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Original Article

The infectivity of pandemic 2009 H1N1 and avian influenza viruses for pigs: an assessment by *ex vivo* respiratory tract organ culture*

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Background Pigs are thought to act as intermediate hosts in the ecology of influenza viruses of both avian and human origin. The recent development of procedures for pig *ex vivo* respiratory organ explants has provided new tools for the assessment of influenza virus infection in pigs.

Objectives To use pig *ex vivo* organ explants to assess the susceptibility of pigs to infection with contemporary viruses, for which there is evidence of human infection and that are thought to pose the greatest threat to pig and human populations.

Methods Pig tracheal, bronchi and lung *ex vivo* organ explants were infected with both highly pathogenic and low pathogenic avian influenza (AI) virus and the pandemic H1N1 [A(H1N1)pdm/09] virus. Successful infection of explants was detected using a positive-sense RNA real-time RT-PCR assay and anti-nucleoprotein immunohistochemistry. The distribution of cell-surface α2-3- and α2-6-linked sialic acid receptors, the avian- and mammalian influenza A virus–preferred host receptors, respectively, was also characterised for the *ex vivo* organ cultures and uninfected pig material following necropsy.

Results The α2-3 and α2-6 sialic acid receptor staining on tracheal, bronchi and lung organ explant sections showed similar distributions to those seen for pig tissue following necropsy. While the pig *ex vivo* organ cultures were susceptible to nearly all viruses tested, lower levels of virus were detected in trachea and bronchi after infection.

Conclusion These results confirm that pigs are susceptible to contemporary viruses that may threaten both veterinary and human health and contribute to the ecology of influenza A viruses.

Keywords *Ex vivo*, H1N1, highly pathogenic avian influenza, pandemic, pigs.

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Introduction

Although evidence for direct avian-to-human transmission of avian influenza (AI) viruses exists, pigs are still considered as potentially important mixing vessels for a range of influenza viruses, as they possess both avian (α2-3) and mammalian (α2-6) sialic acid viral receptors. These receptors are necessary for the preferential attachment of avian and mammalian influenza viruses, respectively, to the target host cell.[1–4] The presence of both receptor types can lead to reassortment events when two distinct populations of influenza virus coinfect an individual pig, creating potentially new variants of influenza virus with pandemic potential.[1,5] Pigs therefore play a major role in the ecology of influenza viruses. Evidence for AI virus infections in pigs in the field has been obtained quite regularly[6–11] with some variants such as the avian-like H1N1 virus continuing to circulate within pig populations to this day. In fact, the ability of AI viruses to infect pigs has also been demonstrated experimentally with nearly all subtypes.[12] The Eurasian lineage highly pathogenic avian influenza (HPAI) virus of H5N1 subtype, first detected in Guangdong Province, China, in 1996,[13] does not cause severe influenza-like disease in pigs.[14,15] However, there is substantial evidence in several South-East Asian countries that infections of pigs with this virus have occurred,[16–19] although some surveys for H5 antibodies in pigs in areas where H5N1 HPAI virus is present in birds have been negative.[19] Experimental infections of pigs with selected Eurasian lineage H5N1 HPAI viruses have been successful, and an influenza-like disease has been observed,[20] while others have shown a

resistance to infection and/or disease.[14,15] The Eurasian lineage H5N1 viruses continue to cause significant losses in wild bird populations and poultry and have become widespread across Asia, Europe and Africa and endemic in poultry in some countries, including the People's Republic of China, Indonesia, Vietnam and Egypt.[21] At the time of writing, HPAI H5N1 virus has also caused 596 human infections, with a mortality rate of approximately 58%.[22] Human infections with this virus appear to be a result of direct contact with infected poultry, with only limited evidence of human-to-human transmission.[23,24] However, infection of pigs could result in these viruses gaining the adaptations necessary to either maintain infection within pigs, thereby providing a further reservoir for human infection, or provide the mutations required to allow transmission in humans, leading to establishment of this virus in the human population.

Reassortment of influenza viruses in pigs is thought to play a major role in the genetic diversity of these viruses and is considered to be the major driving force for the generation of pandemic strains.[25] The recent pandemic H1N1 [A(H1N1)pdm/09] influenza virus is a reassortant of genes from the triple human, avian and swine influenza reassortant viruses from North American (PB1, PB2, HA, NP, NS) and those from the European avian-like H1N1 swine influenza lineages (NA, M).[26,27] The reassortments leading to the emergence of A(H1N1)pdm/09 virus are thought to have occurred in pigs, but the virus is able to infect, transmit efficiently and cause morbidity with significant mortality in infected humans.[26,28] A(H1N1)pdm/09 viruses have continued to infect and circulate in pigs,[29,30] which has resulted in the emergence of second- and third-generation reassortants.[31–33] This further highlights the importance of pigs in the ecology of influenza A viruses. Therefore, understanding the ability of influenza viruses to infect pigs is fundamental, not only for the improvement of surveillance and prevention of infections in pigs but also for the reduction in zoonotic infections and of newly emergent influenza viruses that may have a pandemic potential. The procedures for pig *ex vivo* organ cultures developed recently[34,35] have provided valuable tools for assessing the infectivity of influenza viruses and therefore their potential threat to pig and human populations. In this study, we provide the first, to the best of our knowledge, use of these tools to assess the ability of HPAI and A(H1N1)pdm/09 viruses to infect pigs.

