

Influenza A virus infection of healthy piglets in an abattoir in Brazil: animal-human interface and risk for interspecies transmission

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Asymptomatic influenza virus infections in pigs are frequent and the lack of measures for controlling viral spread facilitates the circulation of different virus strains between pigs. The goal of this study was to demonstrate the circulation of influenza A virus strains among asymptomatic piglets in an abattoir in Brazil and discuss the potential public health impacts. Tracheal samples (n = 330) were collected from asymptomatic animals by a veterinarian that also performed visual lung tissue examinations. No slaughtered animals presented with any noticeable macroscopic signs of influenza infection following examination of lung tissues. Samples were then analysed by reverse transcription-polymerase chain reaction that resulted in the identification of 30 (9%) influenza A positive samples. The presence of asymptomatic pig infections suggested that these animals could facilitate virus dissemination and act as a source of infection for the herd, thereby enabling the emergence of influenza outbreaks associated with significant economic losses. Furthermore, the continuous exposure of the farm and abattoir workers to the virus increases the risk for interspecies transmission. Monitoring measures of swine influenza virus infections and vaccination and monitoring of employees for influenza infection should also be considered. In addition regulatory agencies should consider the public health ramifications regarding the potential zoonotic viral transmission between humans and pigs.

Key words: influenza virus - interspecies transmission - zoonosis - pork industry

Influenza A viruses are classified into the family Orthomyxoviridae. Viral particles are composed by segmented negative-sense RNA enclosed by a helical capsid and lipoprotein envelope. These viruses infect a variety of species including humans, swine, equine species, aquatic mammals and birds (Wright et al. 2007). Influenza A virus taxonomy is based on the antigenic characteristics of the two envelope glycoproteins, haemagglutinin (HA) and neuraminidase (NA). Currently, 16 HA and nine NA subtypes have been defined (Ma et al. 2009) and a distinct influenza A virus lineage was recently detected in bats shown to carry new HA subtypes putatively designated as HA17 (Tong et al. 2012)

Influenza A viruses have remained endemic in pig populations worldwide and represent a common cause of respiratory infections in pigs (Brown 2000). The primary route of virus transmission is via pig-to-pig contact resulting in nasopharyngeal routes of infection following nasal viral secretions that are disseminated through droplets or aerosols. Coughing, nasal discharge, fever, lethargy, difficulty in breathing, depressed appetite and reproductive disorders have been associated with different influenza A virus subtype infections throughout the world (Kuntz-Simon & Madec 2009). Close contact

between pigs, stress, meteorological and environmental factors have been shown to contribute to influenza virus spread (Brown 2000, Williamson et al. 2012).

Pigs are susceptible to infections with either human or avian influenza viruses and have been suggested as intermediate hosts or mixing vessels resulting in the generation of pandemic viruses following reassortment or adaptation to the mammalian host (Ito et al. 1998, Yu et al. 2008, Ma et al. 2009, Kwon et al. 2011, Wu et al. 2011, Moreno et al. 2012). Attachment of influenza A viruses to host cells occurs through a binding pocket on the viral HA head and a terminal specific *N*-acetylneuraminic acid-galactose (NeuAc-Gal) linkage on the host cell membranes. Most influenza A viruses isolated from humans preferentially recognise NeuAc α 2,6Gal linkages, whereas most avian isolates preferentially recognise NeuAc α 2,3Gal. Swine viruses bind equally well to both NeuAc α 2,6Gal and NeuAc α 2,3Gal (with a slightly higher binding affinity for NeuAc α 2,6Gal), therefore they are able to infect both human and avian cells [for review see Ma et al. (2009)]. Evidence exists demonstrating transmission of influenza viruses from humans to pigs (Howden et al. 2009, Adeola et al. 2010, Song et al. 2010, Forgie et al. 2011, Gray et al. 2012, Williamson et al. 2012) as well as from pigs to humans (Robinson et al. 2007, Yassine et al. 2009, CDC 2012). Influenza A virus transmission between humans and swine often results in the emergence of new strains with the potential of spreading among both populations (Myers et al. 2007, Yassine et al. 2009, Xu et al. 2011, Yu et al. 2011).

