Nested RT-PCR method for the detection of European avian-like H1 swine influenza A virus

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Abstract
Swine influenza A virus (swine IAV) circulates worldwide in pigs and poses a serious public health threat, as evidenced by the 2009 H1N1 influenza pandemic. Among multiple subtypes/lineages of swine influenza A viruses, European avian-like (EA) H1N1 swine IAV has been dominant since 2005 in China and caused infections in humans in 2010. Highly sensitive and specific methods of detection are required to differentiate EA H1N1 swine IAVs from viruses belonging to other lineages and subtypes. In this study, a nested reverse transcription (RT)-PCR assay was developed to detect EA H1 swine IAVs. Two primer sets (outer and inner) were designed specifically to target the viral hemagglutinin genes. Specific PCR products were obtained from all tested EA H1N1 swine IAV isolates, but not from other lineages of H1 swine IAVs, other subtypes of swine IAVs, or other infectious swine viruses. The sensitivity of the nested RT-PCR was improved to 1 plaque forming unit (PFU) mL⁻¹ which was over 10⁴ PFU mL⁻¹ for a previously established multiplex RT-PCR method. The nested RT-PCR results obtained from screening 365 clinical samples were consistent with those obtained using conventional virus isolation methods combined with sequencing. Thus, the nested RT-PCR assay reported herein is more sensitive and suitable for the diagnosis of clinical infections and surveillance of EA H1 swine IAVs in pigs and humans.

Keywords: nested RT-PCR, swine influenza A virus, European avian-like H1, HA gene, molecular diagnosis

1. Introduction
Swine influenza A virus (swine IAV) causes acute infectious respiratory diseases in pigs worldwide and also has involved in repeated human infections, including the 2009 human pandemic (Vincent et al. 2014). Three major subtypes of influenza A viruses, including H1N1, H1N2, and H3N2, have become endemic in pigs. Several avian-origin IAVs, such as H3N2, H4N8, H5N1, H6N6 and H9N2 viruses, have also been sporadically isolated from pigs (Li et al. 2003; Cong et al. 2008; Kuntz-Simon and Madec 2009; Zhang et al. 2011; Brown 2012; Su et al. 2012a, b, 2013). Among the H1N1 subtype of swine influenza A virus, two major lineages, classical and European avian (EA)-like swine IAV, co-circulate in swine populations (Dunham et al. 2009; Vijaykrishna et al. 2011; Vincent et al. 2014). The EA H1N1 swine IAV lineage emerged in Europe in 1979 and essentially replaced classical swine IAVs in prevalence (Pensaert et al. 1981; Brown 2000; Maldonado et al. 2006; Vincent et al. 2014). In China, EA H1N1 swine IAVs have been detected since 2001...
and replaced H3N2 swine IAJs and have become dominant from 2005 (Zhu et al. 2013). EA H1N1 swine IAJs also have the capacity to infect humans (Rimmelzwaan et al. 2001; Gregory et al. 2003; Adiego et al. 2009; Zell et al. 2013). In 2010, a three-year-old boy in China’s rural Jiangsu Province was infected with EA H1N1 swine IAV, indicating that this virus also causes human infections in the Asia-Pacific region (Yang et al. 2012). It is therefore important to monitor EA H1N1 swine IAV infections of pigs to prevent viral circulation within pig populations and transmission to humans.

