Evidence for the circulation of genotype 4 Eurasian avian-like lineage swine H1N1 viruses in Korean swine farms using a newly developed one-step multiplex RT-qPCR

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Abstract

Background: Genotype 4 Eurasian avian-like lineage swine H1N1 viruses, which are reassortants containing the pandemic 2009 H1N1 virus lineage, triple reassortant lineage internal genes, and EA lineage external genes, have been reported in China since 2013. These have been predominant in pig populations since 2016 and have exhibited pandemic potential. Objectives: Examination of the circulation of G4 and its-related EA H1N1 viruses in Korea. Methods: We developed a one-step multiplex RT-qPCR that targeted M, HA1, and PB2 genes to detect G4 and its-related EA H1N1 viruses. We performed the analytical sensitivity and specificity of the multiplex RT-qPCR. The developed multiplex RT-qPCR was applied to swine respiratory samples between 2020 and 2022 in Korea. Results: Among the swine samples, a G4 EA H1N1 virus-related positive sample (6IP) was detected. Phylogenetic analysis based on all eight partial gene segments showed that 6IP contains nucleic acids of IAV segmented genes, which are closely related to G4 EA H1N1 viruses in HA, NA, PB2, NS, and NP genes. Conclusions: The developed multiplex RT-qPCR proved the sensitivity and specificity for the efficient surveillance of G4 and its-related EA H1N1 virus in clinical samples. Considering the phylogenetic analysis, additional screening for G4 EA H1N1 or its related viruses should be performed in future epidemiological studies.

Introduction

Influenza A virus (IAV) is a pathogen that can cause severe illnesses in mammals and birds [10]. Because of the segmented RNA genome of the IAV, gene reassortment occurs frequently, resulting in the emergence of novel viruses [24]. Most of the past pandemic IAVs are reassortants resulting from the shuffling of genes between human and animal IAVs [2, 11]. Among the mammals that can transmit influenza viruses, pigs are considered as “mixing vessels” that might contribute to the emergence of novel influenza viruses with pandemic potential, as swine could be infected by both human and avian-origin influenza viruses [16, 27]. Obvious examples include the “Spanish” flu pandemic of 1918 and pandemic 2009 H1N1 (pdm/09 H1N1) [4, 28]. Thus, constant surveillance of IAVs in pig populations should be performed to prepare for future pandemic IAV threats.

Eurasian avian-like (EA) swine H1N1 viruses are derived from an avian influenza virus [21] and have caused diseases endemic to Europe and Asia [5, 13, 20, 29]. Recently, researchers demonstrated that the genotype 4 (G4) EA H1N1 virus, predominant since 2016 in pig populations in China, could be a candidate for
pandemic influenza [25]. The G4 EA H1N1 virus contains the pdm/09 H1N1 lineage, triple reassortant (TR) lineage internal genes and EA lineage external genes [25]. The G4 EA H1N1 viruses can bind to receptors on human cells and effectively replicate progeny viruses in human airway epithelial cells; they have shown effective infectivity and aerosol transmission in animal models [25, 26]. In addition, G4 EA H1N1 viruses have a high risk of spreading to humans because of their low cross-reactivity with trivalent vaccines [25]. Currently, a TaqMan probe-based G4 EA H1N1 virus detection kit exists; however, it is difficult to perform IAVs screening using a single tube as the detection wavelengths of all the probes are identical. To efficiently identify the genotype of IAVs, it is important to select G4 EA H1N1 virus-related samples from swine clinical samples. In addition, despite their pandemic potential, epidemiological studies of G4 EA H1N1 viruses have not been conducted in Eurasian countries other than China. Therefore, we developed a detection platform to select G4 EA H1N1 virus-related samples using a single tube.

Materials and methods

Primers and probes

To detect G4 EA swine H1N1 viruses, we collected 171 HA1 gene sequences of EA-lineage swine H1N1 viruses, 29 PB2 gene sequences of G4 EA swine H1N1 viruses, and 41 PB2 gene sequences of EA swine H1N1 viruses from GenBank or the Global Initiative on Sharing Avian Influenza Data (GISAID) [25]. We performed ClustalW multiple alignments using BioEdit software (7.2.5 version) to target the common sequences of the EA lineage HA1 and PB2 genes of G4 EA swine H1N1 viruses. To determine the type of influenza virus, we used universal primers and a probe targeting the matrix gene designed by the WHO (Supplementary Table 2).