Studies have shown that swine workers are at an increased risk of contracting an influenza A infection

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from pigs (Olsen et al. 2002, Myers et al. 2006, Gray et al. 2007, Yassine et al. 2009, Beaudoin et al. 2012, CDC 2012). Biosecurity and biocontainment measurements might help prevent the spread of pathogens, including influenza viruses from pigs to humans and *vice-versa*. Personal protective equipment (PPE) and proper hygiene practices are recommended for persons with potential exposures to livestock infected with influenza (CDC 2010). However, the most effective protective measure would be to vaccinate both pigs and workers (CDC 2010).

The goal of this work was to demonstrate the circulation of influenza A viruses among asymptomatic piglets in an abattoir in the state of Minas Gerais (MG), Brazil, and discuss its potential impact on public health.

MATERIALS AND METHODS

Specimens - Tracheal swabs (n = 330) were collected from apparently healthy pigs in an abattoir in MG between September 2009-September 2011. The samples were collected after the animals were slaughtered and samples were preserved in viral transport medium (VTM) containing RNAlater® (Life technologies, Austin, TX, USA) and transported in a cooler to the laboratory for influenza A virus detection.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) amplification - Viral RNA was extracted from 300 µL of respective samples in VTM using the Totally RNA Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommendations. RT-PCR and nested-PCR amplifications were performed using specific primers designed to amplify a conserved influenza A virus M gene region as described previously (Ellis & Zambon 2001).

To confirm the specificity of the PCR products, the amplified DNA of positive, randomly selected samples were purified using the Wizard SV gel and PCR Clean-Up system kit (Promega, Madison, WI, USA) and the sequences determined using the BigDye® Terminator Cycle Sequencing Kit and the ABI PRISM® 3100 automated DNA sequencer (Applied Biosystems). DNA sequences were edited using the Chromas software (Technelysium Pty Ltd, Australia) and compared to DNA sequences available in GenBank (ncbi.nlm.nih.gov) using the BLAST tool (ncbi.nlm.nih.gov/BLAST).

Ethics - The Animal Ethics Committees of the Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, approved the study protocol.

RESULTS

Tracheal swabs (n = 330) were collected from 180 day-old piglets weighting approximately 110 Kg. Study animals were never vaccinated against influenza viruses and at the time of sample collection no animal presented with any clinical respiratory signs of disease. Samples were collected by a trained veterinarian that also performed a gross visual examination of the lung tissues. None of the slaughtered animals presented with any noticeable macroscopic signs of influenza infection.

Of the 330 samples examined, 47 samples were collected in 2009, 183 in 2010 and 100 in 2011. Thirty sam-

ples (9%) were positive for influenza A virus by RT-PCR analysis directed to the M protein encoding gene (Table). Sequence analysis confirmed the specificity of the assay and therefore the detection of influenza A virus (data not show). The yearly frequency of influenza virus infections was 12.8% in 2009, 9.8% in 2010 and 6% in 2011.

The conditions of the abattoir regarding biosecurity, biocontainment and use of PPE were analysed. The workers reported that on average 50 animals brought in from different farms in the region (and housed together in a pre-slaughter area for 3-5 days) were slaughtered weekly. Workers involved in animal feeding and slaughtering used special footwear and clothing (disposable coveralls), but no goggles, masks or gloves (Fig. 1). Anti-influenza vaccination was neither mandatory nor reported for either animals or workers. A pet cat lived in the abattoir area that had access to animal waste (Fig. 2).

DISCUSSION

In this study, the prevalence of subclinical influenza A virus infections was 9%. It was not possible to obtain information regarding handling procedures used by respective suppliers of the animals slaughtered during the study period. However, it is clear that these animals represented a source for influenza viruses with the potential of causing infections in otherwise healthy animals resulting in significant economic losses. Although clinical disease is associated with low mortality rates, infections result in high rates of morbidity, causing primarily loss of herd productivity and weight loss. Recent evidence of viral spread among populations of asymptotically infected pigs showed the significant health risk this possess for the pig industry (Forgie et al. 2011, Bowman et al. 2012, Gray et al. 2012, Wong et al. 2012). In Brazil, seroepidemiological evidence demonstrated the spread of influenza A virus among pig herds. A serological survey performed in MG demonstrated that 44.5% and 10.1% of surveyed animals were positive for swine influenza virus strains belonging to subtypes H1N1 and H3N2, respectively, and 38.3% were positive for human