Reverse transcription (RT)-PCR and multiplex RT-PCR are now standard methods for both detection and subtyping of influenza A viruses (Li et al. 2001; Fereidouni et al. 2009; He et al. 2009; Yang et al. 2010). Nested RT-PCR is a variation of standard RT-PCR in which two pairs of PCR primers are used (instead of one pair). The outer pair of primers is initially used to amplify a fragment and the inner pair of primers, called “nested primers” and binds inside the first PCR product to allow for the amplification of a second PCR product, which is shorter than the first. Thus, nested RT-PCR can improve detection sensitivity of conventional PCR (Falchieri et al. 2012). Nested RT-PCR has been used in several applications for which maximum sensitivity is crucial, such as detection of human immunodeficiency virus (HIV) and hepatitis C virus (HCV) in serum samples in which the viral titer is frequently less than 100 viral genomes per mL of serum (Goode et al. 2002). In a previous study, a multiplex RT-PCR method of detecting different lineages of swine influenza A viruses was developed, with the sensitivity of 10−3–50% egg infective doses (EID$_{50}$) mL$^{-1}$ for EA H1N1 swine IAJs (Fu et al. 2010). For pigs infected with swine IAJs, the amount of virus shed in nasal secretions peaks titers ranging from 10$^{3.5}$ to 10$^{7.5}$ EID$_{50}$ mL$^{-1}$ in early infection (days 2–4 post infection) (Janke 2013). However, due to mild or no overt signs of illness (Detmer et al. 2013), the diagnosis of the infection of swine IAJs is often delayed, which results in missing the viral replication peak, and omission of the virus with titers below the limits of detection. Thus, a more sensitive method is needed for routine screening of clinical samples.

In this study, a nested RT-PCR assay targeting the hemagglutinin (HA) gene of EA H1N1 swine IAV was developed for differentiation of EA H1 swine IAV from other swine IAV lineages and subtypes circulating in pig populations in China.

Results of the current work demonstrate that the developed nested RT-PCR technique is more sensitive and suitable for the diagnosis of clinical infections and surveillance of EA H1 swine IAJs in pigs and humans.

2. Results

2.1. Primer design and selection

The primers were designed targeting the conserved regions of HA sequences of EA H1N1 swine IAV strains and not in those of other lineages/subtypes swine IAJs. Among different combinations of outer and inner primer pairs, based on their amplification efficiencies and specificities, two primer pairs were selected for the nested RT-PCR assay: the outer primer set amplified a 726-bp fragment within the HA gene of EA H1N1 swine IAJs, and the inner primers amplified a 462-bp fragment within the first-round amplification product (Table 1).

2.2. Development of the nested RT-PCR

The annealing temperature, extension time, and number of cycles were optimized during the development of nested RT-PCR. Annealing temperatures of 55°C for the outer primers and 56°C for the inner primers resulted in the brightest band after gel electrophoresis (data not shown). Similarly, extension times of 1 min/2 min for the first/second rounds of PCR amplification were found to give the highest product yield. In the cycle-number-optimization assay, four combinations (20/20, 20/30, 30/20, and 30/30) of the first/second rounds were tested. Specific products were observed for each combination with EA H1N1 swine IAV, and no products were observed for the other viruses, including classical H1N1, H1N1pdm09, H1N2, H3N2, and H9N2 subtypes. In order to render the assay as speedy as possible without sacrificing quality, we selected 20 cycles for the first and second rounds of PCR (data not shown).

2.3. Specificity of the nested RT-PCR assay

In the nested RT-PCR assay, 462-bp amplification products were obtained with the 15 2007–2014 EA H1N1 swine IAV strain templates (Fig. 1). No amplification products were

<table>
<thead>
<tr>
<th>Specific primers</th>
<th>Primer sequences (5’→3’)</th>
<th>Tm (°C)</th>
<th>Target gene</th>
<th>PCR products (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EA-HA-270F (outer)</td>
<td>TGGCAACCCCAAATGTGACT</td>
<td>55.8</td>
<td>HA</td>
<td>726</td>
</tr>
<tr>
<td>EA-HA-995R (outer)</td>
<td>GGGGCAATCTCCAAATAGTG</td>
<td>57.6</td>
<td>HA</td>
<td>462</td>
</tr>
<tr>
<td>EA-HA-429F (inner)</td>
<td>AATTTCCTCCAAAAGGCCACC</td>
<td>52.7</td>
<td>HA</td>
<td>462</td>
</tr>
<tr>
<td>EA-HA-890R (inner)</td>
<td>ATCCGGACATCATATACTCCAGAAC</td>
<td>56.6</td>
<td>HA</td>
<td>462</td>
</tr>
</tbody>
</table>

1) F, forward primer; R, reverse primer.
observed with the 12 other lineage/subtype swine IAVs or the three other types of swine infectious viruses and specificity assay results of five representative swine IAVs from other lineage/subtype were shown in Fig. 2. The PCR products obtained were sequenced to evaluate the specificity of the assay. Sequence analysis showed greater than 99% homology between the PCR products and the target gene sequence.

