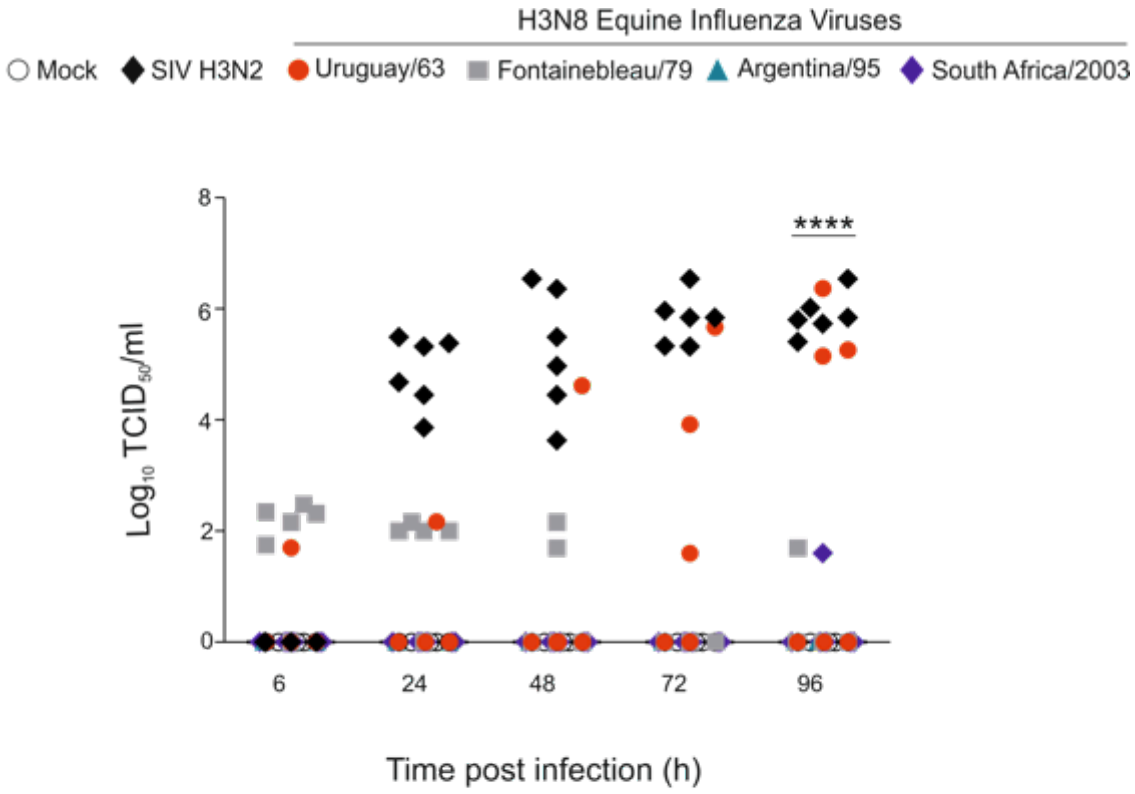

Figure 5. Uruguay/63 replicates in swine lung explants at higher levels than other EIVs. (a) Growth kinetics of SIV H3N2 and H3N8 EIVs in swine lung explants. Dots represent values of individual replicates, **** P<0.0001.

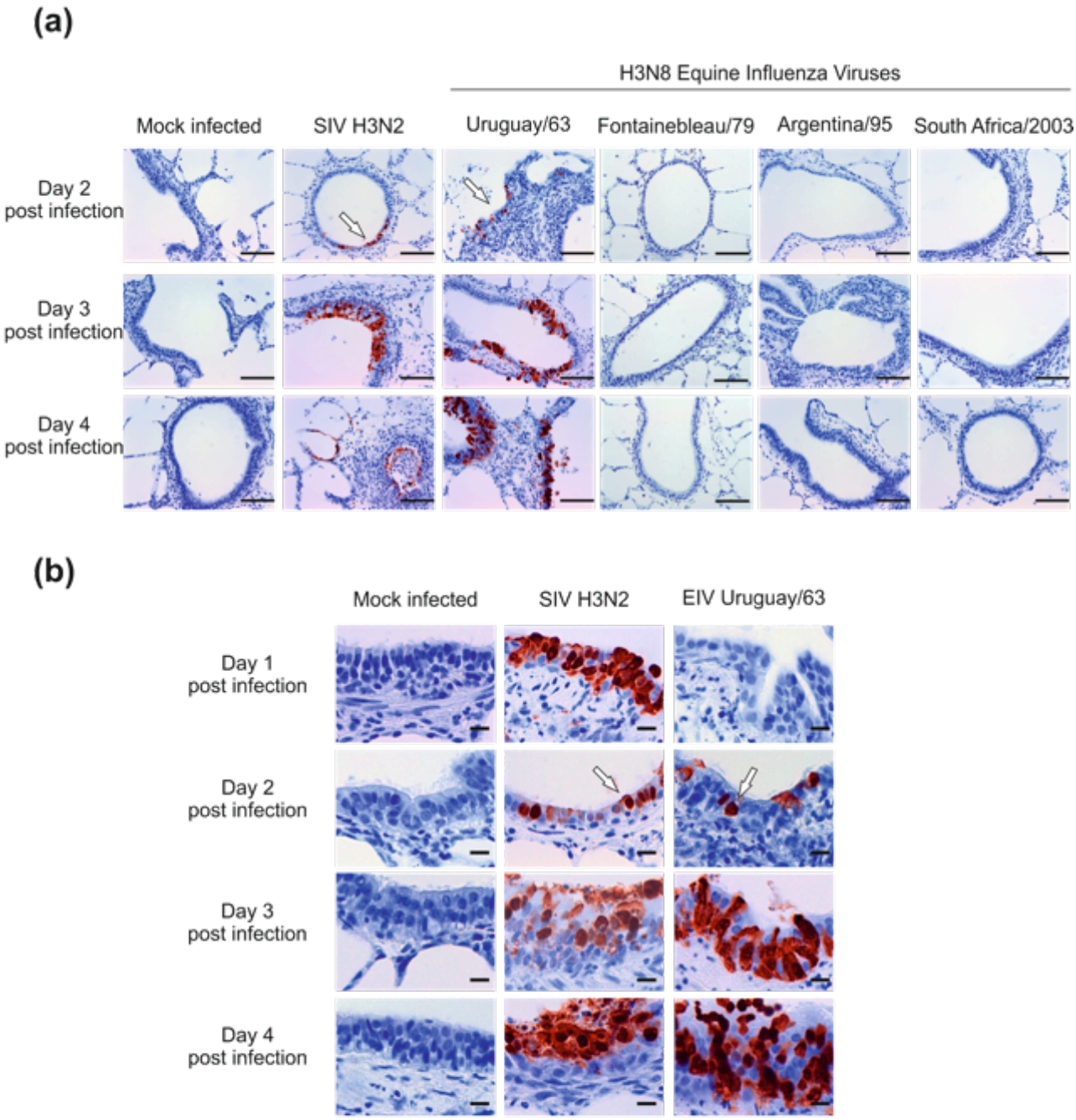

Figure 6. Immunohistochemical detection of viral NP in swine lung explants infected with phylogenetically distinct H3N8 EIVs. (a) Staining of swine lungl explants infected with SIV and a panel of phylogenetically distinct EIVs. Infected cells are stained in brown and indicated by white arrows. (b) Higher magnification of mock, SIV and EIV Uruguay/63 infected explants. Black horizontal bars represent 50 µm.

4.4 Discussion

H3N8 Influenza viruses have been isolated from many animal species such as horses, dogs, pigs, donkeys and seals, and are one of the most prevalent subtypes in the avian reservoir (Karlsson *et al.*, 2014 and references therein). Of particular interest is the isolation of H3N8 equine influenza from several hosts in recent years as well as the wholly equine origin of canine influenza demonstrating a remarkable ability of H3N8 EIVs to cross species barriers, particularly infecting other mammals. Following the detection of EIV in important host such as pigs and given the preliminary evidence on the impact of evolution on the host range of equine influenza we investigated the ability of evolutionary distinct EIVs to infect pig cells and tissues.

We first screened the replication potential of a panel of EIVs, representing phylogenetically distinct clades, in two swine cell lines. NPTr and NSK cells express both α2-3 and α2-6 sialic acid (SA) receptors (Corradi *et al.*, 2003), which are key to influenza virus binding, thus supporting the replication of Influenza A viruses (Ferrari *et al.*, 2003). Despite using a low MOI (0.001), all tested EIVs efficiently replicated in both cultures providing preliminary evidence on the ability of equine influenza to infect swine cells.

Since influenza in pigs is a respiratory disease we then sought to characterize the degree of infection of the different EIVs in the swine respiratory tract. Organ explants represent a unique model to study influenza infection as these maintain the complex architecture of animal tissues and allow a reduction of the number of animals used for experimental purposes.

As expected, SIV consistently exhibited high replication efficiency in nasal mucosa, trachea and lungs. In contrast, EIVs showed variable infection phenotypes depending on the virus isolate and the anatomical region of the respiratory tract tested. The nasal mucosa was the only anatomical region in which no signs of EIV infection and replication were observed. Further along the respiratory tract the only EIV able to infect swine explants was the early isolate Uruguay/63. Despite replicating, a discrepancy between virological and histological results was observed in infected tracheas whereas lungs yielded consistent results. The disparity between virus kinetics in the total culture

extracts and histological changes has been previously described (Gonzalez *et al.*, 2014; Van Poucke *et al.*, 2010) and is likely due to focal infections in limited areas of the explants (Chan *et al.*, 2013).

Our results indicate that despite the ability of all tested EIVs to efficiently replicate in pig cell lines in monolayer cultures -in fact, to similar levels of an H3N2 swine influenza virus- only Uruguay/63 is able to infect and replicate efficiently in distinct anatomical regions of the pig respiratory tract. Explants infected with H3N2 SIV exhibited consistent results in all tested replicas. In contrast, tissues inoculated with EIVs showed some variability in infection despite taking precautions to minimise experimental variability (e.g. by infecting the same tracheal rings of different animals with the same virus, and using exclusively the right apical lobe of the lung). Similar variations in infection phenotypes have been reported in swine tracheal and lung explants infected with human isolates of H5N1 avian influenza virus (Chan *et al.*, 2013). Although the causes of those variable results have not been determined, they could be due to variation in receptor abundance and distribution among explants, as well as variable levels of mucus (in the trachea) and surfactant protein D (in the lungs). All these factors could play important roles in determining the efficiency of influenza virus infection and thus should be taken into account (Hillaire *et al.*, 2013; Matrosovich & Klenk, 2003). Further studies addressing the quantification and variability of such factors in *ex vivo* systems could be important to clarify their potential influence on the experimental variation observed.

