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# Airborne spread and infection of a novel swine-origin influenza A (H1N1) virus

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## Abstract

**Background:** The novel swine-origin influenza A (H1N1) virus (S-O 2009 IV) can cause respiratory infectious diseases in humans and pigs, but there are few studies investigating the airborne spread of the virus. In January 2011, a swine-origin H1N1 epidemic emerged in eastern China that rapidly spread to neighboring farms, likely by aerosols carried by the wind.

**Methods:** In this study, quantitative reverse transcription polymerase chain reaction (RT-PCR) was used to detect viruses in air samples from pig farms. Based on two aerosol infection models (Pig and guinea pig), we evaluated aerosol transmission and infection of the novel S-O 2009 IV isolate.

**Results:** Three novel S-O 2009 IV were isolated from the diseased pig. The positive rate and viral loads of air samples were 26.1% and 3.14-5.72 log<sub>10</sub>copies/m<sup>3</sup> air, respectively. In both pig and guinea pig infection models, the isolate (A/swine/Shandong/07/2011) was capable of forming aerosols and infected experimental animals at a range of 2.0-4.2 m by aerosols, but aerosol route was less efficient than direct contact.

**Conclusions:** The results indicated that S-O 2009 IV is able to be aerosolized by infected animals and to be transmitted to susceptible animals by airborne routes.

**Keywords:** S-O 2009 IV, Epidemic, Airborne transmission, Pig, Guinea pig

## Introduction

In April 2009, swine-origin 2009 A (H1N1) influenza viruses (S-O 2009 IV) were found in Mexico and the United States for the first time, and quickly spread throughout the world, presenting a significant threat to public health [1,2]. S-O 2009 IV is a novel triple-reassortant influenza virus derived from porcine, human, and avian influenza viruses. Different from seasonal influenza viruses, humans lack immunity to this new virus, and thus the virus quickly caused a pandemic [2-5]. As of March 21, 2010, the World Health Organization (WHO) reported that 213 countries or regions were affected and the number of deaths was at least 16,931 people [6].

It has been determined that despite the complex causes of the novel S-O 2009 IV epidemic, airborne spread was one of the major reasons for the pandemic [7]. Aerosols are solid or liquid suspensions in the air and their particle size range is 0.001-100 μm [8]. Once formed, aerosols, including those containing viruses, can rapidly spread to a larger area with the assistance of the wind [9]. However, little is known about the transmission and infection of the novel S-O 2009 IV via aerosols, and there is still some debate about airborne infection of this virus [7,8,10,11].

In early January 2011, a local animal disease prevention and control center in Shandong, China reported that a pig farm in eastern China emerged the suspected novel S-O 2009 IV disease. More importantly, similar infections occurred successively in some downwind pig farms within a week, and workers of these pig farms developed flu-like symptoms. Isolation and identification of pathogens confirmed S-O 2009 IV infection. Thus, we

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speculated that airborne transmission played an important role in the spread of this epidemic. Here, we collected indoor air, pig nasopharyngeal swabs, and blood samples to analyze the positive rate of the S-O 2009 IV; and based on both pig and guinea pig aerosol infection models, airborne infection capacity of S-O 2009 IV isolate was evaluated.

## Results

### Identification of viruses

The seven viral isolates were obtained from the nasopharyngeal swab samples, including three strains of H1N1 and four strains of H3N2. Antigenicity and sequence analysis of the three strains of H1N1 viruses confirmed that the isolated strain was S-O 2009 IV. And the isolate (A/swine/Shandong/07/2011) was used in aerosol infection models to verify its airborne transmission traits.

### Results of air and serum samples

The positive rate of 157 air samples collected from the 40 pig farms was 26.1% (41/157) and the virus content range was 4.09-5.72  $\log_{10}$ copies/m<sup>3</sup> air; hemagglutination and hemagglutination-inhibition (HA-HI) tests showed that the positive rate of serum samples was 28.5% (57/200) and titers were 80–2560. Infection analysis of staff with flu-like symptoms from seven pig

farms found that 43.5% (10/23) were seropositive rate for S-O 2009 IV infection (from CDC) (Table 1).