Generation of recombinant G4 EA H1N1 virus

The G4 EA H1N1 virus was rescued using a reverse genetic system kindly provided by Dr Richard J. Webby (St. Jude Children’s Research Hospital, TN, USA). Eight segments of the A/swine/Shandong/1207/2016 (SW/SD/1207/16) virus [25] were cloned into the pHW2000 vector, and the constructs were used to transfect co-cultured human embryonic kidney (293T, CRL-3216) and Madin–Darby canine kidney (MDCK, CRL-2936) cells [8]. The culture supernatant was collected and used to inoculate 9- to 11-day-old SPF chicken eggs. After virus rescue, the full genome sequences of the rescued viruses were verified by sequencing, and all rescued viruses were stored at -80 °C until use. All viral experiments were conducted at the Korea Research Institute of Bioscience and Biotechnology (KRIBB, South Korea) and approved by and conducted in accordance with the guidelines of the Institutional Biosafety Committee (IBC, approval number KRIBB-IBC-20200213) of the KRIBB.

RNA extraction and cDNA synthesis

Recombinant SW/SD/1207/16 RNA was extracted using the TRIzol LS Reagent (Invitrogen, USA) following the manufacturer’s instructions. RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Promega, USA) with random hexamers, following the manufacturer’s protocol.

Preparation of purified PCR products for analytical sensitivity

To generate a quantitative standard, M, HA1, and PB2 sequences of recombinant SW/SD/1207/16 containing primer and probe binding sites were amplified by conventional PCR using the primers listed in Supplementary Table 2. M, HA1, and PB2 amplicons were separated by electrophoresis using agarose gel and purified with the Expin Combo GP kit (GeneAll, South Korea). Each purified PCR product was quantified using a spectrophotometer (DeNovix, USA). The quantities were converted into copy numbers using the following formula [9]:

\[ \text{copy numbers} = \frac{\text{absorbance} \times \text{concentration}}{\text{absorbance}_0} \]
Then, the purified PCR products were serially diluted with nuclease-free water from $1.5 \times 10^8$ copies/µL to $1.5 \times 10^4$ copies/µL (10-fold).

**One-step multiplex RT-qPCR conditions**

All RT-qPCR reactions were performed using a SensiFAST Probe No-ROX One-step kit (Bioline, USA) at a volume of 20 µL. The mixture contained 4 µL of RNA template, 0.8 µL of each F/R primer (10 pmol), 0.2 µL of each probe (10 pmol), 10 µL of 2X SensiFAST Probe No-ROX One-Step Mix, 0.2 µL of reverse transcriptase, and 0.4 µL of RiboSafe RNase Inhibitor.

Multiplex RT-qPCR was performed with reverse transcription at 45 °C for 20 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s and 54 °C for 30 s. RT-qPCR mixtures were thermocycled using a LightCycler 96 System (Roche, USA). DEPC buffer was used as a no-template control (NTC). Positive results were estimated by analysing the amplification curves produced by each probe within 40 cycles.

**Analytical sensitivity of one-step multiplex RT-qPCR**

To evaluate the limit of detection of the developed multiplex RT-qPCR, we used purified PCR products and RNA templates of recombinant SW/SD/1207/16. The extracted RNA was also quantified by a spectrophotometer and 10-fold serially diluted from 1.15 ng/µL to $1.15 \times 10^{-3}$ ng/µL. We also estimated the sensitivity of the G4 EA H1N1 Swine Flu Virus RT-qPCR Detection kit (ScienCell, USA) following the manufacturer’s manual for comparison with the sensitivity of our developed multiplex RT-qPCR.

**Analytical specificity of one-step multiplex RT-qPCR**

The analytical specificity was determined for different IAV subtypes and other viruses. We used nucleic acid samples extracted from the human H1N1 virus, Eurasian avian lineage H1N1 virus and other subtypes (H3N2, H3N8, and H9N2 viruses), porcine reproductive and respiratory syndrome virus (PRRSV), human coronavirus OC43 (HCoV-OC43) (Korea Bank for Pathogenic Virus, Seoul, South Korea), porcine epidemic diarrhoea virus (PEDV), porcine circovirus type 3 (PCV-3), and mammalian orthoreovirus (MRV) to assess the specificity of the developed one-step multiplex RT-qPCR. The human H1N1 virus and other subtypes were provided by the College of Veterinary Medicine, Chonnam National University.

**Swine clinical samples**

We performed IAV screening using 33 IAV-positive and 30 IAV-negative archived swine respiratory samples provided by OptiPharm Inc. (Osong, South Korea). We performed screening using universal primers and probe sets specific to the IAV matrix gene, followed by screening using our multiplex RT-qPCR. We then compared the results of the first and second screenings.