2.4. Sensitivity of the nested RT-PCR assay

The sensitivity of the nested RT-PCR assay was determined by testing RNA extracted from the 10-fold serially-diluted reference virus. As shown in Fig. 3, the sensitivity of the nested RT-PCR amplification was 1 plaque forming unit (PFU) mL\(^{-1}\), which was 100-fold greater than the sensitivity (10\(^3\) PFU mL\(^{-1}\)) of the single round RT-PCR using only the inner primers, and 10\(^4\)-fold higher than that (10\(^4\) PFU mL\(^{-1}\)) of the previous established multiplex RT-PCR method of detecting different lineages of H1 and H3 swine IAV (Fu et al. 2010).

2.5. Evaluation of the nested RT-PCR assay using clinical samples

To examine the ability of the nested RT-PCR to detect target viruses in the field, 365 nasal mucosal swabs were randomly taken from apparently healthy swine populations in Shandong Province of China in 2012. These samples were tested with both the nested RT-PCR and the classical detection method of viral isolation combined with sequencing. Extensive antigenic cross-reactions among different lineages of H1N1 swine IAVs have been previously observed, so the hemagglutinin inhibition (HI) assay was unable to combine with viral isolation to differentiate EA H1N1 swine IAV from viruses belonging to other lineages of the H1N1 subtype (Vijaykrishna et al. 2011). With the nested RT-PCR, 10 out of the 365 nasal samples from pigs tested positive, and the sizes of the amplicons were as predicted. The same results were obtained from viral isolation combined with sequencing. Thus, the newly designed diagnostic method and the viral isolation method were 100% consistent.

3. Discussion

In infected pigs, the narrow time frame of virus shedding and lack of overt signs of illness usually cause delays in diagnosis, as the viral replication peak is missed. Currently, the dominance of EA H1N1 swine IAVs throughout Chinese swine populations and potential transmission to human highlights the importance of establishment of a sensitive detection assay especially for EA H1N1 swine IAVs. A number of RT-PCR methods for the identification of H1N1, H1N2, and H3N2 swine IAVs have been reported (Chan et al. 2006; Lee et al. 2008; Lorusso et al. 2010; Chiapponi et al. 2012). Among these assays, Chiapponi et al. (2012) reported a multiplex RT-PCR for differentiating EA swine IAVs.
subtypes H1N1, H1N2, and H3N2 circulating in Europe. The genetic characteristics of swine IAVs vary by geographical region, and Chinese viruses differ from viruses isolated in Europe and North America (Van Reeth 2007; Kuntz-Simon and Madec 2009; Vijaykrishna et al. 2011). Thus, it is necessary to establish a method of identifying Chinese-specific influenza A viruses, which would facilitate investigation of the prevalence of this type of swine influenza A virus in pigs and other hosts.

Here, the nested RT-PCR assay targeting HA gene of EA H1N1 swine IAV circulating in China was developed. This assay can be completed in 5–6 h by detecting virus directly in nasal swabs. Existing methods of viral isolation require 5–6 days and must be followed by genetic sequencing for confirmation. The specificity of the nested RT-PCR was confirmed by testing different lineages and subtypes of swine IAVs and other infectious swine viruses circulating in China. Compared with single round RT-PCR and the previously established multiplex RT-PCR, the sensitivity of the nested RT-PCR was greatly improved by introducing a nested amplification step, which was in accordance with the findings of Nguyen et al. (2013). Initially, we examined the ability of the single step PCR to detect target viruses using 10 positive pig nasal samples previously identified by virus isolation. With the single step PCR, six out of the 10 nasal samples from pigs gave negative results (data not shown). So we further employed nested RT-PCR to improve detection sensitivity of single step PCR. This newly developed sensitive assay is expected to show high concordance with classical methods, particularly in the detection of samples with low viral loads. In this study, the detection of EA H1N1 swine IAV in field samples showed 100% concordance between the newly established nested RT-PCR assay and the classical method of virus isolation combined with genetic sequencing.