Here we show that Uruguay/63, the oldest EIV isolate, can infect swine tracheas and lung explants but not the nasal mucosa. Notably, lungs infected with Uruguay/63 showed similar levels of infection to those observed with SIV H3N2, a swine-adapted influenza virus. In contrast, all the other EIVs tested showed an impaired ability to infect any portion of the pig respiratory tract. As previous reports indicate that the swine respiratory tract supports infection and replication of some avian influenza viruses (Löndt *et al.*, 2012; Van Poucke *et al.*, 2010), it is not surprising that Uruguay/63 - the most avian-like virus of the H3N8 EIV lineage -exhibited the highest ability to replicate in pig respiratory tissues. Moreover, the scarce replication in the upper respiratory and the increasing infection ability towards the deeper airways clearly resembles the infection phenotypes described for avian influenza viruses. As only the respiratory part of the

nasal mucosa was used in this study we cannot exclude that EIV might replicate in the olfactory part, which has been shown to harbour receptors to which avian viruses bind (Van Poucke *et al.*, 2013). Culture of the olfactory mucosa was attempted but not successfully achieved for the time being.

It is feasible to think that the initial EIVs were more avian-like and thus able to infect pigs, and that such tropism for the swine respiratory tract was lost when EIV became more adapted to the horse during the initial EIV epidemics. Then, EIV acquired the ability to infect dogs through genetic drift during continuous evolution in horses. Notably, Uruguay/63 and South Africa/2003 displayed very different infection phenotypes in the dog tracheas: South Africa/2003 infects dog tracheas in a similar fashion that canine influenza viruses, whereas Uruguay/63 is highly attenuated (Gonzalez *et al.*, 2014) supporting the view that evolutionary processes result in adaptive changes in the viruses that impact both on host range and viral tropism.

The finding that all EIVs tested replicated at similar levels to an H3N2 swine influenza virus in pig cell lines, whereas distinct infection phenotypes were observed in explants suggests the presence of tissue-specific host barriers at the site of infection that must play a central role in viral pathogenesis and emergence, and highlights the importance of using relevant biological systems to assess changes in host range.

Our study has various limitations. First, we tested a small number of EIVs, albeit the choice of viruses was based on a previous study that showed an association between the infection phenotype of evolutionary distinct EIVs in dog tracheas and the emergence of CIV (Gonzalez *et al.*, 2014). Here we included the same viruses used in that study and also tested other EIV isolates (Fontainebleau/79 and Argentina/95), which represent additional evolutionary distinct clades.

Second, while our results do not necessarily mean that EIV will or could have emerged in pigs, they do not rule out that possibility either. Previous reports on the association of *ex vivo* and *in vivo* infections, together with the isolation of EIV in pigs in Asia (Tu *et al.*, 2009) indicates that natural infection of EIV in swine can (and did) occur. Given the important role of the pig as a contributor to the gene pool of human influenza viruses it is important to determine if other IAVs could expand that gene pool via infections in

swine. Thus, identifying the risk of emergence of currently circulating viruses (or their genes) is important from the point of view of pandemic preparedness.

Third, we did not attempt to identify the genetic determinants that allow EIV to productively infect the respiratory tract of the pig and therefore our study could be considered observational. Future studies using reassortant and mutant viruses generated by reverse genetics will be required to achieve that task. However, the use of these approaches is currently under extensive debate.

In sum, we showed that an evolutionary distinct EIV displays an enhanced tropism for the respiratory tract of the pig compared to other viruses of the same lineage. Our results support the hypothesis that viral evolution during the natural cycles of infection of influenza host populations could result in dynamic changes in their host range. Such changes must be in line with ecological and epidemiological factors in order to allow the establishment of novel lineages in susceptible species.

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Chapter 5

Replication of H3N8 avian influenza viruses isolated from wild birds in Mongolia in equine tracheal explants

5.1 Introduction

With the exception of the newly discovered influenza A viruses in bats, wild waterfowl and shorebirds are recognised as major reservoir of all known subtypes of influenza A viruses (IAVs) (Webster & Bean, 1992) representing a model of long pathogen-host coevolution and adaptation. The main site of replication of these viruses in their reservoir is the epithelial cells lining gastrointestinal tract and thus transmission occurs via through the fecal-oral route. Because of the migratory activity of these hosts viruses can be carried around worldwide and occasionally be transmitted to land based poultry or other animal species.

Transmission of avian influenza viruses (AIVs) from wild birds to mammals may be facilitated by circulation of viruses in land-based poultry such as turkey and quail through which they can acquire advantageous mutations for replicating in mammalian hosts (Makarova *et al.*, 2003). Such theory was hypothesised following the human cases of infection with H5N1 and H9N2 subtypes originating from domestic poultry (Webby & Webster, 2001) and has been supported by several experimental studies of adaptation of influenza viruses in these species (Hossain *et al.*, 2008; Thontiravong *et al.*, 2012).

Inter-species transmission of influenza A viruses (IAVs) is a complex phenomenon that is not yet completely understood. Because of the great concern that human pandemics represent, much attention and scientific effort has been invested in studying transmission of AIVs to relevant hosts such as pigs. Nevertheless historically other introductions of AIVs into mammals have occurred and provide a source of information that needs to be further exploited. Horses are susceptible to infection with avian influenza viruses. The currently circulating H3N8 and the extinct H7N7 equine influenza viruses have originated from the large gene pool maintained in wild birds (Worobey *et al.*, 2014). A second independent introduction of an entirely avian H3N8 that has briefly circulated among horses in China has confirmed the susceptibility of this host to AIVs

(Guo *et al.*, 1992). Moreover by retracing historical data on an equine epizootic, Morens and colleagues proposed it as an early example of highly pathogenic avian influenza (Morens & Taubenberger, 2010a). Interestingly this epizootic was associated with similar disease in birds, dogs and humans (Morens & Taubenberger, 2010b).

Viral characteristics are of primary importance but also ecological settings may be key aspects required for transmission of IAVs. In this context Mongolia is a very interesting study site of avian-to-horse transmission of AIVs because of several peculiarities (Gilbert *et al.*, 2012 and references therein). It is crossed by the two major Asian flyways and it is an important niche for a large number of migratory waterfowl. These avian populations have been shown to harbour both highly pathogenic and low pathogenicity AIVs (Kang *et al.*, 2011; Sakoda *et al.*, 2010). Among all subtypes of influenza circulating in the avian reservoir in Mongolian regions the H3N8 is one of the most prevalent (Kang *et al.*, 2011). There is a large horse population in Mongolia that lives in close proximity to both wild birds and humans. Several epidemics of H3N8 EIV have been documented in Mongolia in the last 40 years (Yondon *et al.*, 2013).

In such ecological context human exposure to IAVs of animal origin may be rather frequent. Despite the likelihood of contact of humans with birds and horses, serological studies have revealed little to no evidence of zoonotic influenza infections and where positivity was detected no specific risk factors were identified (Khurelbaatar *et al.*, 2014). As there have been reports of children showing respiratory signs concomitant with disease in horses, but this age group was not included in the serological survey, the choice of the wrong target population might have influenced the results.

Another interesting aspect of Mongolian regions is the very limited poultry production consisting mainly in egg production in small and relatively bio-secure facilities in urban areas. Such condition is more than unique compared to the intense rearing of domestic poultry in the neighbouring China. For all the aforementioned reasons, Mongolia represents a unique ecosystem to study the possible direct transmission of avian influenza from wild birds to horses.

To understand the mechanisms that rule inter-species transmission it is fundamental to look at several aspects of viral replication at the site of infection. In chapter three we

have described the establishment and validation of an equine tracheal explant system to study influenza infection.

The aim of the present chapter is to evaluate the replication potential of H3N8 AIVs isolated from wild birds in Mongolia between 2012 and 2013 in equine tracheal explants. This *ex vivo* study is the results of a collaboration between the Wildlife Conservation Society and the Centre for Virus Research at Glasgow University and is part of a major study including phylogenetic analyses of AIVs and EIVs circulating in Mongolia and a serological survey conducted on horse sera looking for anti AIV antibodies. The phylogenetic and serological investigations are mentioned but not included as results in this thesis.

5.2 Materials and methods

5.2.1 Explant preparation and infection

Preparation of equine tracheal explants was conducted as described in chapter three. Tracheal explants were infected by pipetting 5-20 µl of viral suspension containing 200 pfu onto the epithelial surface. Experiments were conducted in duplicates in tissues from three different animals. Unfortunately, in one experiment the positive control was not reliable therefore the data reported herein is the result of two independent experiments.

5.2.2 Viruses

A total of ten AIV isolates collected from wild birds in Mongolia and kindly provided by WCS were used in this study. Viruses were chosen based on phylogenetic analyses in order to include a representative isolate for the different clades. H3N8 isolate number 21, 80, 106, 160, 209, 621, 881, 963, 2106, 2076 and 2271 were selected. As positive control the EIV A/equine/South Africa/2003 isolate was used. Viral stocks were produced in 9-to-11 day old embryonated chicken eggs and titrated by standard plaque assay followed by immunostaining as described in chapter three.

5.2.3 Virus quantification, bead clearance assay, histology and immunohistochemistry

The methods used to characterize the infection phenotype are thoroughly described in chapter three.

5.2.4 Statistical analyses

Results were analysed using GraphPad PRISM® Version 6 software for statistical analysis. A two-way Anova with Bonferroni's post test for multiple comparisons was performed. Differences were considered significant when $P<0.05$.

5.3 Results

5.3.1 H3N8 AIVs replicate in equine tracheal explants albeit to a lower level and with different dynamics compared to equine influenza

To investigate under controlled conditions whether the respiratory tract of horses supports the replication of H3N8 AIVs from wild birds we infected equine tracheal explants with several Mongolian isolates collected during surveillance. Following phylogenetic analyses, viruses of different clades were tested and compared with an equine influenza virus (A/equine/South Africa/2003). First of all, to assess the reliability of our system, we quantified the replication of EIV. Consistent replication was recorded among different experiments and within each experiment by comparing individual replicas. Viral replication was recorded as early as day 1 post infection (p.i.) with a peak at day 2 and then a progressive decrease until day 5 p.i.. Titres ranged from 4 to 7.8 $\log_{10}$ pfu/ml.

