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Enhanced infectivity of H5N1 highly pathogenic avian influenza (HPAI) virus in pig *ex vivo* respiratory tract organ cultures following adaptation by *in vitro* passage

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ABSTRACT

Pigs are thought to play a role in the adaptation of avian influenza (AI) viruses to mammalian hosts. To better understand this mechanism and to identify key mutations two highly pathogenic AI (HPAI) viruses (H5N1 and H7N7) were grown in pig cells, To mimic the pressure of an immune response, these viruses were grown in the presence of antiserum to the homologous virus or porcine IFN-γ. Mutations were identified in both viruses grown *in vitro* in the presence and absence of antisera or IFN-γ and included the PB2 mutations, E627K or 627E,D701N, described previously as requirements for the adaptation of AI viruses to mammalian species. Additional mutations were also identified in PB1, HA, NP and M genes for viruses passaged in the presence of immune pressure. The infectivity of these viruses was then assessed using *ex vivo* pig bronchi and lung organ cultures. For lung explants, higher levels of virus were detected in organ cultures infected with H5N1 HPAI viruses passaged in pig cell lines regardless of the presence or absence of homologous antisera or IFN-γ when compared with the wild-type parental viruses. No infection was observed for any of the H7N7 HPAI viruses. These results suggest that the mutations identified in H5N1 HPAI viruses may provide a replication or infection advantage in pigs *in vivo* and that pigs may continue to play an important role in the ecology of influenza A viruses including those of avian origin.

1. Introduction

Despite evidence for direct avian-to-human transmission of influenza viruses (Centres for Disease Control and Prevention, 2008; Lin et al., 2000; Mounts et al., 1999), pigs are still considered as possible transient hosts for the adaptation of AI viruses to other mammalian hosts, as they possess the sialic acid (SA) receptors necessary for the preferential attachment of avian (α2-3 SA) and mammalian (α2-6 SA) influenza viruses to the host cell (Ito et al., 1998; Rogers and D'Souza, 1989; Rogers and Paulson, 1983; Thongratsakul et al., 2010). Therefore infection of pigs with AI viruses could potentially allow for the adaptation and selection of AI viruses able to infect and transmit in other mammalian species to occur. Evidence for infections of pigs with AI viruses has been well described in both the field and under experimental conditions: H1N1 AI virus was first detected in European pigs in 1979 (Pensaert et al., 1981; Scholtissek et al., 1983) and in China in 1993 (Guan et al., 1996), a variant of which continues to circulate to this day; H1N1, H3N3 and H4N6 viruses were identified in Canada between 1999 and 2001 (Karasin et al., 2000, 2004); H9 AI viruses in China in 2003, 2004 and 2006 (Cong et al., 2008; Xu et al., 2004; Yu et al., 2008); and H7N7 in the Netherlands in 2003 (de Jong et al., 2009). The ability of AI viruses to infect pigs, with little evidence of clinical disease, has also been demonstrated experimentally with nearly all subtypes (Kida et al., 1994; Uchida et al., 2011).

A new variant of highly pathogenic avian influenza (HPAI) virus of H5N1 subtype, now termed Eurasian lineage, was first detected in geese in Guangdong, China in 1996 (Xu et al., 1999). This variant has spread throughout South-East Asia, Europe and into Africa, causing significant mortality in wild bird populations, the commercial poultry industry and, at the time of writing, 620 human infections, with a mortality rate of 59% [World Health Organisation (WHO), 2012]. Generally, the Eurasian lineage H5N1 HPAI viruses do not cause influenza-like disease in pigs, but there is substantial though primarily serological evidence in several South-East Asian countries that infection of pigs with this virus has occurred (Choi et al., 2005; Nidom et al., 2010; Shieh et al., 2008). Other surveys for H5 antibodies in pigs in geographical locations where H5N1 HPAI virus is present in birds have been negative (Song et al., 2010). However, experimental infections of pigs with certain Eurasian lineage

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H5N1 HPAI viruses has produced influenza-like disease (Li et al., 2008). Serological evidence during the H7N7 HPAI virus outbreak in poultry farms in the Netherlands in 2003 has supported the infection of pigs with H7 HPAI viruses (de Jong et al., 2009). During this outbreak there was extensive evidence of transmission to humans, with subsequent limited human-to-human transmission and a single fatality also occurred (de Jong et al., 2009; Fouchier et al., 2004; Koopmans et al., 2004).

Human infections with Eurasian lineage H5N1 HPAI virus appear to be a result of direct contact with infected poultry, with limited evidence, if any, of human-to-human transmission (Centres for Disease Control and Prevention, 2008; Mounts et al., 1999). However, there is concern that infection of pigs could provide these viruses with the opportunity to adapt to a mammalian species or reassort with swine influenza viruses or human influenza viruses infecting pigs and increase the risk of infection in mammalian species. This could result in the maintenance of these viruses within the pig population, thereby providing an alternative reservoir for human infection, or the mutations required to establish and maintain this virus variant within the human population. Therefore, identifying the key mutations required by AI viruses to adapt to mammalian hosts may allow for the improved surveillance for these mutations in infected avian species and consequently help in the prevention of infections in pigs. This could also result in the reduction of both zoonotic infections and the emergence of new influenza viruses with pandemic potential. To better understand the mechanisms of adaptation of AI viruses to pigs, and to identify key mutations fundamental to this process, HPAI viruses were passaged in pig cell lines in the presence of an immune pressure supplied artificially. This was done to mimic the pressure of an immune response in an attempt to induce higher levels of mutations than the passage of HPAI viruses in pig cells alone. The infectivity of the resultant mutant viruses was then assessed using *ex vivo* pig organ cultures.