Some other variations of RT-PCR methods are found in the detection of influenza A virus. Multiplex PCR has the advantage of simultaneous determination of different subtypes or lineages, but also has the disadvantage of low sensitivity due to competition among different sets of primers (Ali and Reynolds 2000; Pang et al. 2002; Xie et al. 2006). Real-time RT-PCR is also a sensitive and specific method to detect influenza A viruses (Lorusso et al. 2010; Yang et al. 2010). However, this method requires higher-level technology and expensive equipment, therefore not commonly used in the field. Compared with the above methods, our newly established nested RT-PCR is an economically-sound and highly sensitive method, and can be used to perform routine surveillance in pig farms to detect EA H1 swine IAVs, without sending samples to provincial or national-level laboratories for diagnosis after disease outbreaks. The high sensitivity of this method allows detection as early as possible even when there are no overt signs of illness in pigs.

4. Conclusion

The nested RT-PCR established in this study is both specific and sensitive, and it can be used to identify EA H1 swine IAVs thus investigate infections in pigs and humans.

5. Materials and methods

5.1. Viruses

27 swine IAVs were used in this study, including 15 EA H1N1 swine IAV strains and 12 strains of other lineages and subtypes (Table 2). The EA H1N1 swine IAVs were isolated from 2007 to 2014. The other lineages and subtypes of swine IAVs included classical H1N1, H1N1pdm09, H1N2, H3N2, and H9N2 subtype viruses, which are found in pigs in China (Vincent et al. 2014). Among the swine IAVs, the EA H1N1 viruses of 2011–2014 and H1N1pdm09 virus were identified using a reference RT-PCR method and DNA sequence analysis (Hoffmann et al. 2001). Other
viruses which have been characterized in previous studies (Liu et al. 2009; Bi J et al. 2010; Bi Y et al. 2010; Sun et al. 2010). Swine IAVs were all grown in Madin-Darby Canine Kidney (MDCK) cells, titrated by a plaque assay (Kawaoka et al. 1984), and stored at −80°C until use. Other infectious swine viruses were used for the specificity tests of the nested RT-PCR (Table 2), including porcine reproductive and respiratory syndrome virus (PRRSV), pseudorabies virus (PRV), and porcine circovirus type 2 (PCV-2), which were provided by Dr. Ge Xinna (College of Veterinary Medicine, China Agricultural University, Beijing, China).

5.2. Field samples

To evaluate the nested RT-PCR in detecting field samples, a total of 365 nasal swabs were collected from farmed pigs in Shandong (eastern China) in 2012. No overt signs of illness were found in these sampled pigs. Swab samples were collected and stored in viral transport medium containing antibiotics at 4°C during transportation to the laboratory (Liu et al. 2009).

5.3. Primer design and selection

To determine the conserved regions of HA genes of EA H1N1 swine IAVs, sequence data for the HA genes of different lineage/subtypes of swine IAVs were obtained from the GenBank database (GenBank: http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=database) and aligned using the MegAlign program (DNASTar Inc., Madison, WI, U.S.). Primer pairs, including outer and inner primer sets, were designed according to the conserved regions of the HA genes of EA H1N1 swine IAV. The basic primer design rules were as follows: The primer could have only one target site in the template DNA; the optimal melting temperature for primers was in the range of 52–61°C; the two primers of a primer pair should have closely matched melting temperatures within a difference of 5°C; there should be an absence of primer secondary structures including hairpins, self-dimers, and cross dimers; few of adenine residues in the 3’ end; the inner primer sets within the sequence of the first amplification product. Five outer primer pairs and five corresponding inner primer pairs specific to the HA genes of EA H1N1 swine IAV were designed. Different combinations of outer and inner primer pairs were evaluated through their amplification efficiencies and specificities.