TABLE
Swine influenza virus detection in tracheal swabs collected from asymptomatic pigs from an abattoir in the state of Minas Gerais, Brazil, between September 2009-September 2011

Year	Detection of influenza A virus		
	Positive samples (n)	Negative samples (n)	Positive (%)
2009	6	41	12.8
2010	18	165	9.8
2011	6	94	6
Total	30	300	9

influenza strain H1N1 (Rajão et al. 2012). Another study of pig farms located in eight different states in the South, Southeast and Midwest Regions of Brazil demonstrated that 16.7% and 2.2% of the surveyed animals possessed antibodies against human influenza viruses subtypes H3N2 and H1N1, respectively (Brentano et al. 2002).

Transmission of influenza viruses from pigs to humans has been reported occasionally, but most cases result in mild infections rarely resulting in death (Robinson et al. 2007, Howden et al. 2009, Yassine et al. 2009, Adeola et al. 2010, Song et al. 2010, Forgie et al. 2011, CDC 2012, Gray et al. 2012, Williamson et al. 2012). Swine workers and their family members are at increased risk of contracting swine influenza virus infections (Olsen et al. 2002, Myers et al. 2006, Gray et al. 2007, Robinson et al. 2007, Yassine et al. 2009, Beaudoin et al. 2012, CDC 2012, Wong et al. 2012). In this study, we were unable to document human flu-like illnesses during the period of sample collection. However, continuous exposure of the farm and abattoir workers to animals suffering from subclinical influenza virus infections provided an environment conducive to the occurrence of interspecies infections and the emergence of new potentially pathogenic viral variants.

Recently, Gray et al. (2012) found a 19% prevalence of influenza A viruses among 57 show pigs swabbed during the 2009 Minnesota State Fair during the second wave of the 2009 pandemic. Even though molecular analyses or viral cultures identified the presence of influenza A

virus infection, no pigs presented with clinical signs of influenza illness at the time of sampling, suggesting that exposure to apparently healthy pigs at shows could result in influenza A virus transmission. A survey of individuals working with pigs at the fair identified two persons presenting with influenza-like illness within a seven day period after the fair: an adult, with onset one day after his pigs arrived at the fair (and 4 days before pig swabbing) and his daughter who developed an illness on the last day of the fair (3 days after pig swabbing). In addition, three pigs exhibited by the child tested positive for influenza A viruses (Gray et al. 2012).

Wong et al. (2012) reported a case of an influenza A H3N2 variant (H3N2v) infection in a child who attended an agricultural fair in Pennsylvania, United States of America (USA), in 2011. A retrospective cohort study among members of an agricultural club who attended the fair identified two other confirmed cases, four probable and 82 suspected cases. Among the study members, the risk for suspected case status increased as swine exposure increased from no exposure (4%), visiting swine exhibits (8%) and touching swine (16%). This study concluded that fairs may serve as venues for zoonotic transmission of viruses with epidemic potential; therefore health officials should investigate respiratory illness outbreaks associated with agricultural events (Wong et al. 2012).

A study conducted by Bowman et al. (2012) demonstrated that 22.3% of exhibit pigs at agricultural fairs in Ohio, USA, between 2009-2011, were asymptotically infected with influenza A viruses. The study concluded that subclinical influenza virus A infections in pigs at agricultural fairs may pose a risk to human health and create challenges for passive surveillance programs for influenza virus A in swine herds.

Shinde et al. (2009) reported 11 cases of infection with triple-reassortant swine influenza A (H1) viruses in persons with exposures to pigs in the USA, before the human epidemic caused by influenza viruses of swine-origin.

A survey of the literature conducted by Myers et al. (2007) found 50 cases of apparent zoonotic swine influ-



Fig. 1: abattoir employees involved in animal feeding and slaughtering. Workers used special footwear and disposable coveralls, but no goggles, masks or gloves.