As regards to the H3N8 AIVs replication in equine tracheal explants was detected for all the isolates except for AIV 21 (not included in the graph). Depending on the isolate different replication dynamics were observed as shown in in figure 1. For some isolates virus was detected as early as day 1 p.i. whereas for others at later stages. Furthermore, for some isolates viral presence in the tissues was observed at different time points whereas for others single or double peaks of infection were recorded. Despite replicating, the maximum titres reached by the AIVs were significantly lower than those observed for EIV ($P<0.001$) reaching a peak titre of 4.8 $\log_{10}$ pfu/ml.

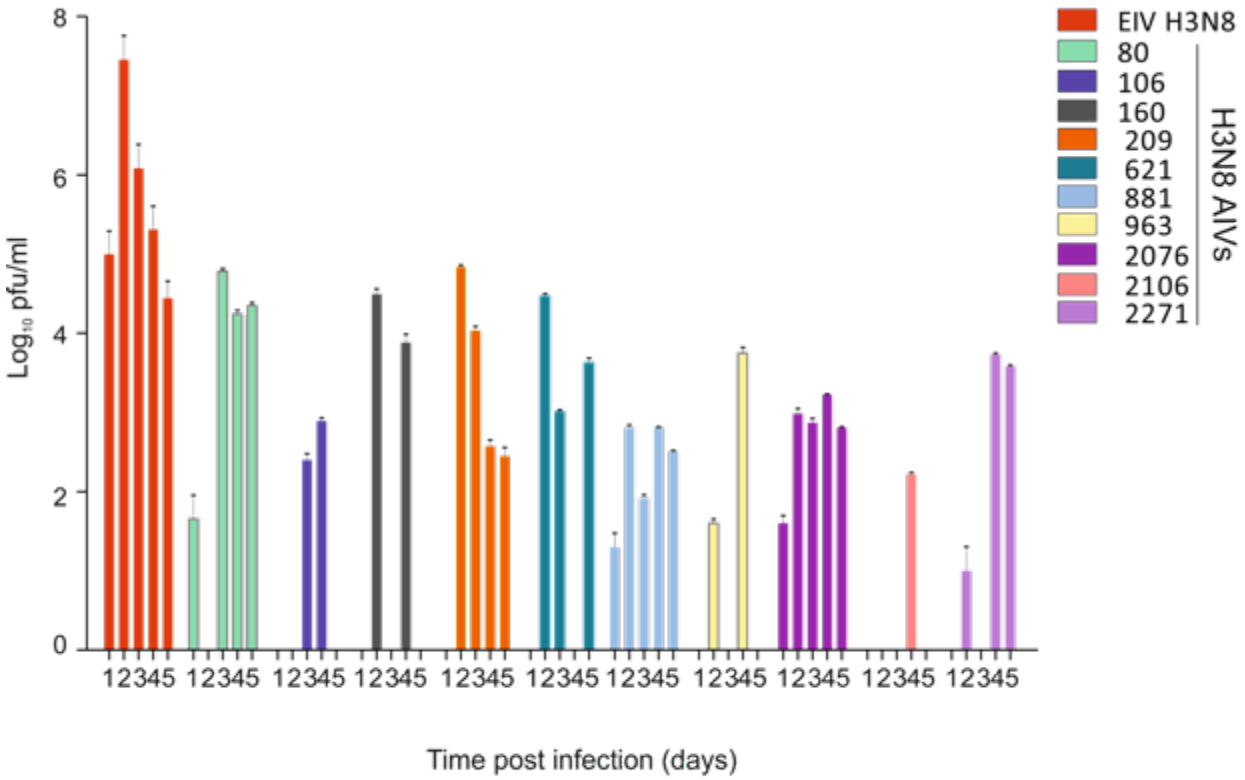

Figure 1. Replication of H3N8 EIV and Mongolian H3N8 AIVs in equine tracheal explants. Virus quantification in infected explants at different time points. Bars represent the mean and SEM of two independent experiments.

5.3.2 Replication of H3N8 AIVs does not affect the ciliary function

To evaluate whether the ciliary function could be affected by infection with H3N8 AIVs we conducted a coloured bead assay on the surface of the explants. To ascertain the reliability of the method we first evaluated such feature in a negative and positive control. Mock-infected explants cleared the beads within five minutes throughout the study period as shown in figure 2 confirming the remarkable vitality of the equine explant system following several days of culture.

Explants infected with EIV showed a progressive decrease in bead clearance starting from day 2 p.i. until the last time point at which the assay was conducted. The altered function observed in these samples was observed as an increase in bead clearance time (which ranged from 10 to 50 minutes) or a complete absence of clearance depending on the sample evaluated and on the day post infection.

Equine explants infected with H3N8 AIVs did not show any sign of altered ciliary function regardless of the isolate. Bead assays conducted on these samples were not significantly different from mock-infected throughout the study period.

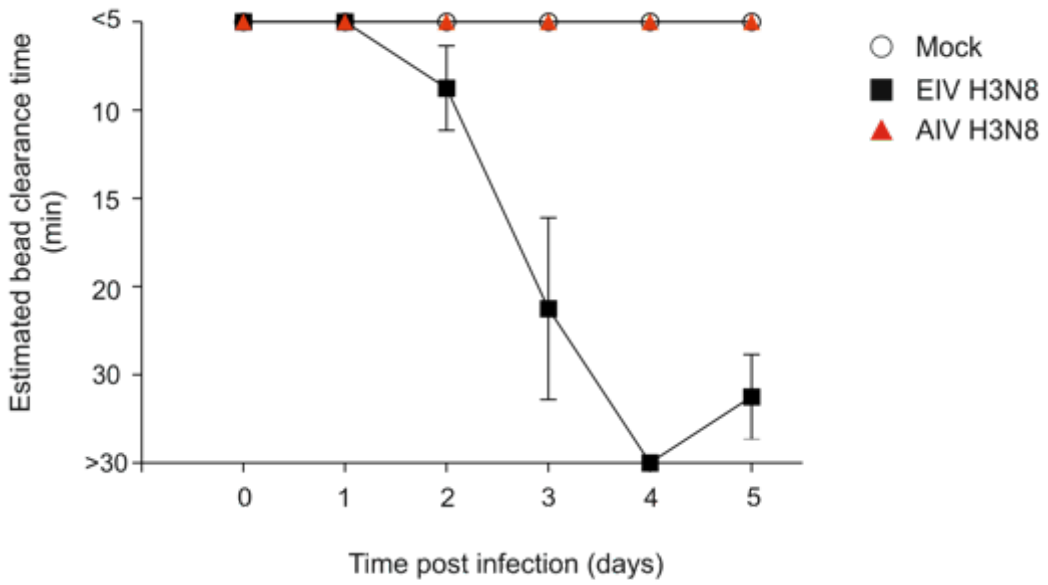

Figure 2. Bead clearance assay in equine tracheal explants infected with H3N8 EIV and Mongolian H3N8 AIVs. Lines represent the average time and SEM in bead clearance in two independent experiments. AIV H3N8 is representative of all AIVs tested.

5.3.3 H3N8 AIVs do not induce visible signs of infection in equine tracheal explants

To investigate whether H3N8 AIVs could induce epithelial changes associated with infection we evaluated infected explants by histological means at 6 hpi (designated as day 0) and then every 24 hours for five days. Mock-infected explants maintained the normal architecture and cellular complexity of a pseudo-stratified ciliated respiratory epithelium throughout the study. In EIV infected samples changes in epithelial morphology compatible with viral infection were evident from day 2 p.i. onwards as shown in figure 3. Typical lesions included loss of cilia at the apical surface, vacuolisation and shrinkage of epithelial cells and a progressive thinning of the epithelium.

Interestingly, none of the tested AIVs induced evident changes in the morphology of the epithelium ascribable to influenza infection (fig. 3). At all time points infected explants revealed an epithelial morphology comparable to mock-infected samples. No alterations in either ciliated or epithelial cells were observed regardless of the virus tested. To increase the chances of detecting alterations, duplicate samples were collected and serially sectioned through.

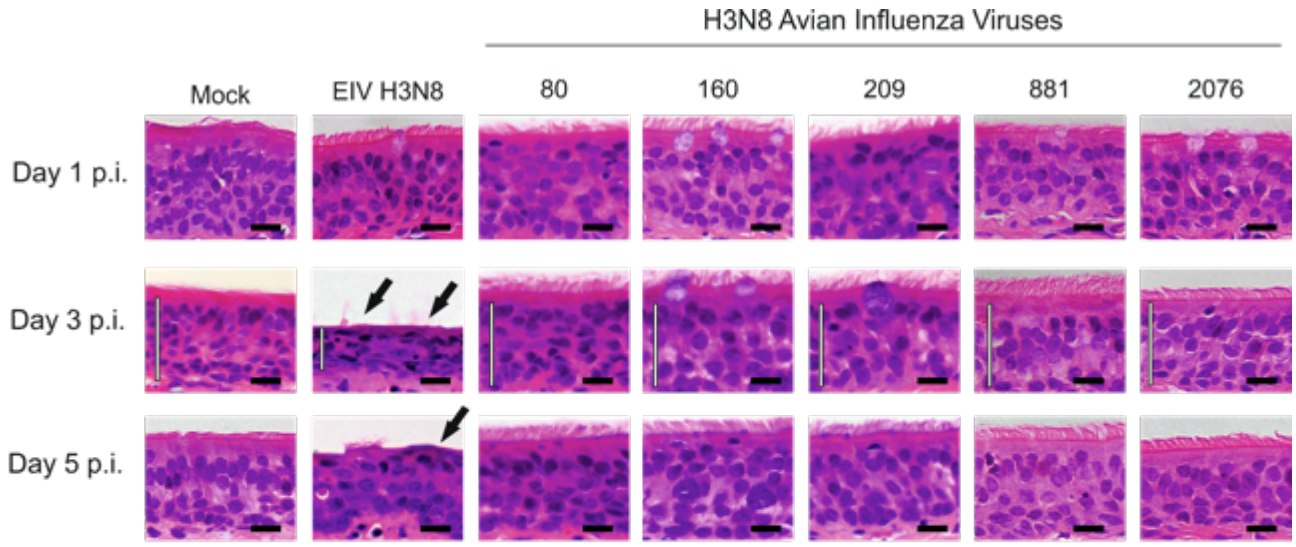

Figure 3. Histological examination of equine tracheal explants infected with H3N8 EIV and Mongolian H3N8 AIVs. Hematoxylin and eosin staining of equine tracheal explants infected with EIV South Africa/03 and several H3N8 AIVs at different times post infection (p.i.). Black arrows show loss of cilia on the epithelial surface. White vertical lines show the epithelial thickness of mock, EIV-infected and AIV-infected explants on day 3 p.i.. Black horizontal bars represent 50 µm.