### Results of S-O 2009 IV aerosol infection models

All experimental pigs and guinea pigs in the challenged groups were shown to shed viruses and seroconverted to S-O 2009 IV. In the swine infection model experiments, viruses were detected in the direct contact groups from nasal secretions of three pigs during the first round of experiment and two pigs during the second round. In the aerosol infection group, two pigs were positive during the first round of experiments and one positive during the second round. Pigs with virus detected in nasal secretions were also positive by serological testing. Serum titers of the direct contact group and aerosol infection group were 320–1280 and 640–1280, respectively (Table 2).

In the guinea pig infection model experiments, viruses were detected in nasal secretions from four guinea pigs in the first round of experiments and three guinea pigs in the second round in the direct contact group. In the aerosol infection group, viruses were detected in nasal secretions from two guinea pigs for the first round of experiments and three guinea pigs for the second round. Serum titers of animals in the direct contact group and aerosol infection group were 320–1280 and 320–1280, respectively (Table 2).

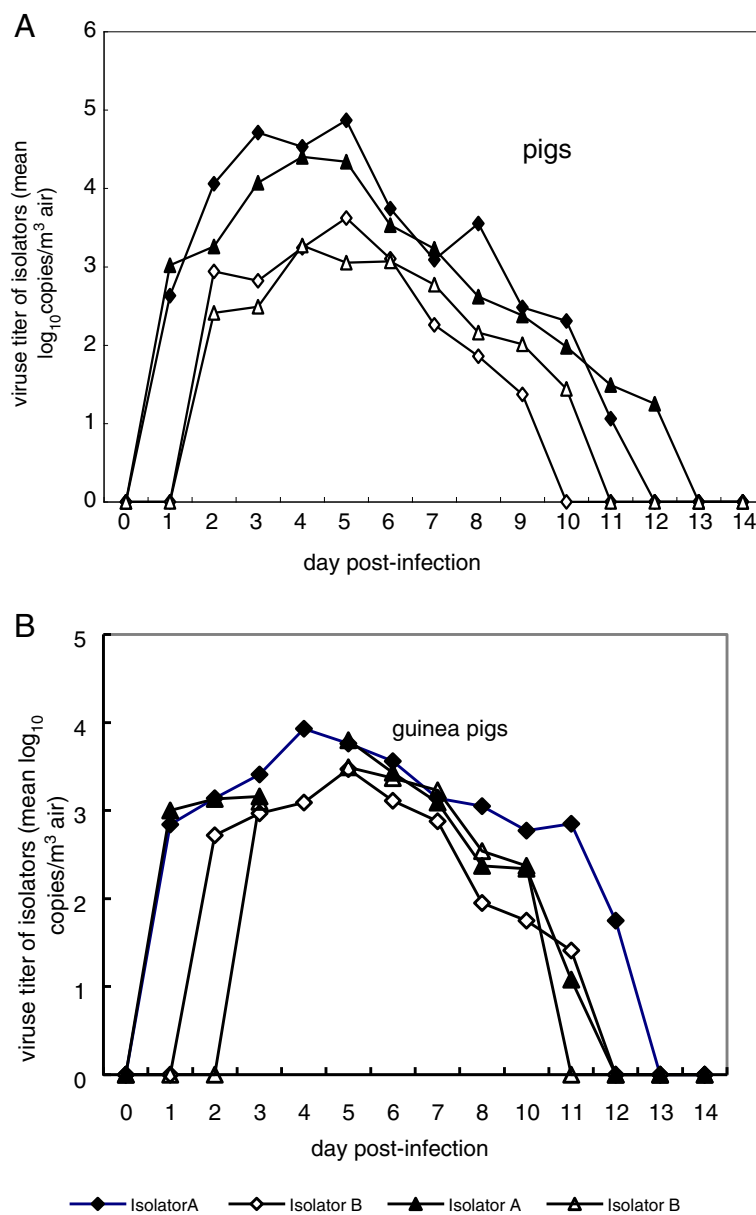
**Table 1 Detection results of airborne S-O 2009 IV in 40 pig farms**