**Partial sequencing and phylogenetic analysis**

We performed conventional PCR using universal HA1 and PB2 primers (Supplementary Table 3) for partial sequencing and analyzed their genetic lineage in swine clinical samples. Sanger sequencing was performed using an Applied Biosystems 3730xl DNA analyser. HA1 partial sequences were analyzed using the Swine H1 Clade Classification Tool in the Influenza Research Database. To examine the genetic lineage in detail, partial PB2 and HA sequences were analyzed using BLASTn (https://blast.ncbi.nlm.nih.gov/) and Molecular Evolutionary Genetics Analysis 11 (MEGA 11). All phylogenetic trees based on nucleotide sequences were formed using the maximum likelihood method with 1000 replicates in MEGA 11. We also performed partial
sequencing of the NA1 and M genes of the Cy5 signal-positive samples. We obtained reference sequences from GenBank and GISAID to construct phylogenetic trees.

**Further sequence analysis of G4 EA H1N1 virus-positive sample**

We regarded the sample that showed all fluorescence signals as a G4 EA H1N1 virus-positive sample. We further analyzed the genetic lineages of the PB1, PA, NP, and NS genes of the suspicious samples via partial sequencing using the primers (Supplementary Table 3).

**Virus isolation**

MDCK cells were grown in minimum essential medium (MEM) with 5% fetal bovine serum (FBS) and 1X antibiotic-antimycotic in 25 cm² flasks. For virus isolation, we inoculated the 0.45 μm syringe-filtered and 10-fold diluted samples that showed Cy5 fluorescent signal to the cell monolayers for 1 h at 37 in 5% CO₂. Then, cells were washed once with DPBS. Then, MEM with 0.3 μg/mL TPCK-trypsin was added to cell monolayers, and cells were incubated for 96 h. After incubation, the cell supernatants were passaged, and the presence of IAVs was confirmed using RT-qPCR.

**Results**

**Analytical sensitivity of one-step multiplex RT-qPCR**

We estimated the sensitivity of multiplex RT-qPCR using purified PCR products, RNA templates and repeated it at least thrice. For the diluted purified PCR products, the detection limit of RT-qPCR was $1.5 \times 10^4$ copies/μL based on the amplification curves produced in singleplex and multiplex conditions (Supplementary Figure 1) and electrophoresis results of singleplex RT-qPCR (Supplementary Figure 2). The standard curves produced by RT-qPCR showed linearity ($R^2 = 0.9903$–0.999, Supplementary Figure 1). In the case of diluted RNA, the detection limit of one-step multiplex RT-qPCR was $1.15 \times 10^{-1}$ ng/μL (Table 2). The NTC did not produce amplification curves. When the multiplex RT-qPCR method was compared with the sensitivity of the detection kit, our multiplex RT-qPCR was 10-fold more sensitive. Considering these results, we considered a positive reaction for the M and HA1 genes when the Cq value was less than 36 and for the PB2 gene when the Cq value was less than 35.

**Analytical specificity of one-step multiplex RT-qPCR**

The specificity of multiplex RT-qPCR was assessed in triplicate using different IAV subtypes and other pathogens. We found that all IAV subtypes were detected by the primer/probe set targeting the matrix gene, while other subtypes and pdm/09 H1N1 lineage human H1N1 viruses were not detected by our HA1 and PB2 primers and probe sets (Table 3). Fluorescent signals were not detected for PRRSV, HCoV-OC43, PEDV, PCV-3, or MRV.

**Application of one-step multiplex RT-qPCR using swine clinical samples**

33 IAV-positive and 30 IAV-negative clinical swine samples provided by OptiPharm Inc. were used to screen for swine IAVs. Positive and negative samples were verified using universal matrix gene primers and probe sets (Supplementary Table 4). The developed one-step multiplex RT-qPCR showed that all the positive samples were IAV-positive, and 2 of the 30 negative samples showed fluorescent signals but did not show the expected DNA band on the agarose gel. Among the positive samples, seven were HEX signal-positive, six were Cy5 signal-positive, and one (named ‘6IP’) showed all the signals (Supplementary Table 4). We regarded 6IP as a sample infected with the G4 EA H1N1 virus.