Fig. 2: pets living in the abattoir area in contact with the animal waste.

enza virus infections involving civilians and military personnel. Most civilian subjects reported exposures to swine. Myers et al. (2006) also observed that occupational exposures may also play important roles in the mixing vessel hypothesis. That study demonstrated that farmers, meat processing workers and veterinarians presented with significantly elevated anti-swine influenza titres compared to control subjects.

Forgie et al. (2011) investigated a human and swine influenza outbreak at a swine research farm in Alberta, Canada, and reported human-to-swine transmission and subsequent influenza epidemics in both pig and human populations. Sixty-seven percent of humans and 94% of swine with positive laboratory tests results had few or no symptoms (Forgie et al. 2011).

Until 2005, sustained circulation of human viruses in pig populations was uncommon. Since then, swine influenza virus strains resulting from reassortment events containing HA gene segments of human origin have been detected (Ma et al. 2009) and the development of weak immune responses against homologous and heterologous strains may be the cause of recurrent infections. It was also observed that infections could occur in piglets even in presence of colostral-derived antibodies against virus subtypes circulating between farm animals (Simon-Grifé et al. 2012).

Several studies have demonstrated the circulation of influenza viruses among pets, particularly cats and dogs. Transmission and subclinical infection of human and avian H3N2 (Houser & Heuschele 1980, Song et al. 2008), equine H3N8 (Crawford et al. 2005), avian H5N1 (Songserm et al. 2006b) and the pandemic 2009 H1N1 (Damiani et al. 2012) influenza virus strains to dogs have been documented. Serological evidence of naturally acquired infections caused by seasonal human H1N1 and H3N2 (Ali et al. 2011), the pandemic 2009 H1N1 (Sponseller et al. 2010, Ali et al. 2011), and the avian H5N1 (Songserm et al. 2006a) influenza virus strains in cats have also been reported. Generally animals show no signs of influenza, although symptomatic infections have been described (Songserm et al. 2006a, b, Harder & Vahlenkamp 2010, Sponseller et al. 2010). Interspecies transmission of canine influenza virus subtype H3N2 to domestic cats has also been described (Song et al. 2011).

Circulation of influenza viruses among companion animals increases the chances of animal-human transmission. A study conducted among pig farms in Malaysia evaluated risk factors associated with swine influenza H1N1 and H3N2 viruses. This study showed that the presence of pet mammals on the farm (such as cats) significantly increased the probability of infection. Pigs from farms where no pets were observed were about 32 times less likely to be seropositive for H1N1 influenza virus compared to pigs raised in farms where cats were present (Suriya et al. 2008). In this study, we observed the presence of a cat in the abattoir area living in close contact with pigs and humans, creating a perfect environment for zoonotic influenza virus transmission.

Interestingly, despite the damage caused by influenza virus infections, these types of infections are not routinely monitored in pig farms (Gray & Baker 2011).

The diagnosis of symptomatic influenza virus infections in pigs has been based mainly on clinical signs and histopathological lesions (not present in subclinical infections). However, once the animal is a virus carrier it becomes a source of viral spread regardless of their clinical status. Despite this animal be deemed suitable for the slaughter, it can contaminate other farm animals that may develop the disease. In addition, the infected animals can also contaminate human beings who are directly involved in the pig handling.

Currently, Brazil is the fourth largest global trader of pig meat with an estimated pig population of 38 million animals. In 2011, Brazil exported over 500 million tons of pork to 60 countries worldwide (ABIEPCS 2011). The standards for the slaughter of pigs are strict and designed to minimise the spread of pathogens. In Brazil, pig slaughter methods and meat handling are regulated by the Ministry of Agriculture, Livestock and Supply (MAPA). This agency regulates procedures implemented before and after slaughter (*ante-mortem* and *post-mortem*) (MAPA 1995). However, MAPA does not require the implementation of PPE or vaccination of the abattoir workers. Since pig farming represents a major source of income for Brazil, implementation of monitoring measures for the identification of pig influenza virus infections would be fundamental to improving the productive parameters of this industry. Similarly, taking into account that influenza viruses can also be transmitted to humans through pigs, vaccination and monitoring of employees for infection should be considered. In addition, the potential of zoonotic transmission between humans, pigs and pets should raise public health concerns that should be considered by regulatory agencies.

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