Farms	Air samples	Virus RNA copies of air samples ( $\log_{10}/m^3$ )	Sero- Conversion	Farms	Air samples	Virus RNA copies of air samples ( $\log_{10}/m^3$ )	Sero- Conversion
Farm 1	4/6	4.14–5.62	3/5	Farm 21	3/6	4.41–5.07	3/5
Farm 2	4/6	4.05–4.93	5/5	Farm 22	0/4	0	0/5
Farm 3	2/5	3.83/5.66	4/5	Farm 23	1/4	4.62	2/5
Farm 4	2/4	4.20/4.54	3/5	Farm 24	1/4	4.40	2/5
Farm 5	2/4	4.75/5.19	2/5	Farm 25	1/3	4.78	1/5
Farm 6	2/4	3.74/4.64	4/5	Farm 26	1/4	3.14	2/5
Farm 7	3/4	4.20–4.63	3/5	Farm 27	0/4	0	0/5
Farm 8	3/4	4.12–5.72	4/5	Farm 28	0/3	0	0/5
Farm 9	3/4	4.55–5.09	3/5	Farm 29	0/3	0	0/5
Farm10	0/4	0	1/5	Farm 30	0/3	0	1/5
Farm 11	0/3	0	0/5	Farm 31	0/3	0	0/5
Farm12	0/3	0	0/5	Farm 32	0/3	0	0/5
Farm13	0/4	0	0/5	Farm33	1/4	4.95	3/5
Farm14	0/4	0	0/5	Farm34	0/4	0	0/5
Farm15	1/3	5.50	0/5	Farm35	0/3	0	1/5
Farm16	0/4	0	0/5	Farm 36	0/4	0	0/5
Farm17	2/4	4.42/5.02	1/5	Farm 37	0/3	0	0/5
Farm18	1/5	5.59	1/5	Farm 38	1/4	4.65	3/5
Farm19	3/4	4.91–5.33	4/5	Farm 39	0/4	0	0/5
Farm20	0/4	0	0/5	Farm 40	0/4	0	1/5

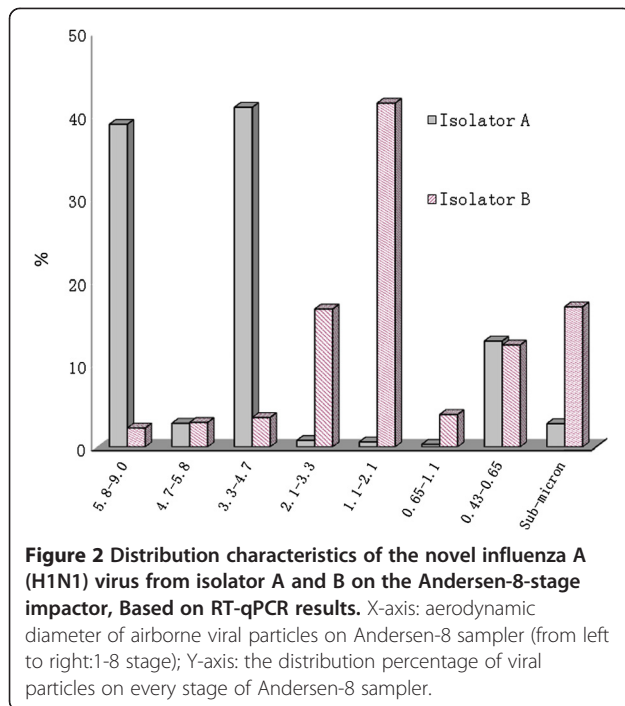
**Table 2 Transmission and infection of 2009 A(H1N1) IV aerosol in pig model and guinea pig model**

	Inoculated animals		DC animals <sup>§</sup>		AI animals <sup>§</sup>	
	Virus in nasal wash <sup>#</sup> (log <sub>10</sub> peak copies/ml)	Sero- conversion <sup>*</sup>	Virus in nasal wash <sup>#</sup> (log <sub>10</sub> peak copies/ml)	Sero- conversion <sup>*</sup>	Virus in nasal wash <sup>#</sup> (log <sub>10</sub> peak copies/ml)	Sero- conversion <sup>*</sup>
Pigs	1 3/3(5.3-6.1)	3/3(640-2560)	3/3(4.9-5.6)	3/3(320-1280)	2/3(5.5-6.0)	2/3(640-1280)
	2 3/3(5.1-5.8)	3/3(640-1280)	2/3(5.1-5.6)	2/3(320-640)	1/3(4.9)	1/3(640)
Guinea Pigs	1 5/5(4.3-5.2)	5/5(640-1280)	4/5(4.2-4.8)	4/5(640-1280)	2/5(3.8-5.1)	2/5(320-640)
	2 5/5(5.4-6.2)	5/5(320-1280)	3/5(4.7-5.8)	3/5(320-1280)	3/5(4.1-6.2)	3/5(320-1280)