5.3.4 Viral nucleoprotein and virus induced apoptosis are hardly detected in H3N8 AIVs infected explants

To look for a cellular target in the equine tracheal epithelium susceptible to H3N8 AIV infection we conducted an immunohistochemical staining for viral nucleoprotein (NP). In EIV infected explants viral NP was detected as early as day 1 p.i. and until the end of the study period (5 days). Infected cells were localized exclusively in the epithelium. With regards to AIVs infected explants these yielded negative results in the majority of the cases despite serially sectioning through the samples. Only in two samples infected with Mongolian AIVs we were able to detect positive cells on day 3 and 5 post infection as shown in figure 4. It must be highlighted that replicate samples collected from the same tracheal ring and infected with the same virus yielded different results as only in 1/2 replicas positive cells were detected.

To further describe the infection phenotype of AIVs in equine tracheal explants we conducted an immunohistochemical staining for apoptosis. Mock-infected samples yielded no apoptotic cells whereas EIV infected explants showed presence of apoptosis from day 1 p.i. persisting until day 5 p.i. as shown in figure 5. No apoptotic cells were detected in AIVs infected samples despite serial sectioning of all replicas (representative samples are shown in figure 5).

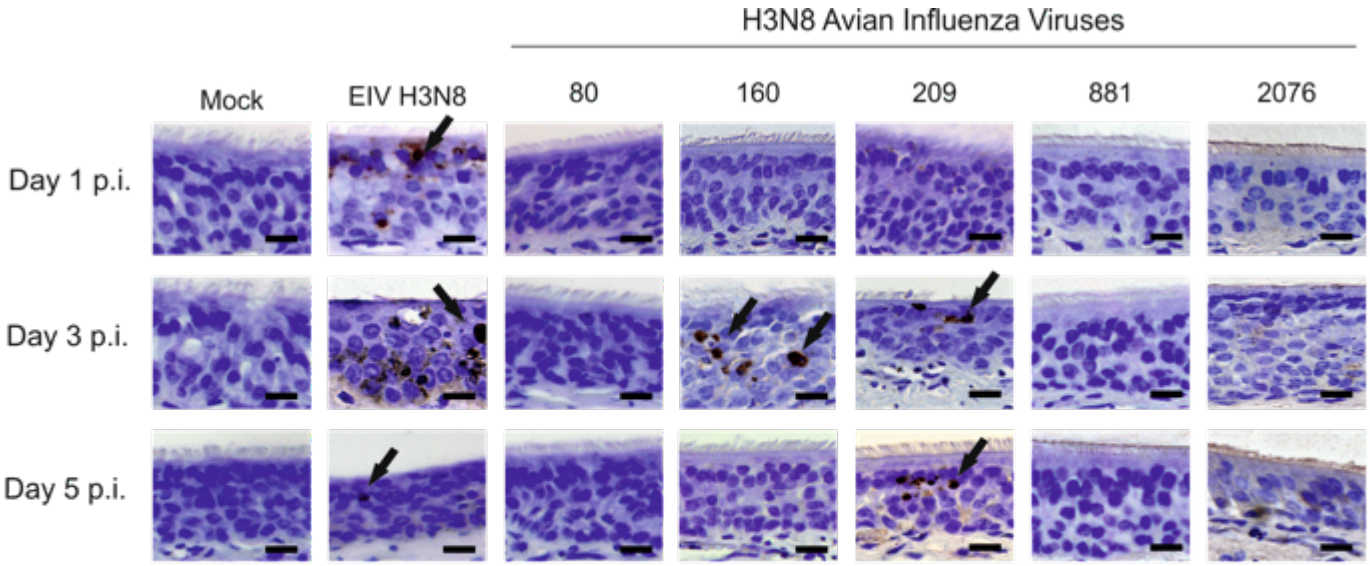

Figure 4. Viral nucleoprotein detection in equine tracheal explants infected with H3N8 EIV and Mongolian H3N8 AIVs. Immunohistochemistry for viral NP in equine tracheal explants infected with EIV South Africa/03 and several H3N8 AIVs. Black horizontal bars represent 50 µm. Positive cells are stained in brown and indicated by black arrows.

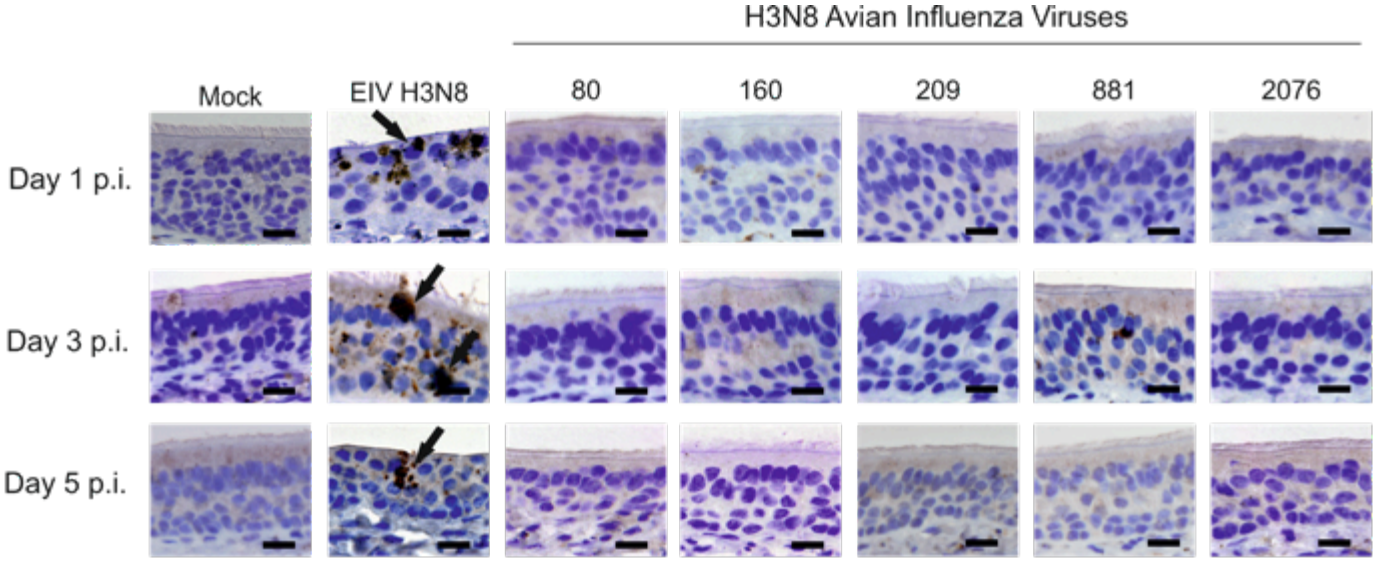

Figure 5. Apoptosis detection in equine tracheal explants infected with H3N8 EIV and H3N8 AIVs. Immunohistochemistry for cleaved caspase 3 in equine tracheal explants infected with EIV South Africa/03 and several H3N8 AIVs. Black horizontal bars represent 50 µm. Positive cells are stained in brown and indicated by black arrows.

5.4 Discussion

The two currently known subtypes of equine influenza virus H7N7 and H3N8 have originated from the avian gene pool (Worobey *et al.*, 2014). Moreover, the H3N8 EIV epizootic that affected horses in China in the 1980's provided further evidence of an avian-origin virus introduced independently into horses (Guo *et al.*, 1992).

The mechanisms that drive cross species transmissions of IAVs, either resulting in dead-end infections or propagation among the novel host, are still largely unknown. Several features such as viral characteristics, host-specific barriers and ecological conditions are likely involved therefore choosing appropriate experimental models and geographical study areas is of paramount importance in tackling disease transmission.

A unique background for studying the direct transmission of AIVs from the wild bird reservoir to horses is found in Mongolia where both populations are present in high numbers and poultry production is limited (Gilbert *et al.*, 2012).

Influenza in horses is a respiratory disease therefore the epithelium lining the airways is a key site of virus-host interaction. As discussed in chapter 3, equine tracheal explants offer a valid method to study such interaction. In the present chapter we compared the infection phenotype of H3N8 AIVs isolated from wild birds in Mongolia with that of an H3N8 equine influenza virus in equine tracheal explants over a five days period.

In first instance we determined viral replication: as expected, and consistent with results presented in chapter three, EIV South Africa/03 infected tracheal explants reaching high viral titres from day 1 p.i. onwards.

As for the AIVs, the infection phenotype displayed differed from that of EIV. Except for one isolate, all tested isolates replicated in tracheal explants albeit to a lower level than EIV and with distinct dynamics according to the isolate. Viral replication results were very consistent for EIV in terms of daily viral detection in explants whereas for AIVs variations depending on the isolate were observed. Host range restriction is determined by several factors. Amongst these recognition and attachment to host receptors mediated by the hemagglutinin protein represents the first step of virus-host interaction. Avian and equine influenza viruses both recognize α2-3 silaic acids (SA)

bound to sugar chains present on the cellular surface. Receptor distribution studies conducted on equine tissues highlighted the dominance of α2-3 sialic acids bound to N-glycolyl neuraminc acid (NeuGc) galactose from the nasal mucosa down to the bronchial epithelium (Muranaka *et al.*, 2011). AIVs preferentially bind to another moiety of SA, namely the α2-3 N-acetyl neuraminc acid (NeuAc) galactose, but several subtypes including H3N8 have been shown to be able to bind also to α2-3 NeuGc (Ito *et al.*, 2000). Moreover, variations in receptor avidity for the two moieties have been reported within the H3N8 subtype depending on the isolate. Such feature might explain the variability observed among the isolates tested in our study and future virus binding assays should be implemented to enrich our findings and improve the understanding of the observed patterns.