2. Materials and methods

2.1. Viruses

Two HPAI viruses were selected to study the adaptation of HPAI viruses in pigs in the presence of an immune pressure supplied artificially – A/turkey/Turkey/1/05 H5N1 and A/chicken/Netherlands/3219-3/03 H7N7. The A/turkey/Turkey/1/05 H5N1 HPAI isolate was selected as it is the first of the current Eurasian lineage (clade 2.2.1) H5N1 viruses to be isolated in Europe. The A/chicken/Netherlands/3219-3/03 H7N7 HPAI virus was isolated during the large disease outbreak in poultry farms in the Netherlands in 2003 (Fouchier et al., 2004). Viruses were propagated in 9- to 10-day-old embryonated specific pathogen free (SPF) fowls' eggs for two passages.

2.2. Cell lines

Newborn swine kidney (NSK) (BS CL 177) and newborn pig trachea (NpTr) (BS CL 176) cell lines (Ferrari et al., 2003) were kindly provided by the Istituto Zooprofilattico Sperimentale della Lombardia e dell'emilia (IZSLER), Italy. The cells were maintained in Eagle's minimum essential medium (Life Technologies Ltd, UK) in Earle's BSS supplemented with 10% foetal calf serum (Autogen Bioclear, AUS).

2.3. Preparation of pig polyclonal sera to H5 and H7 HPAI viruses

Polyclonal pig sera was produced to provide an immune pressure *via* homologous antibody during the passage of A/turkey/Turkey/1/05 (H5N1) and A/chicken/Netherlands/3219-3/03 (H7N7) HPAI viruses in pig tissue culture monolayers. Briefly, three-month-old, high health status, Landrace cross-breed pigs were housed in a biosafety containment level (BCL) 2 facility and provided with food and water as required. Prior to immunisation, nasal swabs and blood samples were taken from the pigs to determine the absence of infection with influenza virus and/or previous exposure to swine influenza by matrix gene real-time RT-PCR (RRT-PCR) and haemagglutination inhibition (HI) tests. The latter included assays for a selection of representative swine influenza viruses - classical H1N1, avian-like swine H1N1, H1N2 and human-like H3N2 using standard methods (OIE, 2008). All sera were raised prior to the appearance of A(H1N1)pdm/09 in the UK human or swine populations. To produce polyclonal sera, two pigs for each virus were each inoculated intramuscularly (IM) with β-propiolactone inactivated A/turkey/Turkey/1/05 (H5N1) or A/chicken/Netherlands/3219-3/03 (H7N7) HPAI virus supplemented with Montanide ISA 50V2 adjuvant (Seppic, UK). Each pig received three booster doses at 3-week intervals by IM injection of homologous, inactivated virus supplemented with the above adjuvant. The production of antibodies was monitored by HI assay on sera collected prior to each booster dose and after terminal bleeds of each pig using standard methods (OIE, 2008). Since discrepancies between ELISA and HI tests for sera from pigs infected with AI viruses have been reported (Kida et al., 1994), possibly due to differences in haemagglutinin (HA) antigenic sites between avian, human and swine viruses, the terminal sera were also tested by virus microneutralisation assay (VNA).

2.4. Virus microneutralisation assay

The presence of neutralising antibodies in pig sera to H5N1 and H7N7 HPAI viruses was determined by a VNA developed at the AHVLA, Weybridge in collaboration with the Health Protection Agency (HPA), UK. This method was based on the World Health Organisation protocol for the detection of neutralising antibodies specific to seasonal influenza A viruses in human sera (WHO, 2002), with modifications and was optimised for the detection of neutralising antibodies in pig antisera specific for HPAI viruses. Briefly, virus-neutralising antibody titres were determined in MDCK cells. The allantoic fluid stocks of A/turkey/Turkey/1/05 (H5N1) and A/chicken/Netherlands/3219-3/03 (H7N7) HPAI viruses were titrated in MDCK cells and the median tissue culture infectious dose ($TCID_{50}$) determined for each. A 10-fold serial dilution of pig sera were incubated with 100 TCID50 of homologous virus, in 96 well plates, for 1 h at room temperature. A 5×10^4 MDCK cell suspension in Eagles minimal essential medium (EMEM) (Life Technologies Ltd, UK) supplemented with 1% (v/v) foetal calf serum (Autogen Bioclear, AUS), 1× non-essential amino acids, 50 mg/ml gentamycin, 25 mM Hepes (Life Technologies Ltd, UK) and 2 mM L-glutamine (Life Technologies Ltd, UK), was added to each well. The plates were then left for 16–18 h in a humid incubator at 37 °C with 5% (v/v) CO_2. The cells were fixed with methanol, and the presence of virus assessed using a horseradish peroxidise (HRP)-based ELISA with a mouse anti-influenza A NP monoclonal (Oxford Biotechnology Limited, UK) followed by a rabbit anti-mouse, HRP-conjugated monoclonal. The OD of the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was measured at 450 nm which was normalised against the background OD measured at 620 nm. Back titration controls were performed and positive and negative control sera were included with each assay. Neutralising antibody titres were expressed as the reciprocal of the serum dilution that inhibited 50% of viral antigen detection of 100 $TCID_{50}$ of virus, calculated using the Reed and Muench method (Reed and Muench, 1938).