**Partial sequencing and phylogenetic analysis**

Partial sequences of swine influenza viruses were deposited in GenBank (Supplementary Table 1). We performed conventional PCR using the primers (Supplementary Table 3) for partial sequencing and examined the genetic lineage of the HA1 and PB2 partial genes using the MEGA 11 program for all swine clinical samples. We further sequenced and analyzed the positive samples using BLASTn and MEGA 11 programs (Supplementary Table 4 and Supplementary Figure 3). We also analyzed the HA1 and PB2 partial sequences.
in 13 positive samples that showed fluorescent signals. Among the eight HEX signal-positive samples, five were assigned to the 1C:2.3 clade by the Influenza Research Database. Among the six Cy5 signal-positive samples, five belonged to the pdm/09 H1N1 lineage. In the case of NA partial sequencing, three samples belonged to the pdm/09 H1N1 lineage, and one sample belonged to the EA lineage. In the case of M partial sequencing, four samples belonged to the pdm/09 H1N1 lineage, and one sample belonged to the EA lineage (Supplementary Table 4 and Figure 1).

Further phylogenetic analysis about 6IP sample showed that the clade of the PB1 and NP genes of 6IP belonged to the pdm/09 H1N1 lineage, whereas the PA and NS clades belonged to the avian and TR lineages, respectively. Partial PB2, HA, NP, and NS sequences seemed to be related to G4 EA H1N1 viruses (Figure 2). We tried to isolate the G4 EA H1N1 virus-related swine influenza viruses using MDCK cells; however, this was unsuccessful and excluded from further studies.

Discussion

The goal of this study was to efficiently detect G4 and related EA H1N1 viruses and confirm the circulation of G4 EA H1N1 viruses in pigs in South Korea. EA swine H1N1 viruses have been circulating in pig populations on the Eurasian continent [20]. Most human infections caused by H1N1 viruses are derived from direct exposure of pigs, and EA swine H1N1 viruses occasionally cause severe human infections [5, 17, 19]. A previous study also demonstrated that EA H1N1 viruses could become more harmful pathogens by accumulating mutations in the polymerase gene [18]. While EA swine H1N1 viruses have been circulating in Eurasian countries, G4 EA H1N1 viruses have emerged in China and have been predominant since 2016 [26]. G4 EA H1N1 viruses have severe pathogenicity, enhanced replication, and transmission ability in vivo and can be transmitted from pigs to humans [6, 14, 25].

Despite their pandemic potential, few G4 EA H1N1 virus detection tools exist. Genotyping IAV is required several times as the genetic lineage of all eight segments needs to be identified. To reduce surveillance time, we needed to select G4 EA H1N1 virus-positive samples among clinical samples. qPCR based on TaqMan probes has high sensitivity and specificity and good reproducibility for molecular diagnosis in clinical fields [3]. Thus, we developed a multiplex RT-qPCR method for surveillance of IAVs using a single tube. In a previous study, the PB2 and HA genes were shown to be major determinants of the host specificity of IAV [15]. The general RT-qPCR method targeting matrix genes is the gold standard for the rapid molecular detection and determination of influenza virus types [23]. We considered these studies to design primers and probes. M, HA1, and PB2 genes were used to identify influenza virus types, verify serotypes, and narrow the host range of IAV, respectively.

The developed primers and probes could detect G4 EA H1N1 viruses at 1.5 × 10^4 copies/μL when using purified PCR products as templates (Table 1 and Supplementary Figure 1). Under singleplex conditions, we observed the target DNA band on agarose gel at 1.5 × 10^4 copies/μL when compared with NTC (Supplementary Figure 2). In triplex conditions, the limit of detection was the same as that in singleplex conditions (Table 1 and Supplementary Figure 1). When RNA was used as the template, the detection limit of multiplex RT-qPCR was up to 1.15 × 10^−1 ng/μL (Table 2). Our detection method was approximately 10-fold more sensitive than the RT-qPCR detection kit. Based on previous results that the detection limit of triplex RT-qPCR for the detection of avian IAVs was 5.0 × 10^4 copies/μL [12], the developed multiplex RT-qPCR can be applied in clinical diagnostics.