Ex vivo experiments allow to gain insights into numerous aspects of the biology of viruses. Replication of EIV caused a progressive ciliostasis as a consequence of intense epithelial damage. As ultimate expression of efficient infection viral nucleoprotein and virus-induced apoptosis were abundantly observed in EIV infected explants. Of particular interest is the finding that despite replicating, none of the AIVs caused evident epithelial damage and the ciliary activity was not affected. Furthermore viral nucleoprotein detection was achieved in only two samples on day 3 and 5 post infection and no apoptotic cells were detected. Such feature has been commonly described in infections of swine (see chapter 3 and (Van Poucke *et al.*, 2010)]) and canine tracheal explants (Gonzalez *et al.*, 2014) with non-species adapted viruses. A likely explanation is that these viruses induce very focal infections, which may be hard to detect histologically. In our opinion even if not adequately proved in these studies (because it was not the purpose) these findings may be an expression of the lack of adaptation of tested viruses to the host. In fact such discrepancies were never reported in the case of positive control samples (infection of explants with a species adapted virus). Further analyses of the differences observed at the site of infection, may reveal important aspects of the viral adaptation process. Increasing the sample collection in the early phases of infection as well as investigating the interaction of viruses with the mucus present on the epithelial surface, that has been shown to vary between viruses with different receptor

specificity (Van Poucke, 2013), could provide a further understanding of the phenotypes described herein.

Despite not being consistent for all AIVs, our staining of viral nucleoprotein provided preliminary evidence that both AIVs and EIV target tracheal epithelial cells. Genetic reassortment may be an accessible pathway of adaptation in the likelihood of two viruses infecting a same cell. Having proved a common site for replication, a more careful analysis of the cellular types targeted will allow us to assess the possibility of viral reassortment.

This *ex vivo* study demonstrates that H3N8 AIVs can replicate in equine tracheal explants and is supported by historical evidence of avian H3N8 introductions into horse populations. A recent study aiming at evaluating the replication potential of several subtypes of AIVs different from H3N8 in the horse highlighted incongruent results between *ex vivo* and *in vivo* data (Chambers *et al.*, 2013). Following a preliminary study of viral replication in equine tracheal explants Chambers and colleagues infected ponies with AIV isolates of the H1, H6 and H7 subtype and they all failed to replicate. The information provided on the abundance (e.g. number of replicas) of *ex vivo* experimental samples is limited and the infection phenotype only relied on virus quantification without additional proofs of viral infectivity (e.g. immunohistochemistry). Furthermore high viral loads were used for infecting explants (10^6 EID_{50} per explant) which may have influenced the outcome of the experiment. In fact, the titres reached in explants by the AIVs chosen for *in vivo* infections were higher than those observed for one of the EIVs included as positive control and just slightly lower than the other control EIV.

The first airways (nasal mucosa) and associated immune system may not be suitable for viral replication and prevent the virus from reaching the trachea. To our knowledge SA receptor distribution in horse nasal mucosa and trachea is similar (Muranaka *et al.*, 2011) but further characteristics such as glycan topology and modifications (e.g. fucosylation or sulphation) can affect viral affinity (Reperant *et al.*, 2012 and references therein) and should be accurately studied in the horse. To overcome such discrepancies, where possible, *ex vivo* studies on viral tropism should include tissues of the different

parts of the respiratory tract to have a broader picture on replication competency. It must be ultimately acknowledged that the barriers present in the living host may be key in hampering viral replication and draw a line on the limits of the *ex vivo* cultures.

Adding to historical evidence, our results are also strongly supported by serological surveys conducted among Mongolian horses in which anti H3N8 AIVs antibodies were detected (Pablo Murcia, personal communication).

Overall the results provided by our *ex vivo* study indicate that H3N8 AIVs isolated from wild birds can replicate in the trachea of horses with different infection dynamics and to a lower extent compared a horse adapted virus such as EIV. As reassortment events may occur in this host, further studies addressing the exact cellular sites and the molecular determinants required for an AIV to efficiently replicate in the horse trachea may be very useful in shedding light on the emergence of equine influenza from viruses circulating in the avian reservoir.

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Chapter 6

General discussion

Influenza A viruses (IAVs) are zoonotic pathogens that annually infect humans and several animal species epidemically and at unpredictable intervals generate pandemics. Their heterogeneous gene pool is maintained in wild aquatic birds, the natural reservoir. The segmented RNA genome and the error prone polymerase of these viruses allow a frequent appearance of mutations and a tendency to reassort. Both mechanisms are essential for virus evolution and have been involved in host switching events whose understanding represents the leading research front in influenza. Inter species transmission, either resulting in well-established infections in a new host population or occasional spill-over events, are frequent. Several factors are seemingly involved such as probability of contact between two species, virus-receptor interaction, ability to replicate in the infected cell, successful release and escape from host immunity (Reperant *et al.*, 2012). Because of public health concerns the most studied transmission involves humans as final recipients, and birds and pigs as virus donors. Nevertheless, such events also occur between animal populations. This offers the opportunity to understand the mechanisms that underpin viral emergence in multiple ecological contexts.

The H3N8 subtype harboured in the avian reservoir has been capable of entering different mammalian hosts (horses and seals [Karlsson *et al.*, 2014]) and of being further transmitted between mammals, as proven by the emergence of H3N8 canine influenza from equine influenza (Crawford *et al.*, 2005). Studying disease dynamics in the target tissues of the host is fundamental to gain insight into host barriers that may hamper viral replication and establishment. *Ex vivo* culture of respiratory tissues allows to investigate the infection phenotype of influenza viruses under controlled conditions. Therefore, this thesis aimed at investigating H3N8 IAVs disease dynamics at the site of infection in two relevant host species, the pig and the horse.

We first sought to characterize host-adapted viruses in swine and horses providing an extensive description of multiple aspects of swine and equine influenza pathogenesis in respiratory explants of naïve animals (chapters two and three respectively). For this

purpose we adopted previously published techniques of *ex vivo* cultures, adding minor modifications, and monitored the dynamics of infection over time through an infection phenotype featuring viral (e.g. quantifying viral growth) and host characteristics (e.g. tissue damage and response such as apoptosis). Similar experiments had been previously described (Lin *et al.*, 2001; Nunes *et al.*, 2010; Van Poucke *et al.*, 2010). Nevertheless, our purpose was to gain further insights by expanding the study period, the viruses tested, the analyses conducted and the tissues involved. Moreover, by comparing and discussing pre-existing literature we chose culture and infection methodologies that more realistically resemble the host-pathogen interaction *in vivo*. Explants were cultured at the air-liquid interface, infected with a low quantity of virus (200 pfu) and monitored over a time period similar to that observed in natural infections. In pigs the entire respiratory tract was collected (nasal mucosa, trachea and lung). Unfortunately, for practical reasons, in horses we were only able to work on the trachea.

Viral replication was consistent in both swine and equine explants from 24 hpi onwards and manifested as a progressive decrease in the ciliary activity of the tracheal epithelium. Through histological and immunohistochemical examination we identified the epithelium lining the respiratory tract as the major target of infection for both SIV and EIV. Typical features of infection included loss of cilia at the apical surface, cellular disruption and a striking thinning of the epithelium. Immunohistochemistry supported this last finding by showing how infection of few cells (24 hpi) increasingly spreads across the epithelium affecting its entire thickness. Importantly, by comparing several replicas obtained from the same animal and among independent experiments we evidenced a different degree of infection depending on the individual explant observed. As the exact number of susceptible cells in the explants cannot be controlled, we have hypothesized that this factor along with the innate immunity may both play a role. The innate immunity is triggered by the infection of epithelial cells and the magnitude of its effect also depends on the number of cells in each explant. Therefore, when using this technique an appropriate experimental design including several replicas for all the different analyses should be implemented.

Our extensive examination has added substantial information to previous literature and confirmed that swine and equine *ex vivo* systems respectively support swine and equine influenza replication and are suitable to study virus pathogenesis and at the same time observing the 3Rs ethos. Finally, the lesions observed in the respiratory epithelium closely resembled those of naturally and experimentally infected animals (Janke, 2014; Sutton & Viel, 1997).

Following the validation of the two systems with host-adapted viruses we then addressed our interest to H3N8 IAVs. During the last decade the emergence of CIV and the sporadic detections of EIV in other hosts have raised concerns on the tropism of H3N8 for other mammals. Recently it has been suggested that EIV evolution along time could have played an important role in the emergence of CIV (Gonzalez *et al.*, 2014). As EIV spill-over infections were detected after CIV emergence, we sought to investigate whether changes in host range along the evolutionary history of EIV included other animal species besides dogs.

Despite not having yet disentangled the scientific reasons behind the susceptibility of pigs to IAVs of different origin, there is a general consensus on their crucial role in influenza ecology. H3N8 EIV was detected in pigs during 2004-2006 influenza surveillance in China (Tu *et al.*, 2009). Therefore we wanted to characterize the infection phenotype of phylogenetically distinct EIVs, each representing a different evolutionary period, in relevant biological systems representing the pig (chapter four).

To have a first insight, we infected two primary swine cell lines (NPTr and NSK) that had been previously shown to support IAV replication (Ferrari *et al.*, 2003). Despite using a low MOI (0.001), all tested EIVs efficiently replicated in both cultures to comparable levels to an H3N2 SIV providing preliminary evidence on the ability of EIV to infect swine cells. On the other hand, when testing our panel of EIVs on swine explants, striking differences were observed among EIVs and between EIV and SIV.