Materials and methods

Pig *ex vivo* tracheal, bronchi and lung organ cultures

All animal work was performed in accordance with the AHVLA committee for ethical studies and the UK 1986 Animal Scientific Procedure Act and AHVLA code of practice for performance of scientific studies using animals (project licence number, 70/7062). Pigs were sourced from a high-health-status herd. Before tissue collections were performed, nasal swabs and blood samples were taken to test for current infection with or previous exposure to influenza virus by matrix gene real-time RT-PCR (RRT-PCR) and HI assay, respectively, using standard methods.[36] Pigs were killed humanely by exsanguinations following electrical stunning, and trachea, bronchi and lung were collected and transported in a 1:1 mixture of Dulbecco's modified Eagle medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 media (Life Technologies Ltd, Paisley, UK) supplemented with antibiotics – penicillin (100 U/ml), streptomycin (50 μg/ml), l-glutamine (10 mm) and amphotericin (2·5 μg/ml) (Life Technologies Ltd).

Tracheal and bronchi explants

Pig *ex vivo* tracheal organ cultures were produced via interactions with the Cambridge Infectious Disease Consortium (CIDC), UK, using the methods described[34] with slight modifications. An identical protocol was applied to bronchi *ex vivo* organ cultures. Briefly, the trachea and bronchi were washed 3–4 times in DMEM (Life Technologies Ltd) supplemented with antibiotics, cut into 0·5-cm^3 sections and placed on filter paper wick–covered, 1% w/v agarose plugs in six-well tissue culture plates containing DMEM (Life Technologies Ltd) supplemented with antibiotics. The viability of the tracheal and bronchi sections was assessed by epithelial cilial activity using 1-μm polybead polystyrene microsphere beads.

Lung explants

The lung organ culture method was kindly provided by Kristien van Reeth and Sjouke van Poucke at Gent University, Belgium.[35] All lung explants were generated according to this protocol with slight modifications. Briefly, the apical lobe was filled with 1% w/v agarose and allowed to set at 4°C. The agarose-filled lobe was then cut longitudinally to form strips that were further set within 4% w/v agarose at 4°C, followed by cross-sectional slicing to form 1 cm^3 lung sections that were placed fully submerged in six-well tissue culture plates containing DMEM supplemented with penicillin (100 U/ml), streptomycin (0·1 mg/ml), l-glutamine (0·3 mg/ml) and gentamycin (0·1 mg/ml) (Life Technologies Ltd), and incubated overnight at 37°C with 5% v/v CO_2. The explants were then washed with warm PBS and placed fully submerged in free six-well tissue culture plates with the same DMEM/antibiotics media described earlier.

All tracheal, bronchi and lung *ex vivo* organ cultures were fixed in 10% v/v buffered formalin for a minimum of 48 hours prior to histological evaluation. Cell morphology and organ culture viability were assessed by haematoxylin and eosin (H&E) staining. The distribution of sialic

acid-linked receptors was also determined by immunohistochemistry (IHC).

Detection of sialic acid receptors by IHC

The distribution of cell-surface glycoproteins or glycolipids containing terminal sialyl–galactosyl residues linked by α2-3-linked and α2-6-linked sialyl–galactosyl moieties, the avian- and mammalian influenza A virus–preferred host receptors, respectively, was characterised for *ex vivo* tracheal, bronchi and lung organ cultures and uninfected pig material following necropsy. Comparisons were made specifically between receptor distributions for the uninfected and the *ex vivo* organ explant material to determine whether the receptor distributions in the organ cultures post-harvest would reflect the natural distributions observed in pig tissue.

Samples for histology were fixed in 10% v/v neutral buffered formalin (VWR, East Grinstead, UK) and processed routinely through graded alcohols and chloroform and embedded in paraffin wax. Four-micrometre-thick sections, cut on a rotary microtome, were stained with H&E or used for immunohistochemical detection of influenza A nucleoprotein. Briefly, sections for host receptors IHC were dewaxed in xylene and passed through graded alcohols to Tris-buffered saline solution with 0·05% Tween (TBSt) (0·005 m Tris, pH 7·6, 0·85% w/v NaCl). Endogenous peroxidase activity was quenched with a methanol/hydrogen peroxide block (VWR) for 15 minutes. Slides were assembled into Shandon coverplates to facilitate IHC using the Shandon Sequenza system (Thermo Fischer Scientific, Runcorn, UK), and primary antibody cross-reactivity with tissue constituents was prevented using a normal immune serum block. Samples were subsequently incubated with biotinylated *Maackia amurensis* lectin II (1/100) (Vector Laboratories, Peterborough, UK) for the α2,3 avian receptor or biotinylated *Sambucus nigra* (Elderberry) bark lectin (1/1000) (Vector Laboratories) for the α2,6 mammalian receptor for 1 hour, and VECTASTAIN Elite ABC-peroxidase reagent (Vector Laboratories) for 30 minutes, at room temperature. Sections were washed three times with TBSt between incubations. The immunohistochemical signal was visualised using 3,3 diaminobenzidine (Sigma-Aldrich, Poole, UK), and sections were counterstained in Mayer's haematoxylin (Surgipath, Peterborough, UK), dehydrated in absolute alcohol, cleared in xylene and mounted using dibutyl phthalate xylene (DPX) and glass coverslips.