<sup>§</sup>DC direct contact; AI aerosol infection; <sup>#</sup>Virus titers are expressed as mean log<sub>10</sub>peak copies/ml; <sup>\*</sup>Hemagglutination inhibition(HI) assay was performed with homologous virus and turkey red blood cells. No. of positive animals and HI range indicated.



**Figure 1 Concentration of the SO 2009(H1N1)IV aerosol produced by pigs and guinea pigs in isolators A and B.** The diamond represents the first round of experiment; triangle represents the second. Black represents isolator A, white represents isolator B. (B) Not detected in the first round of experiment 4dpi Day 0: animals in group A were intranasally inoculated with 10<sup>6</sup> pfu of the novel influenza A (H1N1) virus.



During the two rounds of swine infection model experiments, S-O 2009 IV aerosols were detected at the beginning of 1 day post-infection (dpi) in isolator A, peaked (4.87, 4.40  $\log_{10}$ copies/ $m^3$  air) at 5 and 4 dpi, and was absent from 12 or 13 dpi. In isolator B, S-O 2009 IV aerosols were detected at 2 dpi, peaked (3.62, 3.27  $\log_{10}$ copies/ $m^3$  air) at 5 and 4 dpi, and was absent after 10 or 11 dpi (Figure 1A).

For the guinea pig experimental infection model, the results of the two rounds of experiments indicated that virus shedding was detected at 1 dpi of the challenged group; S-O 2009 IV aerosols were detected at 1 and 2 dpi, peaked at 4 and 5 dpi (in isolator A and in isolator B) and disappeared at 11–13 dpi. In the two rounds of guinea pig infection model experiments, S-O 2009 IV aerosols were detected at 1 dpi in isolator A, peaked at 4 and 5 dpi (3.93, 3.80  $\log_{10}$ copies/ $m^3$  air), and was absent from 12–13 dpi. In isolator B, SO 2009 IV aerosols were detected at post-inoculation 2 and 3 days, peaked at 5 dpi (3.47, 3.49  $\log_{10}$ copies/ $m^3$  air), and was absent at 11 or 12 dpi (Figure 1B).

#### Diameter of S-O 2009 IV aerosol particles

In isolator A, the distribution ratio of viral aerosol particle sizes was highest for a particle diameter of 3.3–4.7  $\mu m$  (40.95%), and lowest for 0.65–1.1  $\mu m$  (0.30%). In isolator B, the distribution ratio of viral aerosol particle sizes was highest for particle diameters of 1.1–2.1  $\mu m$  (41.45%), and lowest for 5.8–9.0  $\mu m$  (2.28%) (Figure 2).

## Discussion

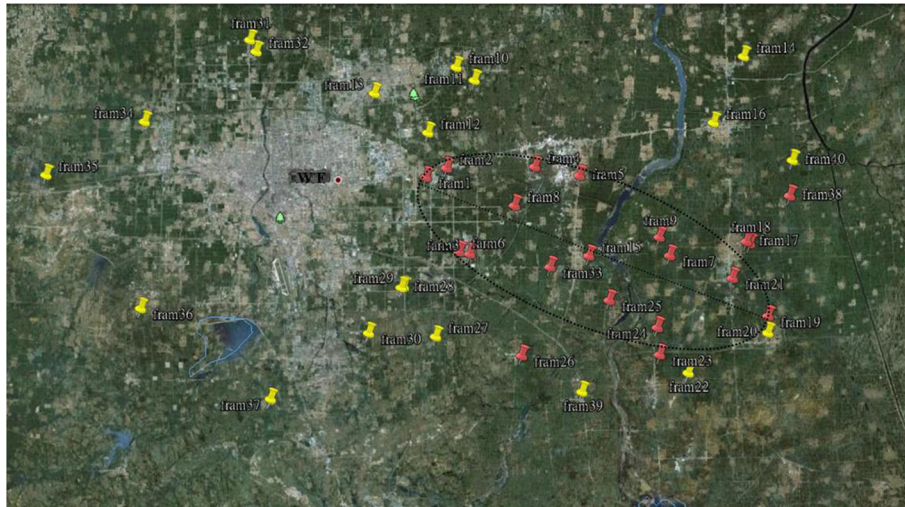
Since early January 2011, the S-O 2009 IV epidemic was first identified in two pig farms of eastern China that quickly spread to nearly 20 farms within a week. A geographical analysis of these pig farms showed that these farms were distributed in a close ellipsoid zone with a central axis in line with prevailing northwesterly winds. A collection of 157 air samples from 40 pig farms revealed that 26.1% contained virus with almost 50% of pig farms in the area affected. This transmission was presumed to be via downwind dissemination of S-O 2009 IV aerosols. It appears that the virus was spread by airborne transmission and our findings in experimental models support this.