The early isolate Uruguay/63 was the only EIV able to infect and replicate efficiently in distinct anatomical regions of the pig respiratory tract showing a greater tropism for the lungs compared to the primary airways. The following infection phenotype was

observed: little to no replication in the upper respiratory tract with negative immunohistochemistry for viral NP versus higher titres in the lungs and NP stained bronchiolar cells. This phenotype recalled that described for AIVs in the same explant system by other authors (Chan *et al.*, 2013; Van Poucke *et al.*, 2010). As EIV originated from an avian H3N8 we have hypothesised that the initial EIVs were more avian-like and thus able to infect pigs, and that such tropism for the swine respiratory tract was lost when EIV became more adapted to the horse during the initial EIV epidemics. Such adaptation could have resulted in a shift in receptor preference. The N-glycolyl neuraminc acid (NeuGc) is the dominant sialic acid (SA) species in the equine respiratory tract and increased affinity for this moiety has been reported for EIVs isolated after 1979. In turn earlier isolates showed a major affinity for N-acetyl neuraminic acid (NeuAc) which is the dominant SA species in pigs and birds (Suzuki *et al.*, 2000). It has been argued that this difference could have also been caused by extensive replication of the early EIV isolates in embryonated chicken eggs and this cannot be ruled out. Nonetheless, as all of the viruses used in our study were isolated and egg-grown at least 10 years prior to conducting the experiments and because of the increasing evidence that receptor recognition itself does not imply a functional relevance in infection (Air, 2014; Van Poucke, 2013a) we hypothesize that further steps in viral replication may have been involved in the observed differences. Such a hypothesis may be further investigated by reverse genetics studies aimed at understanding the molecular determinants of EIV Uruguay/63 pathogenesis. It must be acknowledged that our results on a limited panel of viruses do not rule out the possibility that currently circulating EIVs could emerge in pigs. In fact, detection of EIV in pigs has occurred proving that numerous factors contribute to disease transmission and that natural occurrences are not always retraceable under experimental settings.

The finding that all EIVs tested replicated to similar levels to an H3N2 SIV in pig cell lines, whereas distinct infection phenotypes were observed in explants suggests the presence of tissue-specific host barriers at the site of infection that must play a central role in viral pathogenesis and emergence. Lin and colleagues (2010) infected bovine primary cell cultures with H3N8 EIV and observed replication. When they next infected calves with the same virus it failed in infecting the animals. This further supports our vision on the

importance of using relevant biological systems to assess viral tropism and the presence of tissue-specific host barriers. Even though we did not have a final *in vivo* confirmation, the data provided by our *ex vivo* study clearly represent a big step forward (compared to *in vitro* data) in terms of screening the replication potential of several isolates and narrowing down eligible candidates for *in vivo* experiments.

Parallel to our studies of H3N8 EIV in pigs, we further pursued our investigation of H3N8 disease transmission between different animal species by collaborating with the Wildlife Society in Mongolia. This region represents a unique ecological setting for studying the direct transmission of AIVs from the wild bird reservoir to horses. Activities included phylogenetic analysis of H3N8 isolates from wild birds, serological surveys on equine sera aimed at detecting antibodies to H3N8 AIVs and an *ex vivo* evaluation of the replication potential of selected isolates in equine tracheal explants.

With one exception, all H3N8 AIVs tested replicated in equine tracheal explants yet displaying different infection dynamics in terms of days of isolation and number of consecutive days in which viral replication was recorded. AIVs replicated to a lower extent when compared to EIV as shown by significantly lower maximum titres ($P<0.001$). This was also confirmed by the fact that none of the isolates induced visible epithelial damage and viral NP detection was sporadic and not consistent among different replicas infected with the same virus. Once more, our results highlighted an important difference in infection phenotype and its consistency between host adapted and non-adapted viruses. Through this *ex vivo* study we have demonstrated that H3N8 AIVs can infect the equine trachea and serological evidence of infection in horse sera (Pablo Murcia, personal communication) also supports the occurrence of sporadic infections in the field. Nonetheless, the difference in infection phenotype between AIV and EIV reported herein and the fact that avian H3N8 has efficiently established in horses only twice, suggest that, for a successful establishment to occur, specific adaptation may be necessary.

Some considerations on the use of explant cultures were raised from our experiments. Swine explants infected with H3N2 SIV as well as equine explants infected with H3N8 EIV exhibited consistent infection in all tested replicas (within and among experiments). In

contrast, tissues inoculated with non-host-adapted viruses showed some variability in infection despite taking precautions to minimise experimental variability (e.g. by infecting the same tracheal rings of different animals with the same virus, and using exclusively the right apical lobe of the lung). Similar variations in infection phenotypes have been reported in swine tracheal and lung explants infected with human isolates of H5N1 avian influenza virus (Chan *et al.*, 2013). Currently, the causes of such observations have not been determined. There are multiple barriers that a virus encounters at the site of infection. The presence of mucus covering the epithelial surface of the airways as well as surfactant protein D in the lung have been shown to play a role in determining the efficiency of IAV infection (Hillaire *et al.*, 2013; Matrosovich & Klenk, 2003; Van Poucke, 2013b; Roberts *et al.*, 2011). The relative abundance of such factors (e.g. how many mucus producing cells are present in each explant) as well as the variation in receptor distribution among explants have not been determined and may all play important roles in explaining why in some explants viruses are able to replicate and in others not. Host-adapted viruses may be advantaged in overcoming such barriers. Further studies addressing the quantification and variability of such factors in *ex vivo* systems could be important to clarify their potential influence on the experimental variation observed.

Moreover, when testing non-host-adapted viruses, there was inconsistency between virological and histological results in both tracheal explants systems. Epithelial damage was not observed and viral NP detection was hardly achieved despite quantifying viral replication. Previous studies have attributed this inconsistency to the presence of very focal infections (Chan *et al.*, 2013) that are hard to detect histologically. Alternatively, we have proposed that this phenotype could be an expression of the lack of adaptation of the virus to the specific host. In fact, Gonzalez *et al.* (2014) reported that an EIV isolated at the time of CIV emergence replicated in canine tracheal explants displaying a similar phenotype to CIV whereas earlier isolates, hypothetically non adapted to dogs, replicated to lower levels and did not cause epithelial damage. It also deserves to be mentioned that in host-adapted infections NP positive cells were detected as early as 24 hpi (and possibly earlier) whereas in non-host-adapted infections (swine lung explants infected with H3N8 EIV Uruguay/63 or equine tracheal explants infected with H3N8 AIVs

160 and 209) these were found only from 48 hpi or later onwards. This finding suggests that cellular internalization may require longer and therefore viral particles are more likely to be counteracted by the immune system.

The lack of replication of EIV Uruguay/63 in the nasal mucosa coupled with an increasing affinity for the trachea and even greater for the lungs underlines the advantage of using an explant system covering the entire respiratory tract. Other reported evidence also stresses the importance of using different tissues when assessing viral tropism, namely, the incongruity between viral replication in equine tracheal explants and *in vivo* studies in ponies (Chambers *et al.*, 2013). Furthermore, it is important to include the primary airways, as studying viral behaviour in this site is fundamental not only to reveal the virus primary site of entry, but also to assess the likelihood of the virus exiting the host (Shinya *et al.*, 2006). Using the entire respiratory tract is not always feasible and this pinpoints one of the limits of the interpretation of *ex vivo* studies and the necessity of secondary *in vivo* comparisons. Nonetheless, when coupled with epidemiological evidence of infection or complementary field studies such as our serological survey in horses, the explant technique becomes stronger and can indeed enhance knowledge on the host-pathogen interaction picture.

In summary, the use of *ex vivo* cultures of the respiratory tract allowed us to describe in detail the infection dynamics of host-adapted influenza viruses under experimental conditions and subsequently compare them to non-host-adapted viruses highlighting important differences at the site of infection and along the respiratory tract. Within the context of this thesis, the use of organ explants has yielded strong and consistent results when using host-adapted viruses. On the other hand, results obtained when testing other viruses were more variable and had some discrepancies. These findings, that match those of similar previous studies, raise challenging questions about the biological reasons behind them and open new pathways of disease investigation at the site of infection. Ultimately, we have described differences in viral replication according to the tissue involved. Different anatomical sites could be used for receptor affinity studies and to explore tissue-associated viral quasispecies, the latter being an innovative approach to studying disease dynamics in organ explants. Despite having stressed and discussed

some of its limitations, we have confirmed that the *ex vivo* system bridges very important gaps between *in vitro* and *in vivo* experiments.

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Appendix

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Journal of General Virology

Phylogenetically distinct equine influenza viruses show different tropism for the swine respiratory tract.

--Manuscript Draft--

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Short Title:	EIV host range and tropism
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Abstract:	Influenza A viruses circulate in a wide range of animals. H3N8 equine influenza virus (EIV) is an avian-origin virus that has established in dogs as canine influenza virus (CIV) and has also been isolated from camels and pigs. Previous work suggests that adaptive mutations acquired during EIV evolution might have played a role in CIV emergence. Given the potential role of pigs as a source of human infections, we determined the ability of H3N8 EIVs to replicate in pig cell lines and in respiratory explants. We show that evolutionary distinct EIVs display different infection phenotypes along the pig respiratory tract, but not in cell lines. Our results suggest that EIV displays a dynamic host range along its evolutionary history, supporting the view that evolutionary processes play important roles on host range and tropism, and also underscore the utility of using explants cultures to study influenza pathogenesis.

Phylogenetically distinct equine influenza viruses show different tropism for the swine respiratory tract.

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SUMMARY

Influenza A viruses circulate in a wide range of animals. H3N8 equine influenza virus (EIV) is an avian-origin virus that has established in dogs as canine influenza virus (CIV) and has also been isolated from camels and pigs. Previous work suggests that mutations acquired during EIV evolution might have played a role in CIV emergence. Given the potential role of pigs as a source of human infections, we determined the ability of H3N8 EIVs to replicate in pig cell lines and in respiratory explants. We show that phylogenetically distinct EIVs display different infection phenotypes along the pig respiratory tract, but not in cell lines. Our results suggest that EIV displays a dynamic host range along its evolutionary history, supporting the view that evolutionary processes play important roles in host range and tropism, and also underscore the utility of using explant cultures to study influenza pathogenesis.

Influenza A viruses (IAVs) viruses circulate in various animal hosts, and cross-species transmissions of IAVs have led to either spillover infections or emergence and sustained transmission in new host populations. Avian H3N8 viruses show a remarkable ability to cross species barriers, particularly in infecting mammals. For example, H3N8 equine influenza virus (EIV) is an avian-derived IAV (Worobey *et al.*, 2014) that was first reported in the early 1960s and is still circulating in horses. A second introduction of a different H3N8 avian influenza virus into the horse population took place in 1989 (Guo *et al.*, 1992) but this virus only circulated in equines for a couple of years (Guo *et al.*, 1995). Further, an avian-derived H3N8 IAV has recently caused an outbreak in seals in North America (Karlsson *et al.*, 2014). Finally, the currently circulating H3N8 EIV crossed the species barrier and established in dogs as canine influenza virus (CIV) in early 2000s (Crawford *et al.*, 2005) and natural spillover infections of H3N8 EIV have been reported in pigs (Tu *et al.*, 2009) and camels (Yondon *et al.*, 2014).