Infection of tracheal, bronchi and lung organ cultures

Viruses used in the infections were propagated in 9- to 10-day-old embryonated specific pathogen-free (SPF) fowls' eggs. Pig *ex vivo* tracheal, bronchi and lung organ cultures were infected with 5 μl of infective allantoic fluid of A/chicken/Netherlands/3219-3/03 (H7N7) HPAI virus; A/turkey/Turkey/1/2005 (H5N1) HPAI virus; A/chicken/England/4054/06 (H7N3) low pathogenic avian influenza (LPAI) virus; two viruses isolated during the recent H1N1 pandemic A/England/195/09 [A(H1N1)pdm/09] and A/California/7/09 [A(H1N1)pdm/09] with a dose range of $10^{6·7}$–10^{8} EID_{50}/0·1 ml. As a positive control, tracheal, bronchi and lung organ cultures were also infected with $10^{6·5}$ EID_{50}/0·1 ml of a swine influenza isolate A/swine/England/195852/92 (avian-like H1N1). Replicate samples were collected at 24 and 48 hours post-infection (hpi) for H&E staining for cell morphology and necrosis in addition to anti-NP IHC and matrix gene and (+)RNA RRT-PCR testing for the detection of replicating virus.

Viral RNA isolation and detection by RRT-PCR

A suspension was made for each tissue replicate in 1 ml of brain–heart infusion broth (BHIB) (15% w/v) supplemented with antibiotics (penicillin G, 10 000 U/ml; amphotericin B, 20 μg/ml; gentamycin, 1 mg/ml) (Life Technologies Ltd). A volume of 140 μl from tissue suspensions was mixed with 420 μl of AVL Buffer (Qiagen, Crawley, UK) in a Qiagen S-block and purified by automated viral RNA extraction using the Universal Biorobot system (Qiagen).

Two separate RRT-PCR assays were performed to detect virus and viral replication. First, to confirm the presence of virus in the pig *ex vivo* explants, the matrix (M) gene RRT-PCR was performed using one-step RT-PCR kits (Qiagen) following standard methods.[36] For the M gene RRT-PCR, a positive extraction control of A/chicken/Scotland/59 (H5N1) RNA of known C_t value from inactivated, freeze-dried, egg-grown material was included. Quantitative standards of five 10-fold dilutions of extracted RNA from infective allantoic fluid of a 10^{7} EID_{50} dose of A/turkey/Turkey/1/05 were also included.

Second, a novel RRT-PCR method was developed to detect positive-sense RNA (+RNA). This was used to assess the presence of replicating virus by the detection of messenger (mRNA) and thereby differentiate it from parental viral inoculum within the *ex vivo* organ explant system. However, the design of the assay does not exclude the potential simultaneous detection of viral complementary RNA (cRNA).

The influenza matrix gene was selected as a target for the positive-sense RRT-PCR because of its abundance in influenza virus particles. RT and PCR primers and probes were designed using the lasergene8 primer select software (DNAstar Inc, Madison, WI, USA) based on consensus sequences generated from 592 avian and 163 swine influenza M gene sequences obtained from the BioHealth Influenza Database (http://www.fludb.org). The RT primer was designed to the positive-sense, non-coding 3′ flanking