5.4. Viral RNA extraction and the nested RT-PCR assay

Viral RNA was extracted from virus-containing cell culture supernatants and nasal swabs using a modified version of a previously described process (Wei et al. 2006). Briefly, 300 μL of cell culture supernatant or specimen was mixed with 900 μL of TRIzol LS reagent (Invitrogen, U.S.) and placed on ice for 10 min. Chloroform (200 μL) was added and the mixture was placed on ice for a further 5 min. The suspension was then centrifuged for 15 min at 12,000×g. The RNA was precipitated from the aqueous phase with an equal volume of isopropanol and centrifuged at 13,000×g for 15 min at 4°C. The RNA pellet was washed with 1 mL of 75% ethanol and centrifuged at 12,000×g for 5 min at 4°C. The supernatant was removed and the RNA dried and re-suspended in 12 μL of diethyl pyrocarbonate (DEPC)-treated deionized water. The RT reactions were performed with the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania), according to the manufacturer’s protocol. The PCR reaction included two rounds of amplification (first and second). Each 25 μL reaction mixture for first round amplification contained 5 μL of 5× reaction buffer, 0.5 μL of

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Table 2  Swine influenza A virus (IAV) and other infectious viruses used in this study

<table>
<thead>
<tr>
<th>Virus</th>
<th>Subtype</th>
<th>Lineage</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/swine/Fujian/20/2007</td>
<td>H1N1</td>
<td>European avian-like</td>
</tr>
<tr>
<td>A/swine/Zhucheng/82/2007</td>
<td>H1N1</td>
<td>European avian-like</td>
</tr>
<tr>
<td>A/swine/Beijing/21/2008</td>
<td>H1N1</td>
<td>European avian-like</td>
</tr>
<tr>
<td>A/swine/Beijing/26/2008</td>
<td>H1N1</td>
<td>European avian-like</td>
</tr>
<tr>
<td>A/swine/Shandong/187/2008</td>
<td>H1N1</td>
<td>European avian-like</td>
</tr>
<tr>
<td>A/swine/Zhucheng/D60/2011</td>
<td>H1N1</td>
<td>European avian-like</td>
</tr>
<tr>
<td>A/swine/Zhucheng/201/2011</td>
<td>H1N1</td>
<td>European avian-like</td>
</tr>
<tr>
<td>A/swine/Zhucheng/458/2011</td>
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<td>European avian-like</td>
</tr>
<tr>
<td>A/swine/Shandong/4/2012</td>
<td>H1N1</td>
<td>European avian-like</td>
</tr>
<tr>
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<td>European avian-like</td>
</tr>
<tr>
<td>A/swine/Shandong/20/2012</td>
<td>H1N1</td>
<td>European avian-like</td>
</tr>
<tr>
<td>A/swine/Shandong/13/2012</td>
<td>H1N1</td>
<td>European avian-like</td>
</tr>
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<td>A/swine/Shandong/22/2012</td>
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<td>European avian-like</td>
</tr>
<tr>
<td>A/swine/Shandong/269/2014</td>
<td>H1N1</td>
<td>European avian-like</td>
</tr>
<tr>
<td>A/swine/Shandong/153/2014</td>
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<td>European avian-like</td>
</tr>
<tr>
<td>A/swine/Guangdong/61/2006</td>
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<td>Classical</td>
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<tr>
<td>A/swine/Guangdong/446/2006</td>
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<td>Classical</td>
</tr>
<tr>
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<td>Classical</td>
</tr>
<tr>
<td>A/swine/Shandong/731/2009</td>
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<td>Pandemic 2009</td>
</tr>
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<td>A/California/04/2009</td>
<td>H1N1</td>
<td>Pandemic 2009</td>
</tr>
<tr>
<td>A/swine/Guangdong/1222/2006</td>
<td>H1N2</td>
<td>North American triple reasortant</td>
</tr>
<tr>
<td>A/swine/Guangdong/7/2006</td>
<td>H3N2</td>
<td>–</td>
</tr>
<tr>
<td>A/swine/Guangdong/211/2006</td>
<td>H3N2</td>
<td>–</td>
</tr>
<tr>
<td>A/swine/Guangdong/223/2006</td>
<td>H3N2</td>
<td>–</td>
</tr>
<tr>
<td>A/swine/Shandong/FJN/2003</td>
<td>H9N2</td>
<td>–</td>
</tr>
<tr>
<td>A/swine/Henan/2/2004</td>
<td>H9N2</td>
<td>–</td>
</tr>
<tr>
<td>A/swine/Henan/3/2004</td>
<td>H9N2</td>
<td>–</td>
</tr>
<tr>
<td>JXwn06 (PRRSV)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Fa (PRV)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>BF (PCV-2)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