Historical and epidemiological evidence support the view that pigs play an important role in influenza ecology (Vincent *et al.*, 2014) and might be a source of reassortant viruses with zoonotic potential. Therefore, it is important to determine if IAVs that are endemic in other domestic animals could potentially infect pigs and contribute via reassortment to the IAV gene pool that could eventually transfer to humans, as happened during the 2009 pandemic (Smith *et al.*, 2009).

It has been suggested that EIV evolution could have played an important role in the emergence of CIV: EIVs that circulated in the 1960s displayed a highly attenuated phenotype in dog tracheas, whereas an EIV isolated in 2003 -around the time of emergence of canine influenza virus- exhibited an infection phenotype indistinguishable from that of CIV (Gonzalez *et al.*, 2014).

We wanted to investigate whether changes in host range along EIV evolutionary history included other animal species besides dogs, and focused on pigs because this species can behave as a “mixing vessel” (Ma *et al.*, 2009) and also because EIV has been isolated from pigs in nature (Tu *et al.*, 2009). To this end, we inoculated two different swine cell lines (newborn pig trachea cells and newborn swine kidney cells) with an Eurasian H3N2 swine influenza virus A/swine/Italy/8088/2006 (SIV) and compared its growth kinetics to those of a panel of H3N8 EIVs: A/equine/Uruguay/1963 (Uruguay/63), A/equine/Fontainebleau/1979 (Fontainebleau/79), A/equine/Argentina/1995 (Argentina/95), and A/equine/South Africa/2003 (South Africa/2003). These viruses represent phylogenetically distinct clades of EIV and despite the unavailability of complete passage history they were chosen for specific reasons: Uruguay/63 is the oldest known H3N8 EIV (Murcia *et al.*, 2011), South Africa/03 represents the clade of viruses circulating at the time

of CIV emergence and the other two isolates included represent intermediate clades. The swine cell lines (provided by the Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna [Ferrari *et al.*, 2003]) were maintained at 37°C with 5% CO_2 in Minimum Essential Medium (Gibco) supplemented with 10% Fetal Calf Serum (Euroclone), 1% 200mM L-glutamine (Sigma) and 1% of Penicillin-Streptomycin (P/S, Gibco) and Nystatin (Sigma). In this first study we compared the growth kinetics of the different viruses over a 72-hour time course (MOI 0.001). Viral titres in the supernatants from triplicate wells of three independent experiments were calculated by the Tissue Culture Infectious Dose ($TCID_{50}$) assay according to the Reed and Muench formula. Briefly, samples were serially diluted in MEM supplemented with 1% P/S, 1% L-glutamine and 1 µg/ml of TPCK-treated trypsin (infection medium) and 50 µl of each dilution were inoculated in quadruplicate wells of confluent MDCK cells seeded in 96-well plates. After a two-hour incubation at 37°C, 100 µl of infection medium were added to each well and plates were further incubated for 72 hours. Plates were checked for the presence of CPE and immunostained as described previously (Gonzalez *et al.*, 2014). Although there were slight differences in replication dynamics observed at 6 and 24 hours post infection (hpi), no significant differences were detected between SIV and any of the EIVs tested at later time points ($p>0,05$ two-way Anova with Bonferroni's post-test for multiple comparisons). These results show that all tested EIVs can readily infect and replicate in swine cell lines to similar levels of an H3N2 swine influenza virus (Figures 1a and 1b).

Since influenza in pigs is a respiratory disease we then sought to characterize the infection phenotypes of the different EIVs in the swine respiratory tract. Tissue explants represent a suitable model to study influenza pathogenesis at the site of infection and also allow for a reduction of the number of animals used in research. Experiments were approved by the ethical committee of IZSVe (Approval number CE IZSVe 11_2012). We cultured explants from various anatomical regions of the swine respiratory tract: nasal mucosa (respiratory section), trachea (all tracheal rings were used) and lung (right apical lobe). Tissues were harvested from six to eight-week-old commercial hybrid piglets that were IAV seronegative. Explants were prepared as previously described (Nunes *et al.*, 2010; Van Poucke *et al.*, 2010), and inoculated with 200 plaque-forming units (PFU) of SIV H3N2, or with the aforementioned EIVs. Infection phenotypes were determined at 6, 24, 48, 72 and 96 hours post-inoculation based on virus growth kinetics ($TCID_{50}$/ml as described above), histopathological lesions, changes in ciliary beating and viral antigen detection by immunohistochemistry as described in Gonzalez *et al.*, (2014). Each virus was tested in duplicate in tissues obtained from three different pigs.

As expected, SIV consistently exhibited high replication efficiency in tracheas (Fig. 2b), lungs (Fig. 3b) and nasal mucosa (not shown). Histological damage was evident in the nasal

mucosa displaying lesions such as epithelial disruption and vacuolization (Fig. 2a). Infected tracheas exhibited loss of cilia, reduction in epithelial thickness (Fig. 2d) and decreased ciliary function (Fig. 2c). SIV H3N2 antigen was also readily detected in all infected explants regardless of the anatomical location (Fig. 2a, 2d and 3a).

In contrast, EIVs showed variable infection phenotypes depending on the virus isolate and the anatomical region of the respiratory tract tested. The nasal mucosa was the only anatomical region in which no signs of EIV infection and replication were observed. In the trachea, only one of the four EIVs tested (Uruguay/63) replicated at higher titres than the other EIVs, but still at lower levels than SIV (Fig. 2b). Uruguay/63 exhibited a peak in viral replication (up to 5 $\log_{10}$ $TCID_{50}$/ml) at 72 hpi in four out of six replicas. As for the other EIVs, virus titres were always lower or equal to the initial inoculum suggesting that they replicated at very low levels. The differences observed between Uruguay/63 and the other EIVs were significant at 72 hpi ($p<0,05$ two-way Anova with Bonferroni's post-test for multiple comparisons). However, despite evidence of viral replication, Uruguay/63 did not cause histological changes or alterations in bead clearance (Fig. 2c and 2d, respectively). Further, no viral antigen was detected in the infected tracheas examined despite extensive serial sectioning of infected explants (Fig. 2d). The absence of lesions in explants infected with IAVs that replicate at high levels has been previously described (Gonzalez *et al.*, 2014; Van Poucke *et al.*, 2010). Focal infections in limited areas of the explants have been suggested to be the cause of such disparity (Chan *et al.*, 2013).

In lung explants the difference in infection phenotypes was more evident: Uruguay/63 displayed higher replication efficiency by day two post-inoculation, with up to four log increases and peaking at day four, although some inter-animal differences in replication dynamics were observed (Fig. 3b). In fact, in some explants viral titres of Uruguay/63 at 96 hpi were similar to those exhibited by SIV H3N2 and significantly higher than those observed for the other EIVs ($p<0.0001$ two-way Anova with Bonferroni's post-test for multiple comparisons). Moreover, based on immunohistochemical detection of the viral nucleoprotein, we observed that Uruguay/63 infected the bronchiolar epithelium and rare alveolar cells at comparable levels to SIV H3N2 (Fig. 3a). The other EIVs tested did not replicate (or replicated at very low levels) in infected lung explants and no viral antigen was detected by immunohistochemistry despite testing multiple serial sections.

Overall, our results indicate that despite the ability of all tested EIVs to efficiently replicate in pig cell lines in monolayer cultures -in fact, to similar levels of an H3N2 swine influenza virus- only Uruguay/63 is able to infect and replicate efficiently in distinct anatomical regions of the pig respiratory tract. Explants infected with H3N2 SIV exhibited consistent results in all tested replicas (Figures 2b and 3b). In contrast, tissues inoculated with EIVs showed some variability in infection despite taking precautions to minimise

experimental variability (e.g. by infecting the same tracheal rings of different animals with the same virus, and exclusively using the right apical lobe of the lung). Similar variations in infection phenotypes have been reported in swine tracheal and lung explants infected with human isolates of H5N1 avian influenza virus (Chan *et al.*, 2013). Although the cause/s of those variable results have not been determined, it has been suggested that they could be due to variation in receptor abundance and distribution among explants, as well as variable levels of mucus (in the trachea) and surfactant protein D (in the lungs). All these factors could play important roles in determining the efficiency of influenza virus infection and thus should be taken into account (Hillaire *et al.*, 2013; Matrosovich & Klenk, 2003). Further studies addressing the quantification and variability of such factors in *ex vivo* systems could be important to clarify their potential influence on the experimental variation observed.

Here we show that Uruguay/63, the oldest EIV isolate, can infect swine tracheal (albeit with some variability) and lung explants but not the nasal mucosa. Notably, lungs infected with Uruguay/63 showed similar levels of infection to those observed with SIV H3N2, a swine-adapted influenza virus. In contrast, all the other EIVs tested showed an impaired ability to infect any portion of the pig respiratory tract. As previous reports indicate that the swine respiratory tract supports infection and replication of some avian influenza viruses (Löndt *et al.*, 2012; Van Poucke *et al.*, 2010), it is not surprising that Uruguay/63 - the most avian-like virus of the H3N8 EIV lineage - exhibited the highest ability to replicate in pig respiratory tissues. It is feasible to think that the initial EIVs were more “avian-like” and thus able to infect pigs, and that such tropism for the swine respiratory tract was lost when EIV became more adapted to the horse during the initial EIV epidemics. Then, EIV acquired the ability to infect dogs through genetic drift during continuous evolution in horses. Notably, Uruguay/63 and South Africa/2003 displayed very different infection phenotypes in the dog tracheas: South Africa/2003 infects dog tracheas in a similar to fashion that of canine influenza viruses, whereas Uruguay/63 is highly attenuated (Gonzalez *et al.*, 2014) supporting the view that evolutionary processes result in adaptive changes in the viruses that impact both on host range and viral tropism.

The finding that all EIVs tested replicated at similar levels to an H3N2 swine influenza virus in pig cell lines, whereas distinct infection phenotypes were observed in explants suggests the presence of tissue-specific host barriers at the site of infection that must play a central role in viral pathogenesis and emergence, and highlights the importance of using relevant biological systems to assess changes in host range.