sequence of the matrix gene and the poly-A tail of mRNA. Therefore, this primer will bind mRNA preferentially, but we cannot exclude the possibility that it also binds to the positive-sense, intermediate, genomic copy of influenza virus – complementary RNA (cRNA). Regardless of the relative binding efficiencies of this RT primer, the detection of both cRNA and mRNA is indicative of replicating virus. Candidate (+)RNA RT and PCR primers for the matrix gene were optimised using a Quantitect™ Sybr® Green PCR kit (Qiagen). Successful amplification was confirmed by 2% w/v agarose gel (Life Technologies Ltd) electrophoresis, PCR amplicon purification using QIAquick gel extraction kits (Qiagen) and BigDye® Terminator v3·1 Cycle sequencing (Life Technologies Ltd). RRT-PCR hydrolysis probes (5′-FAM and 3′-TAMRA) were designed and optimised using the Quantifast™ Probe PCR without ROX kit (Qiagen). The reverse transcription reaction was performed with 5 μm of RT primer NCRev1 5′-TTTTGATGGAACAAAGATGA-3′ and 2·5 μl of template RNA incubated at 95°C for 2 minute followed by 30 second on ice before adding, to a final concentration, 2·5 U Molony murine leukaemia virus reverse transcriptase (MMLV RT) (Promega, Southampton, UK), 1× MMLV RT buffer (Promega), 0·2 μm dNTPs each (Qiagen), 1 U RNAse inhibitor and RNAsin (Promega) and incubated at 37°C for 1 hour. The PCR was performed using the 2× Quantifast Probe PCR mix without ROX (Qiagen) containing 1X ROX (Qiagen), 0·4 μm of forward primer M806F 5′-GCAGATGCAGCGATTCAAG-3′, reverse primer M1003R 5′-CACTCTGCTGTTCCTGCCGA-3′, 0·2 μm of probe 5′-[6FAM]AGGCCCTCTTTTCAAACCGTATT[TAM] and 2 μl of cDNA. RRT-PCRs were performed using Stratagene MX3000 thermocycler, and data were analysed with MxPro software (Agilent Technologies, Wokingham, UK). Thermal cycle conditions were 3 minutes at 95°C, followed by 40 cycles of 10 second at 94°C, 30 second at 55°C and 30 second at 72°C. The fluorescence data were collected during the 55°C annealing step using the ROX and FAM filters. The assay was standardised using a 10-fold dilution series of viral RNA extracted from MDCK cells infected with A/turkey/Turkey/1/2005 (H5N1) HPAI of known C_t value. Positive-sense RNA levels were expressed as 40 – C_t values. The specificity of the assay for replicating virus was determined *in vitro* by infection using either live or inactivated HPAI virus. Briefly, Madin–Darby canine kidney (MDCK) cells were maintained in DMEM (Life Technologies Ltd) supplemented with 10% foetal calf serum (FCS) (Autogen Bioclear, Calne, UK), penicillin (100 U/ml), streptomycin (50 μg/ml), l-glutamine (10 mm) and amphotericin (2·5 μg/ml) (Life Technologies Ltd). These cells were infected with 1 ml of either live (10^6 EID_{50}/0·1 ml) or β-propiolactone-inactivated A/turkey/Turkey/1/2005 (H5N1) HPAI virus. After 1-hour incubation at 37°C with 5% CO_2, the cells were washed and overlaid with fresh DMEM supplemented with penicillin (100 U/ml), streptomycin (0·1 mg/ml), l-glutamine (0·3 mg/ml) and gentamycin (0·1 mg/ml) (Life Technologies Ltd). RNA was extracted at 1, 2, 4, 8, 16 and 20 hpi using QIAmp® Viral RNA Mini RNA Extraction Kit (Qiagen). A similar test for assay specificity was performed on RNA, from newborn pig tracheal (NpTr) cells infected with A/turkey/Turkey/1/2005 (H5N1) HPAI virus, pre- and post-purification of mRNA using the Oligotex Direct mRNA mini kit (Qiagen).

Histopathology and detection of influenza A NP by IHC

Triplicate samples from pig *ex vivo* explants were fixed in 10% buffered formalin for a minimum of 48 hours. Samples for histology were routinely processed through graded alcohols and chloroform and embedded in paraffin wax. Four-micrometre-thick sections, cut on a rotary microtome, were stained with haematoxylin and eosin or used for immunohistochemical detection of influenza A nucleoprotein as described.[37] For IHC results, the number of immunolabelled cells was assessed semiquantitatively in each individual organ and scored on a scale ranging from 0 to 5 (0, negative; 1, occasional presence of immunolabelled cells; 2, small number of cells; 3, moderate; 4, numerous; 5, widespread immunolabelling). This allowed for comparisons to be made between sections from each organ explant.

Results

Development of a (+)RNA RRT-PCR assay

A positive-sense RNA RRT-PCR assay was developed to detect the presence of replicating virus by the detection of viral mRNA. To assess the specificity of the assay, (+)RNA RRT-PCR levels were compared in total RNA extracted from supernatants of MDCK cells infected with live and inactivated viruses (Figure 1A) and NpTr cells infected with live viruses with and without mRNA purification (Figure 1B). No (+)RNA was detected in cells infected with inactivated virus, while a marked increase in (+)RNA levels was detected from 1 hpi in MDCK cells infected with the live virus, confirming the detection of replicating virus only (Figure 1A). Similar results were observed for the detection of (+)RNA before and after mRNA purification (Figure 1B), indicating that mRNA was detected preferentially.

Characterisation of cell receptors in *ex vivo* tissue and organ cultures

The distribution of cell-surface glycoproteins or glycolipids containing terminal sialyl–galactosyl residues linked by avian α2-3- and mammalian α2-6-linked sialyl–galactosyl

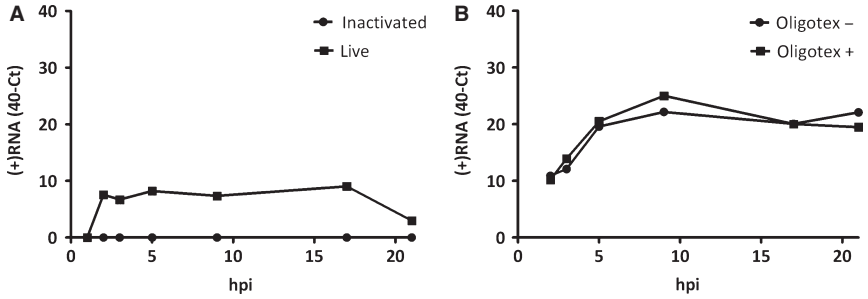

Figure 1. (+)RNA matrix gene real-time RT-PCR detection from samples taken from tissue culture infected with live and inactivated A/turkey/Turkey/1/2005 (H5N1) highly pathogenic avian influenza (HPAI) virus (A), and live A/turkey/Turkey/1/2005 (H5N1) HPAI virus with (oligotex +) and without (oligotex –) mRNA purification (B). (+)RNA levels are expressed as 40 – C_t values. (+)RNA was detected from cells infected with live virus only confirming the detection of replicating virus (A), while similar results were observed for the detection of (+)RNA before and after mRNA purification (B), indicating that mRNA was detected preferentially.