1) A/swine/Fujian/204/2007 was used as the reference strain in the development of the nested RT-PCR. PRRSV, porcine reproductive and respiratory syndrome virus; PRV, pseudorabies virus; PCV-2, porcine circovirus type 2.
dNTP mix (10 mmol L⁻¹ of each dNTP), 1 μL of 25 mmol L⁻¹ MgSO₄ (Promega, Madison, WI, U.S.), 2.5 U of Taq DNA polymerase (Promega), 0.5 μL of each outer primer (20 μmol L⁻¹ of each primer), and 2 μL of cDNA template. The amplification conditions were as follows: initial denaturation at 94°C for 5 min, followed by 20 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min.

An aliquot (2 μL) of the first round amplification product was used as the DNA template for the 25 μL second round amplification reactions. Inner primers were used, the annealing temperature was 56°C, and the extension was done at 72°C for 2 min. Other constituents and reaction conditions were identical to those of the first round amplification. The final amplified products (4 μL) were loaded onto 1% (w/v) agarose gels containing 0.5 μg mL⁻¹ ethidium bromide. Electrophoresis was conducted using 1× TAE buffer and PCR products were visualized under UV transillumination.

5.5. Optimization of nested RT-PCR

The annealing temperature and extension time were optimized separately using a reference EA H1N1 swine IAV strain (A/swine/Fujian/204/2007). For the annealing temperature, a univariate method was used and a gradient of alternatives, as follows: 1) outer primer annealing temperatures (49, 51, 53, 55, or 57°C); 2) annealing temperatures of the inner primers (52, 54, 56, 58, or 60°C). The appropriate annealing temperature was selected based on the intensity of the PCR product bands in the electrophoresis gel. For the extension time, those of the first/second rounds of PCR amplification were set to 1 min/1 min, 1 min/2 min, 2 min/1 min, and 2 min/2 min. The appropriate extension time was determined based on the intensity of the PCR product bands.

The numbers of cycles were optimized using EA H1N1 swine IAV strain (A/swine/Fujian/204/2007) and other lineage/subtype viruses (classical H1N1, H1N1pdm09, H1N2, H3N2, and H9N2 subtype swine IAVs). The EA H1N1 virus was used at a low concentration of 10⁻¹ to 10⁻² PFU mL⁻¹ and the other lineage/subtype swine IAVs were used at a high concentration of 10⁻⁶ to 10⁻⁶.5 PFU mL⁻¹. The virus was 10-fold serially diluted in PBS from 10⁴ to 10⁻² PFU mL⁻¹.

5.7. Performance evaluation of the nested RT-PCR on field samples

A total of 365 field samples were tested with the nested RT-PCR assay. Viral RNA was isolated from nasal swabs of pigs and nested RT-PCR was performed as described above. All the 365 clinical samples were also subjected to viral isolation and sequencing. Briefly, these samples were incubated with MDCK cells at 37°C for 48 h, then cell culture supernatants were tested in a hemagglutination test (Webby et al. 2000). Hemagglutination-positive cell cultures were used for viral RNA extraction and confirmed to be EA H1N1 swine IAVs by RT-PCR and DNA sequence analysis. The primers used for PCR amplification were universal primers for all subtype influenza A viruses (Hoffmann et al. 2001). Finally, the results were compared with those of the nested RT-PCR.

5.8. Sequencing

PCR products were purified from agarose gels using an AxyPrep DNA Gel Extraction Kit (Axygen Scientific Inc., Union City, CA, U.S.), according to the manufacturer’s instructions. The size-specific PCR products were sequenced at the Beijing Genomics Institute (China) and aligned using the MegAlign program (DNAStar Inc.) to evaluate the specificity of the assay.

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References


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