Our study has various limitations. First, we tested a small number of EIVs, albeit the choice of viruses was based on a previous study that showed an association between the infection phenotype of phylogenetically distinct EIVs in dog tracheas and the emergence of CIV (Gonzalez *et al.*, 2014). Here we included the same viruses used in that study and also

tested other EIV isolates (Fontainebleau/79 and Argentina/95), which represent additional phylogenetically distinct clades. Second, while our results do not necessarily mean that EIV will or could have emerged in pigs, they do not rule out that possibility either. Previous reports on the correlation of *ex vivo* and *in vivo* infections (Van Poucke *et al.*, 2010), together with the isolation of EIV in pigs in Asia (Tu *et al.*, 2009) indicates that natural infection of EIV in swine can (and did) occur. Given the important role of the pig as a contributor to the gene pool of human influenza viruses it is important to determine if other IAVs could expand that gene pool via infections in swine. Thus, identifying the risk of emergence of currently circulating viruses (or their genes) is important from the point of view of pandemic preparedness. Third, we did not attempt to identify the genetic determinants that allow EIV to productively infect the respiratory tract of the pig and therefore our study could be considered observational. Future studies using reassortant and mutant viruses generated by reverse genetics will be required to achieve that task.

In conclusion, we showed that a phylogenetically distinct EIV displays an enhanced tropism for the respiratory tract of the pig compared to other viruses of the same lineage. Our results support the hypothesis that viral evolution during long-term transmission of influenza viruses in host populations could result in dynamic changes in their host range. Such changes must be in line with ecological and epidemiological factors in order to allow the establishment of novel lineages in susceptible species.

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FIGURE LEGENDS

Figure 1. Growth kinetics of evolutionary distinct H3N8 EIVs in swine cell lines. (a) Newborn pig trachea cells. (b) Newborn swine kidney cells. Vertical bars show the average and standard deviation of three independent experiments.

Figure 2. Infection of explants derived from swine nasal mucosa and trachea with evolutionary distinct H3N8 EIVs. (a) Histological features of swine nasal mucosa explants infected with H3N2 SIV (positive control) and various H3N8 EIVs (Uruguay/63, Fontainebleau/79, Argentina/95, and South Africa/2003). Lesions are shown in sections stained with haematoxylin and eosin. Infected cells were detected by immunohistochemical staining of the NP viral protein. Positive cells are stained in brown. Black horizontal bars represent 50 μm. (b) Growth kinetics of the viruses described in (a) in swine tracheal explants. Dots represent values of individual replicates, * $P<0.05$. (c) Graphical representation of bead clearance assays in infected and control swine tracheal explants. Lines represent the average time to clear the beads in three independent experiments. (d) Histological features of swine tracheal explants infected with the viruses described in (a). Lesions and infected cells are described as in (a). Panels (a) and (c) show explants at day two post-infection.

Figure 3. Uruguay/63 replicates in swine lung explants at higher levels than other EIVs. (a) Immunohistochemical detection of viral nucleoprotein in lung explants infected with the same viruses as in Figures 1 and 2. Positive cells are stained brown. (b) Growth kinetics of the viruses in swine lung explants. Dots represent values of individual replicates, ****$P<0.0001$.

Figure 1

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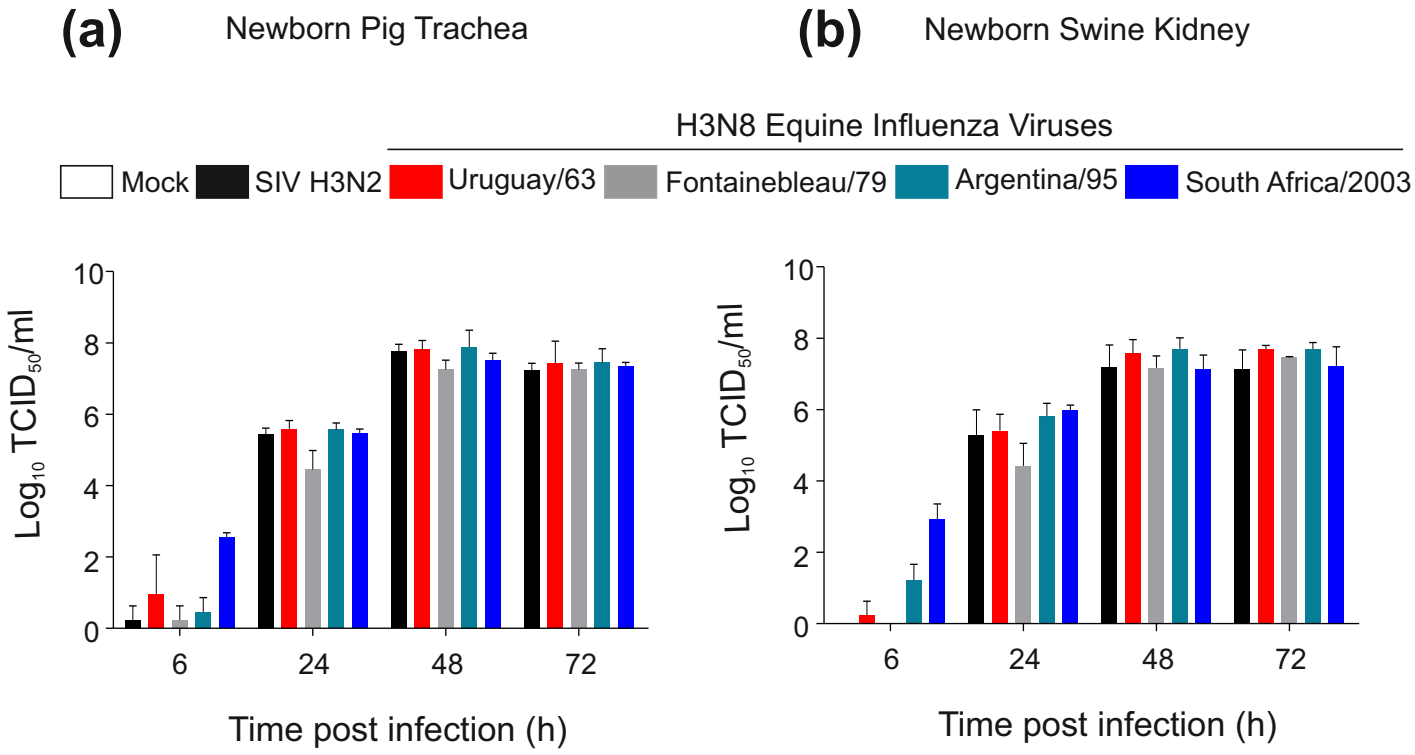

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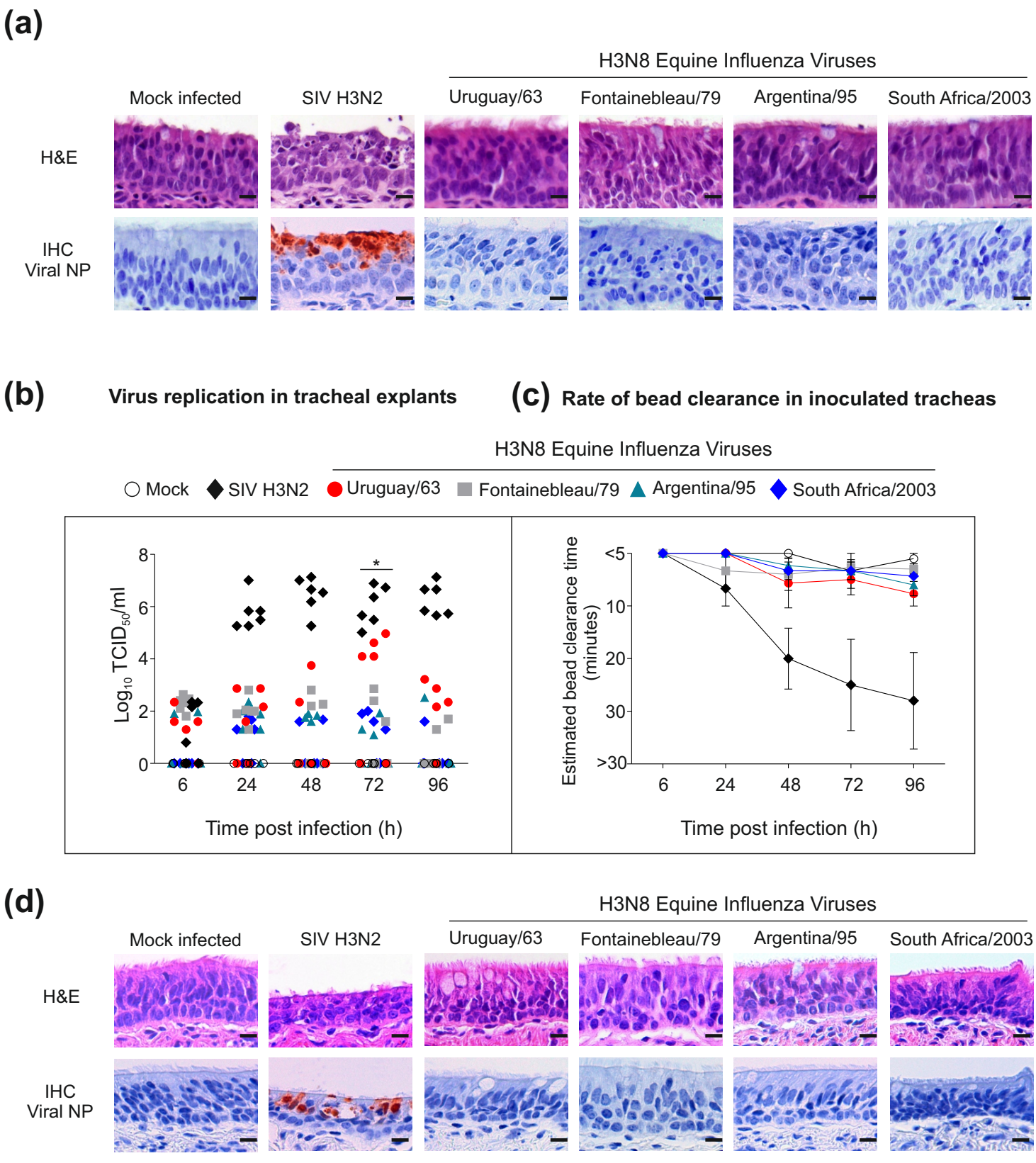

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