moieties was characterised on uninfected *ex vivo* tracheal, bronchi and lung organ culture sections and control pig material following necropsy. Comparisons were made to determine whether receptor distributions on the organ cultures were representative of fresh pig tissue following necropsy. Receptor staining of pig tracheal tissue following necropsy identified strong α2-6 receptor labelling on the epithelial cells surface and moderate labelling in the submucosal glands, while very limited α2-3 receptor staining was identified (Figure 2). Receptor staining of pig bronchi tissue following necropsy identified scattered, minimal α2-3 receptor staining on epithelial cells, while abundant α2-6 receptor labelling was identified on epithelial cells and submucosal glands. Abundant α2-3 and α2-6 receptor labelling was identified on bronchiole epithelial cells and type I and II pneumocytes of the alveoli in pig cranial lung lobe tissue. Alveolar macrophages also showed signs of α2-6 receptor labelling.

The α2-3 and α2-6 receptor staining on tracheal, bronchi and lung organ explant sections showed very similar distributions to those seen in fresh pig tissue following necropsy. Limited α2-3 receptor labelling and strong and numerous α2-6 receptor staining in tracheal explants were observed, while minimal α2-3 receptor staining and abundant α2-6 receptor labelling were observed in bronchi explants. Only lung explants showed a slight decrease in α2-3 and α2-6 receptor staining compared to fresh necropsy tissue whereas moderate labelling was observed for α2-3 receptors in bronchiolar epithelial and alveolar cells, while strong α2-6 receptor labelling of bronchiolar epithelial cells and only moderate labelling of alveolar cells were observed.

Infection of pig organ cultures to determine their relative susceptibility to selected influenza A viruses

The *ex vivo* pig organ culture systems were established to assess their infection with influenza A viruses. In general, good morphological preservation of the tracheal, bronchi and lung organ cultures was observed at 24 and 48 hours after their establishment. However, after 72 hours, some tissue degradation was observed. For IHC results, the number of immunolabelled cells was assessed semiquantitatively in each individual organ. To assess the permissiveness to infection, *ex vivo* pig explants were infected with two A(H1N1)pdm/09 viruses, a selection of AI viruses and an avian-like H1N1 swine influenza virus. Successful infection of the tracheal, bronchi and lung organ cultures was confirmed by both (+)RNA RRT-PCR and IHC for the H7N3 LPAI and H5N1 HPAI viruses (Figure 3), the pandemic H1N1 2009 strains (except for tracheal organ cultures infected with A/England/195/09) (Figure 4) and the avH1N1 swine influenza viruses (Figure 4). However, we were unable to detect infection of all organ cultures with A/chicken/Netherlands/3219-3/03 (H7N7) HPAI virus (data not shown).

In general, more viruses were detected by IHC and (+)RNA RRT-PCR in lung than in bronchi and tracheal organ cultures (Figures 3 and 4), suggesting greater virus replication. However, for infections with A/chicken/England/4054/06 (H7N3) LPAI virus, viral antigen detected by IHC in lung explants was higher than in tracheal and bronchi explants, but similar levels of viral RNA were detected by (+)RNA RRT-PCR in all three tissue explants (Figure 3). Although infection of tracheal explants with A/England/195/09 A(H1N1)pdm/09 virus would appear to have been unsuccessful, as no antigen or viral RNA was detected, the infection levels, detected by IHC, observed in bronchi explants were higher than those seen in lung (Figure 4). These results mirror the level of α2-3-receptor staining observed within these cultures. For all viruses tested, when virus detection was observed in tracheal and bronchi explants, this infection was confined to epithelial cells. For lung explant infection, virus detection was primarily in the bronchiole epithelial cells, but infection of some alveolar pneumocytes was also observed in a small number of cases.