During the experiments, pigs and guinea pigs were housed in positive- and negative-pressure isolators, effectively avoiding the contamination with exogenous microbes. Aerosol transmission experiments were conducted so that the air in isolator B came exclusively from isolator A, which were connected using a 2 m closed tube. Therefore, any infection in isolator B would be determined as aerosols (Figure 3).

During the experimental infections in pigs, S-O 2009 IV was detected at 1–2 dpi in isolators A and B, and the maximum amount of virus detected in A and B were and 4.40–4.87  $\log_{10}$ copies/ $m^3$  air and 3.27–3.62  $\log_{10}$ copies/ $m^3$  air, at 4 and 5 dpi, respectively. However, during the two rounds of experiments, the amounts of virus detected were consistently lower in B than in A and virus was not detectable at 12–13 dpi in A and at 10–11 dpi in B. The duration of detectable S-O 2009 IV aerosols in isolator B was shorter and the amounts of virus were lower than those in isolator A, which was likely related with the deposition, survival time, and removal of aerosol particles.

There is a 2m pipe between the two isolators (upper distal end, 5.3m from the challenged group; Figure 4); therefore, infection in isolator B occurred not via droplet but by aerosolized virus particles. Meanwhile the certain wind velocity in the pipe can well simulate natural wind between different pig farms, even between different pig houses in the same farm. Additionally, aerosol infection group and direct contact group were both serologically positive and nasal secretions had evidence of virus shedding. Nonetheless, the number of infections in aerosol infection group was smaller than in direct contact group, there was no difference in the extent of virus shedding and serum titers of infected pigs. These data demonstrated that infection with these viral strains can induce airborne infection.

Our data indicated that infected pigs located in up-wind farms can generate viruses in nasal and respiratory secretions and spread via the wind to downwind farms. Thus, this type of transmission is dependent on weather, where changes in wind force, wind direction, ultraviolet



**Figure 3** The map is the part of East China area, red points represent the positive pig farms, and yellow ones represent the negative pig farms.

light, temperature or humidity are all likely to affect transmission.

Since the respiratory tract of guinea pigs is very similar to that of humans, guinea pigs were regarded as an appropriate mammalian model for use in this study [12]. In the two repeated trials on guinea pigs, the rates of virus detection and seroconversion to the S-O 2009 IV in aerosol infection group were 2/5 and 3/5 (Table 2), indicating that aerosol infection group was infected by viral aerosols in isolator A and the virus had the capability of airborne transmission among mammals. Therefore, it can be inferred that S-O 2009 IV is likely to be a health risk for farm staff and may be an important point for the prevention and control of new zoonoses, especially swine influenza viruses. It has been reported that the S-O 2009 IV is still persistent among pigs in China and thus remains a major threat to human health [13,14].

At 4 and 5 dpi, the virus content in air samples reached its peak in the isolator along with the amount of virus in nasal secretions and the number of pigs infected in different groups. These data indicated that the amount of virus contained in aerosols is related to the virus shedding capacity and number of infected animals. Until 11–13 dpi, no airborne S-O 2009 IV was detected in the two isolators. Correspondingly, animals in all the experimental groups no longer shed virus. These results suggest that the viruses produced in nasal secretions by animals formed aerosols.