In terms of analytical specificity, the developed multiplex RT-qPCR can work only with influenza viruses. Even though A/California/04/2009(H1N1), A/Puerto Rico/8/1934(H1N1), and A/wild bird/Korea/SK14/2014(H1N1) viruses have identical subtypes, the multiplex RT-qPCR detects only the Eurasian avian lineage, which is closely related with the EA lineage [1, 7] or EA lineage HA1 gene (Table 2 and 3). Interestingly, our PB2 primer and probe set specific to G4 EA swine H1N1 viruses did not work with A/California/04/2009(H1N1) virus, although the genetic lineage of the PB2 gene of the virus was the same as that of the G4 EA H1N1 virus [25] (Table 2 and 3). This suggested that the PB2 primers and probe could specifically detect swine origin-IAVs.
On comparing the IAV screening results with multiplex RT-qPCR results, matrix gene primers and probe sets showed 100% agreement in 33 IAV-positive samples; however, among the 30 IAV-negative samples, two showed false-positive reactions (Supplementary Table 4). Our HA1 primer and probe set showed HEX fluorescent signals in eight positive samples. After partial sequencing, the five positive samples belonged to the 1C.2.3 clade (EA lineage) [1], and three positive samples were HA1 subtype-negative (Supplementary Table 4 and Figure 1). The non-specific reactions might be derived from the low annealing temperature and mixed bases in the probe sequence. Six IAV-positive samples showed Cy5 fluorescence. After partial sequencing, five positive samples belonged to the pdm/09 H1N1 lineage, and one belonged to the TR lineage (Figure 1). The genetic lineage could be detected because the ancestor of the 2009 pandemic H1N1 viruses was related to the TR lineage of the swine influenza viruses [22]. Considering all partial sequencing results, our primers and probe set targeting the EA lineage HA1 gene produced a relatively higher rate of false positive and negative reactions than our primers and probe set targeting the pdm/09 H1N1 lineage PB2 gene (Table 4).

We selected a G4 EA H1N1 virus-positive sample (6IP) and performed a phylogenetic analysis. It consisted of pdm/09 H1N1 lineage PB2, PB1, NP genes, EA lineage HA, NA, M genes, TR lineage NS gene, and avian lineage PA gene (Figure 2). Partial PB2, HA, NP, NA, and NS sequences of 6IP might be related to G4 EA H1N1 viruses. As a suspicious sample was collected on January 27, 2022, in South Korea, the results might imply a reasortment of G4 EA H1N1 viruses circulating in swine populations in Asia. A gene reassortment event with the G4 EA H1N1 virus genome was reported in China in 2020 [30]. G4 EA H1N1 viruses have been mainly reported in China since 2016. However, in countries other than China, no case of detection and isolation of G4 EA H1N1 viruses from swine populations has been reported. Through phylogenetic analysis of the 6IP sample, we found evidence of the circulation of G4 EA H1N1 viruses in swine populations in South Korea. However, because of the unsuccessful isolation of the G4 EA H1N1 virus-related swine IAVs, we could not obtain whole genome sequences of the 6IP sample for a more precise analysis of the reassortment of G4 EA H1N1 viruses. Detailed molecular and serological surveillance in pigs in countries other than China considering genotypes of the EA lineage H1N1 viruses is required to illuminate the global state of circulation and distribution of G4 EA H1N1 viruses in swine populations.

In conclusion, we developed and validated a one-step multiplex RT-qPCR, which could help screen potential G4 EA swine influenza viruses. Applying one-step multiplex RT-qPCR for viral screening in swine respiratory samples in Korea between 2020 and 2022, we detected one G4 EA H1N1-related virus-positive sample (6IP). Although we failed to isolate the virus, phylogenetic analysis based on all eight partial gene segments showed that the positive sample 6IP contained nucleic acids of IAV-segmented genes closely related to G4 EA H1N1 viruses in HA, NA, PB2, NS, and NP genes. This suggests that the G4 EA H1N1 virus or its related reassortant viruses might be circulated in the swine population of Korea. Therefore, additional screening for G4 EA H1N1 or its related viruses should be performed in future epidemiological studies.

Acknowledgments
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Disclosure statement
The authors declare that there are no conflicts of interest.

Data availability statement
The sequences generated in this study were deposited in the GeneBank database under accession numbers (Supplementary Table 1).

References


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génétiques et antigéniques des virus grippaux A zoonotiques et mise au point de virus vaccinaux candidats pour se préparer à une pandémie. Weekly Epidemiological Record= Relevé épidémio-logique hebdomadaire 97:120-132.