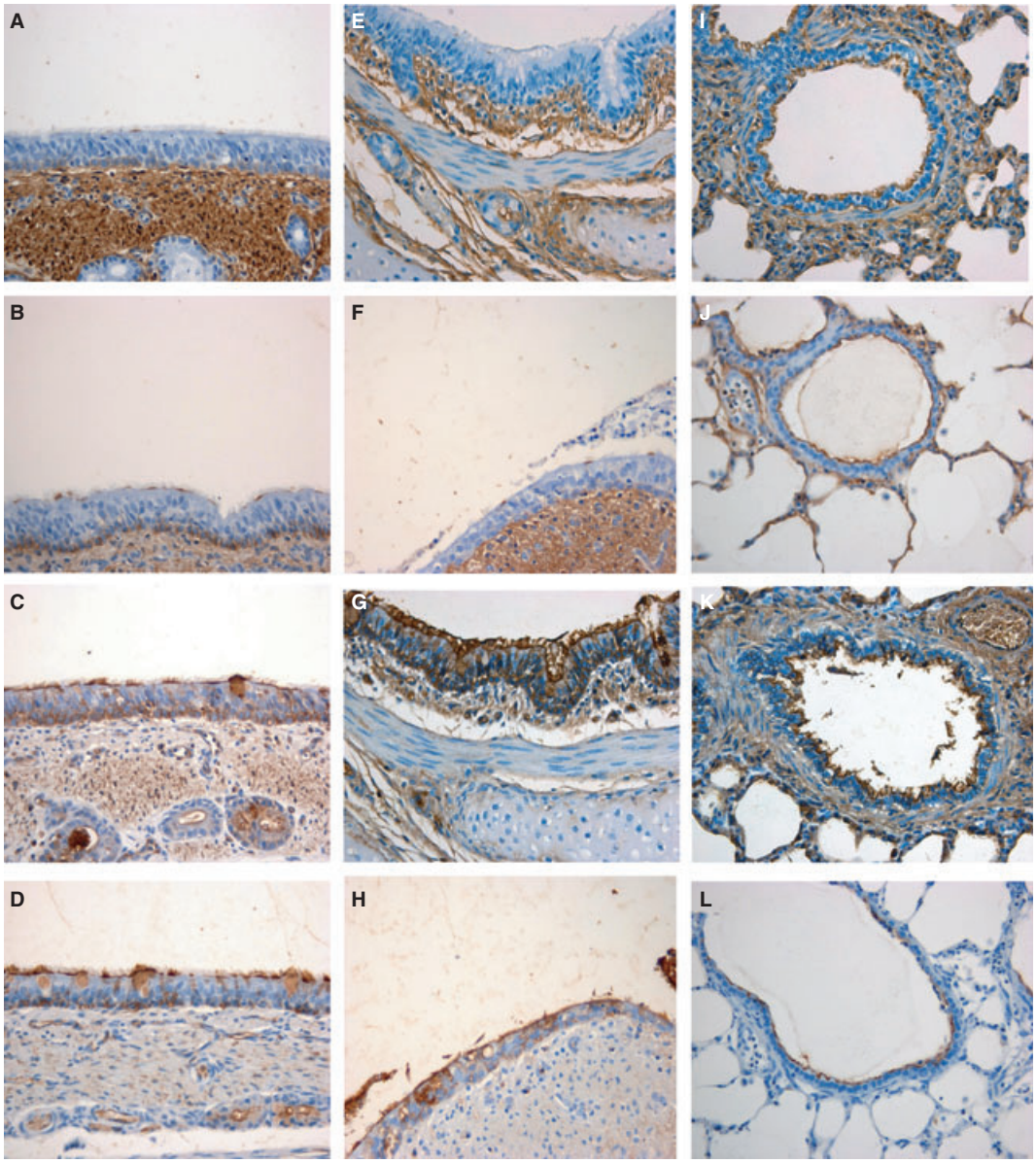

Figure 2. Receptor staining by IHC using *Maackia amurensis* lectin II for the α2,3 sialic acid linked receptor (A, B, E, F, I, J) and or biotinylated *Sambucus nigra* for the α2,6 sialic acid linked receptor (C, D, G, H, K, L) on uninfected *ex vivo* organ culture sections – trachea (B, D), bronchi (F, H) and lung (J, L) – and uninfected fresh pig tissue following necropsy – trachea (A, C), bronchi (E, G) and lung (I, K). IHC, immunohistochemistry.

Discussion

Influenza A virus ecology continues to be influenced by the role of pigs as a reservoir or transient hosts. In the present study, we successfully developed a RRT-PCR assay to detect the presence of replicating virus. The (+)RNA RRT-PCR and the IHC showed similar results for relative quantification of viral replication, indicating the usefulness of this tool. We have confirmed α2-3 and α2-6 sialic acid receptor distributions in pig *ex vivo* tracheal, bronchi and lung organ explants, and *ex vivo* organ explants closely match those seen in pig tissue following necropsy as described.[35,38] We have also successfully infected *ex vivo* organ cultures with viruses considered to pose a significant threat to both veterinary and human health. The results appear to mimic, quite closely, what has been observed in both natural and experimental infections of pigs with A(H1N1)pdm/09 and AI viruses[37,39] (S.M. Brookes, B.Z. Löndt, S.C. Essen AHVLA unpublished data). A(H1N1)pdm/09 viruses and an HPAI virus of H5N1 subtype were able to infect pig *ex vivo* organ cultures, and the detection of virus was higher in the tissues of the lower respiratory tract (i.e. lungs). Endemic swine