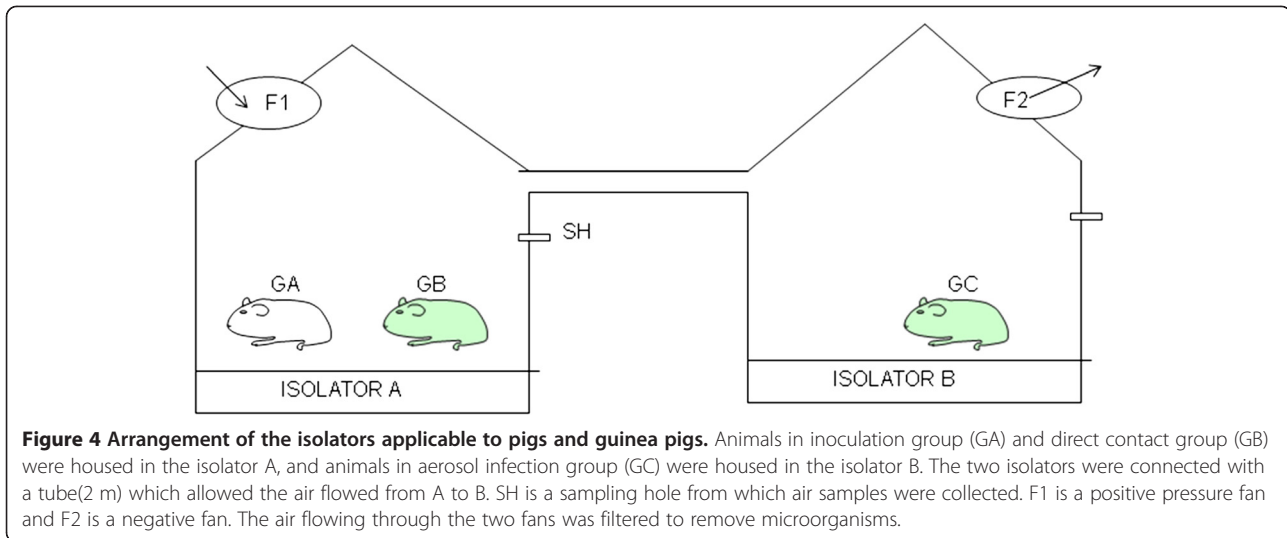
Although the detectable time and concentrations of airborne virus in the two animal experiments were different, the viral aerosol concentration increased or decreased coincidentally. In addition, the viral aerosol concentration was affected by the ventilation rate and the number of infected animals.

The aerodynamic diameter ranges of aerosols in isolator A were determined to be 3.3–4.7  $\mu\text{m}$  and 5.8–9.0  $\mu\text{m}$ ; however, in isolator B particle size was typically 1.1–2.1  $\mu\text{m}$ . The loss of large particle droplets was mainly due to sedimentation, and the loss of aerosol particles  $<5\mu\text{m}$  was mainly due to the inactivation of virus particles and ventilation [15], indicating that smaller particles gathered in isolator B under the effect of ventilation. In the two isolators, particles were typically  $\leq 4.7 \mu\text{m}$ , showing that a higher proportion of viral aerosols can enter the lower respiratory tract via the nasal cavity, whereas particles  $\leq 1 \mu\text{m}$  can enter bronchioles and alveoli, and deposit in the alveoli, increasing the risk of serious infection. Gustin et al. [16] indicated that when discharged by breathing or sneezing in ferrets, influenza virus particles were typically  $\leq 4.7 \mu\text{m}$ . During the flu season, the less than 4  $\mu\text{m}$  (aerodynamic diameter) influenza viral aerosol particles accounted for 53% of the airborne influenza viral count in hospital environments [17–19], consistent with the experimental results of this study. In conclusion, animals infected with S-O 2009 IV can form aerosols that can lead to airborne transmission.