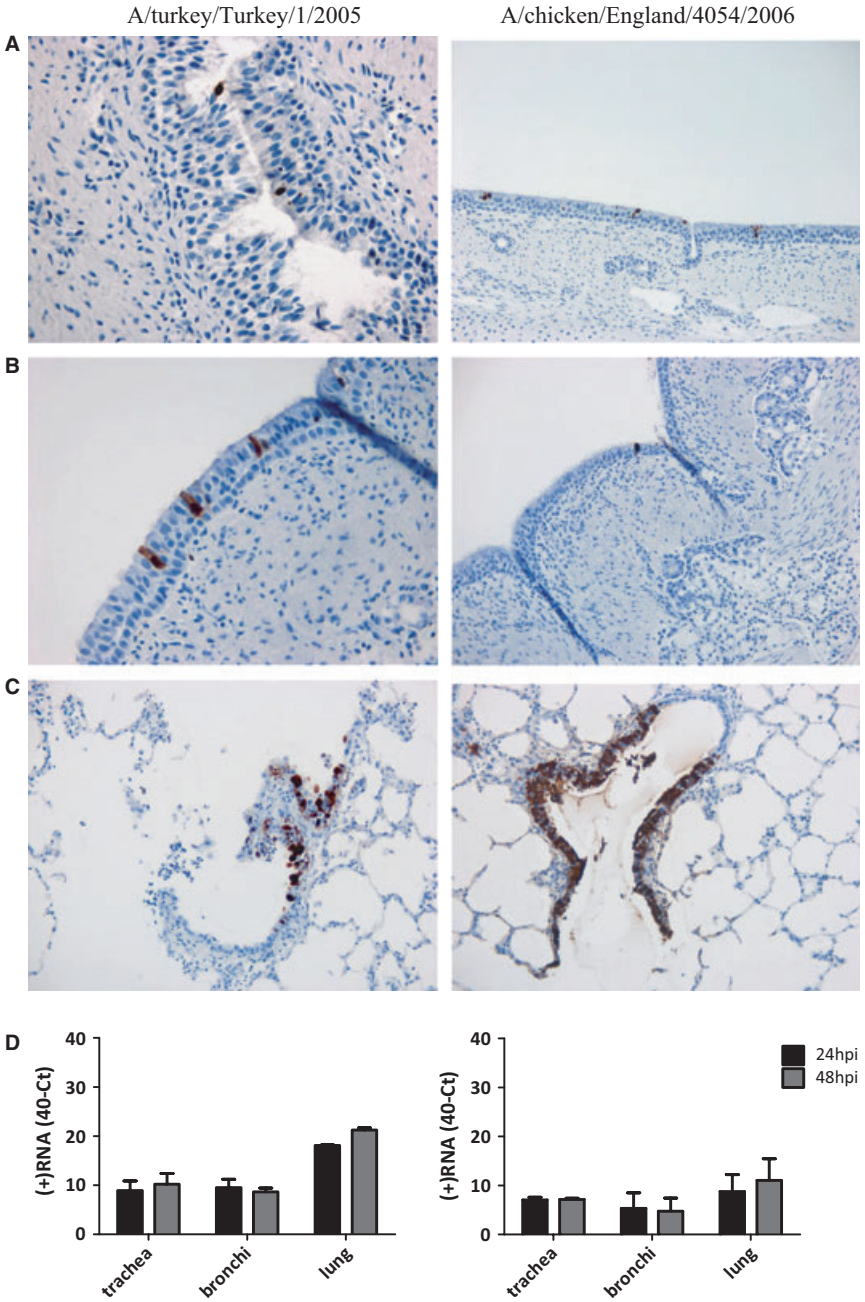

Figure 3. Anti-NP immunohistochemistry staining of pig trachea (A), bronchi (B) and lung (C) *ex vivo* organ cultures, infected with A/turkey/Turkey/1/2005 (H5N1) HPAI and A/chicken/England/4054/2006 (H7N3) LPAI virus, with confirmation of infection by (+)RNA matrix gene real-time RT-PCR (D). Error bars indicate SE. HPAI, highly pathogenic avian influenza; LPAI, low pathogenicity avian influenza.

influenza virus was able to replicate to higher levels, measured by (+)RNA RRT-PCR, in trachea when compared to avian and A(H1N1)pdm/09 viruses. A(H1N1)pdm/09 and AI viruses appeared to replicate preferentially in the bronchi and lung tissue explants. This may be due to the increase in α2-3 receptor density or the number of susceptible cells in the lower respiratory tract. The finding that receptor staining for the α2-6 sialic acid receptor was abundant within the respiratory tract and lungs, while α2-3 sialic acid receptor staining increased towards the lower respiratory organs, closely matches previous reports,[35,38] although lower α2-3 receptor staining has been identified in lung tissue.[4] Lung explants also showed a slight decrease in α2-3 and α2-6 sialic acid receptor staining compared to fresh necropsy tissue.