## Materials and methods

### Outbreak of the epidemic and virus isolation and identification

The swine flu epidemic occurred in eastern China (119° E, 36.30° N), a warm temperate semi-humid monsoon climate zone, with cold, dry winters dominated by north-westerly winds [20]. In early January 2011, a suspected new swine flu epidemic suddenly emerged in a few pig farms of this region and within a week similar epidemics emerged successively in some pig farms in the downwind



**Figure 4 Arrangement of the isolators applicable to pigs and guinea pigs.** Animals in inoculation group (GA) and direct contact group (GB) were housed in the isolator A, and animals in aerosol infection group (GC) were housed in the isolator B. The two isolators were connected with a tube (2 m) which allowed the air flow from A to B. SH is a sampling hole from which air samples were collected. F1 is a positive pressure fan and F2 is a negative fan. The air flowing through the two fans was filtered to remove microorganisms.

direction of this pig farm (Figure 3). A total of 120 nasopharyngeal swabs taken from infected pigs were collected for virus isolation and identification, as previously described by Cong et al. [21]. Samples were processed in a BSL -2+ laboratory.

#### Collection and process of air, nasopharyngeal swab and blood samples

A total of 157 air samples were collected using AGI-30 (All Glass Impinger) placed in the middle of the pig farm, at 1.5 m from the ground, using 20 mL phosphate buffered saline (PBS) sampling medium, with a flow velocity of 12.5 L/min for 40 min [22,23]. Virus was detected in samples using the RT-qPCR methods [24]. Serum samples from pigs (n=200) and pig farm staff (n=23) displaying flu-like symptoms were collected and processed by the local centers for disease control (CDC) [25].

#### Establishment of S-O 2009 IV aerosols transmission and infection models

Weaned piglets (8–10 kg) and guinea pigs (250–300 g) seronegative for S-O 2009 IV were purchased from the Shandong Taibang Biological Product Co., Ltd. and used for experimental infections. All prevailing local, national and international regulations and conventions, and normal scientific ethical practices have been respected in this study. Two positive- and negative-pressure isolators A and B (Model C.C.JH-1; Tianjin Jinhang Purified Air Conditioning Engineering Company, China) were connected using a closed tube 2 m in length and 8 cm in diameter, to adjust air flow from A to B, which not only prevented direct contact of animals in the aerosol infection group with those in the inoculation group, but also prevented the propagation of droplets (Figure 4). Three pigs or five guinea pigs were intranasally

inoculated with  $10^6$  plaque-forming units (PFU) of viruses were placed in isolator A, and were designated inoculation group. After 24 h, another three pigs or five guinea pigs were placed into isolator A, and were designated direct contact group. Three pigs or five guinea pigs were placed into isolator B, and were designated aerosol infection group [26,27]. The isolator temperature was maintained at  $20 \pm 1$  °C,  $30 \pm 4$  % relative humidity (RH), and 0.05–0.2 m/s wind velocity [28]. Pig and guinea pig experiments were conducted independently and repeated. “The Society for Animal Health Feedings & Animal Welfare of Shandong Province approved the study of “Airborne spread and infection of a novel swine-origin influenza A(H1N1) virus” and agreed to examine the serum samples from 200 pigs and 23 pig farm staff for the experiments of that.

#### Collection of aerosols, nasal washes, and blood in infection experiments

After sequencing and analysis of eight fragments from several virulent strains, three strains were identified as the S-O 2009 IV and one of them was used for the aerosol dissemination experiment. Virus shedding and serum antibodies of nasal secretions in experimental animals were monitored and detected to verify if the experimental animals were infected. Nasal secretions from each animal were collected every other day after virus inoculation and after RNA extraction, virus nucleic acids were detected by RT-qPCR [29]. At 7 and 14 dpi, blood from each experiment animal was collected to test for specific antibodies [30].

After inoculation, the AGI-30 air collection device was used to collect air samples in isolators A and B on each day [26] and the virus content in samples was detected using RT-qPCR. To determine the proportion and distribution of aerosol particles of different sizes, at 4 dpi of

the second round of the guinea pig model experiments, the Andersen-8-level collector [18] was used to collect aerosolized viral particles in isolators A and B, using a sterile 1% gelatin glycerol (1% gelatin PBS and glycerol 1:1 mixed) as the medium, at 28.3 L/min flow velocity for 40 min, and the virus content of every level was detected by RT-qPCR.

#### Competing interests

The authors declare they have no competing financial interests.

#### Authors' contributions

HZ and XL are contributed equally to this work. RM, XL, YZ, JG, HY, ZM to participate in the writing of this paper. HD, XL, QL, MZ, ZL, BW, MC, HW and PH participate in the operation of the experiment. All authors read and approved the final manuscript.

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