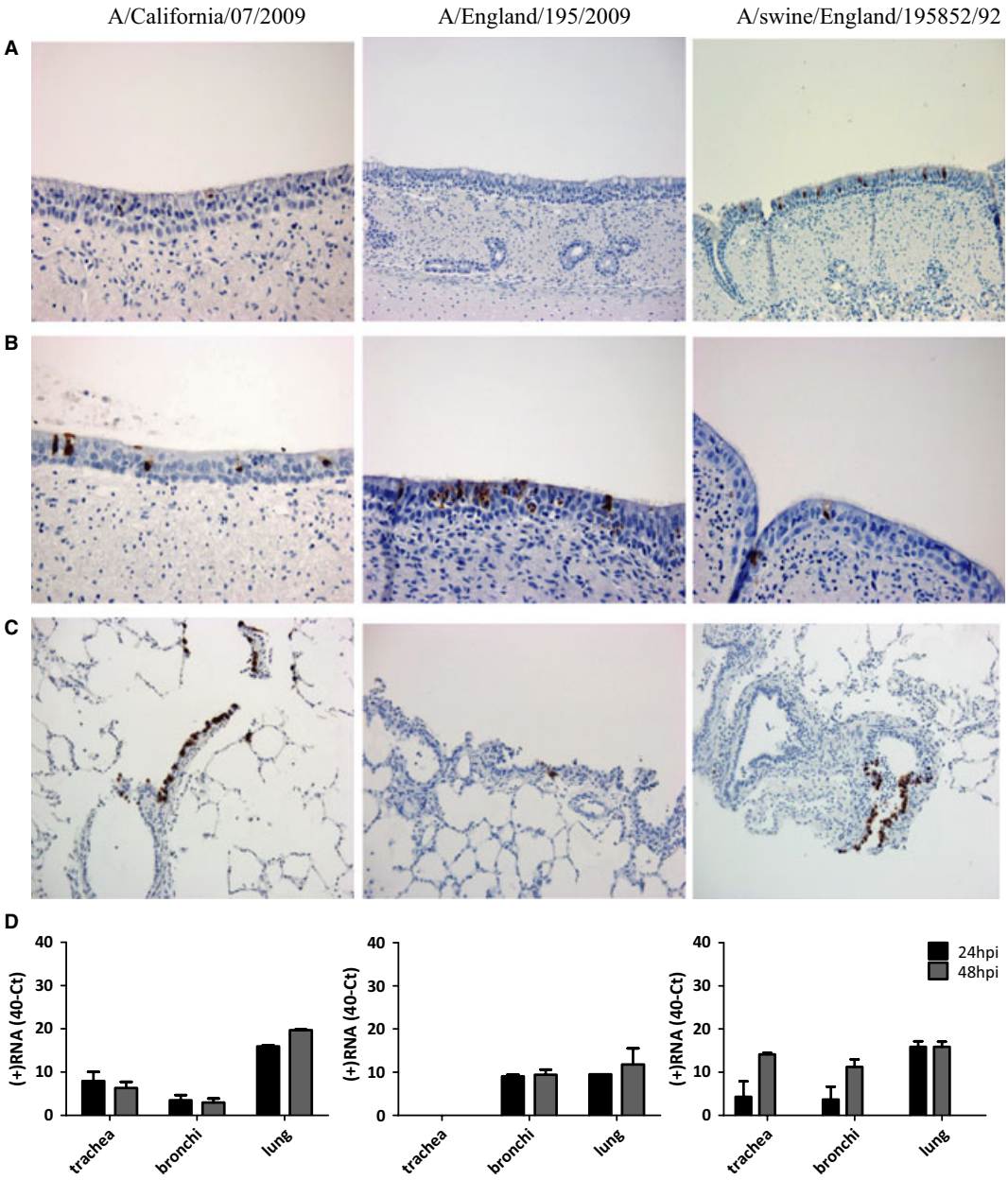

Figure 4. Anti-NP immunohistochemistry staining of pig trachea (A), bronchi (B) and lung (C) *ex vivo* organ cultures, infected with A/California/07/2009 and A/England/195/2009 A(H1N1)pdm/09 virus and A/swine/England/195852/92 avian-like H1N1 swine influenza virus (avH1N1 swIV), with confirmation of infection by (+)RNA matrix gene real-time RT-PCR (D). Error bars indicate SE.

This reduction in labelling may have been the result of degenerative changes associated with the manipulation and *in vitro* culture of the tissue. In general, good morphological preservation of the tracheal, bronchi and lung organ cultures was seen initially. However, after 72 hours, some tissue degradation was observed, which is contrary to similar work reported previously where tracheal *ex vivo* organ cultures were maintained for up to 7 days after establishment.[34]

Previous use of organ cultures to study influenza virus infections reported the infection of pig tracheal, bronchi and lung *ex vivo* organ cultures with avian (LPAI), human (isolated prior to the emergence of the pandemic 2009 H1N1) and swine influenza viruses,[35] and the infection of pig tracheal *ex vivo* organ cultures with swine influenza viruses.[34] However, in the present study, we have described the first infections of similar pig organ cultures, with HPAI and A(H1N1)pdm/09 viruses. Despite

the exceedingly high mortality (approximately 60%) in infected humans, the barrier for HPAI H5N1 viruses of the Eurasian lineage to attain pandemic status has always been the low avian-to-human infection rate and its inability to establish itself within the human population because of an extremely low human-to-human transmission rate.[23] The opposite is true for A(H1N1)pdm/09 viruses, which have been able to transmit efficiently from human-to-human but have displayed only a low mortality rate[40,41] The successful infection of the pig explants, albeit to a higher level in the lower respiratory tract, with Eurasian lineage HPAI H5N1 viruses and the A(H1N1)pdm/09 viruses does suggest that both these viruses could infect pigs with the potential to reassort within the pig, possibly resulting in a highly virulent, easily transmissible virus for the human population.

The work presented here confirms the ability of a newly developed (+)RNA RRT-PCR to detect a range of influenza viruses including A(H1N1)pdm/09, HPAI and LPAI virus. The similar results obtained for IHC and (+)RNA RRT-PCR further support the validity of the assay for replicating virus. More importantly, this work also confirms that different influenza viruses are able to infect pig *ex vivo* organ explants, and these may therefore represent a valuable tool for screening the viruses for their ability to infect pigs prior to *in vivo* studies. Ultimately, pig *ex vivo* organ explants may be able to reduce the numbers of animals used for *in vivo* experimentation.

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