Tables

Table 1. Limits of detection (LOD) under singleplex and triplex conditions

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Singleplex (Triplex replicates) LOD (copies/μL)</th>
<th>Singleplex (Triplex replicates) Cq value (mean ± SD)</th>
<th>Singleplex (Triplex replicates) CV (%)</th>
<th>Triplex (Quintuple replicates) LOD (copies/μL)</th>
<th>Triplex (Quintuple replicates) Cq value (mean ± SD)</th>
<th>Triplex (Quintuple replicates) CV (%)</th>
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</thead>
<tbody>
<tr>
<td>M</td>
<td>1.5 × 10¹</td>
<td>33.23 ± 0.65</td>
<td>0.019</td>
<td>1.5 × 10¹</td>
<td>34.52 ± 0.65</td>
<td>0.032</td>
</tr>
<tr>
<td>HA1</td>
<td>1.5 × 10¹</td>
<td>32.13 ± 1.14</td>
<td>0.035</td>
<td>1.5 × 10¹</td>
<td>35.46 ± 0.45</td>
<td>0.012</td>
</tr>
<tr>
<td>PB2</td>
<td>1.5 × 10¹</td>
<td>31.03 ± 0.53</td>
<td>0.017</td>
<td>1.5 × 10¹</td>
<td>33.56 ± 0.61</td>
<td>0.018</td>
</tr>
<tr>
<td>Positive rate was 80% (4 of 5).</td>
<td>* Positive rate was 80% (4 of 5).</td>
<td>* Positive rate was 80% (4 of 5).</td>
<td>* Positive rate was 80% (4 of 5).</td>
<td>* Positive rate was 80% (4 of 5).</td>
<td>* Positive rate was 80% (4 of 5).</td>
<td>* Positive rate was 80% (4 of 5).</td>
</tr>
</tbody>
</table>

Table 2. Sensitivity analysis of one-step triplex RT-qPCR
Developed one-step multiplex RT-qPCR assay

G4 EA H1N1 virus detection kit (ScienCell, USA)

+ Cq mean value ± SD. ? Positive rate was about 67% (2 of 3). ? Targeting human β-actin housekeeping gene.

Table 3. Specificity analysis of one-step triplex RT-qPCR using various viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cq mean value</th>
<th>Cq mean value</th>
<th>Cq mean value</th>
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<tr>
<td></td>
<td>FAM</td>
<td>HEX</td>
<td>Cy5</td>
</tr>
<tr>
<td>H3N2*</td>
<td>14.15</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A/equine/Kyonggi/SA01/2011(H3N8)</td>
<td>14.87</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H9N2*</td>
<td>18.20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A/California/04/2009(H1N1)</td>
<td>15.73</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A/Puerto Rico/8/1934(H1N1)</td>
<td>14.66</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A/wild bird/Korea/SK14/2014(H1N1)</td>
<td>11.57</td>
<td>17.68</td>
<td>-</td>
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<tr>
<td>PRRSV*</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>HCoV-OC43 (strain KUMC-8)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PEDV*</td>
<td>-</td>
<td>-</td>
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<tr>
<td>PCV-3*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MRV (strain BatMRV/B19-02)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Strain information was unknown.</td>
<td>* Strain information was unknown.</td>
<td>* Strain information was unknown.</td>
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</table>

Table 4. Multiplex RT-qPCR results from swine clinical samples

<table>
<thead>
<tr>
<th>Primers and probe set name</th>
<th>Primers and probe set name</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Developed multiplex RT-qPCR assay</td>
</tr>
<tr>
<td></td>
<td>G4PB2 primer/probe set</td>
</tr>
</tbody>
</table>

Developed multiplex RT-qPCR assay

a Primers in Supplementary Table 3 were used for partial sequencing. ? Eurasian avian-like lineage HA1 gene. ? Two positive samples (22IP and 30IP) were excluded (Supplementary Table 4).

Figure legends

Figure 1. Phylogenetic analysis of partial HA, PB2, NA and M gene of clinical samples which showed fluorescent signal. A phylogenetic tree based on nucleotide sequences was formed by the Maximum likelihood method with 1000 replicates in MEGA 11. ‘G4’ represented G4 EA H1N1 viruses. Sequences labelled black triangle represented outgroups. Bootstrapping values lower than 50% were hidden. Clade assignment of swine clinical samples using Influenza Research Database (https://www.fludb.org) (A).

Figure 2. Phylogenetic analysis of partial PB1, PA, NP and NS gene of the G4 EA H1N1
virus-infected sample. A phylogenetic tree based on nucleotide sequences was formed by the Maximum likelihood method with 1000 replicates in MEGA 11. ‘G4’ represented G4 EA H1N1 viruses. Sequences labelled black triangle represented outgroups. Bootstrapping values lower than 50% were hidden.

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Figure 1.docx available at https://authorea.com/users/623991/articles/646505-evidence-for-the-circulation-of-genotype-4-eurasian-avian-like-lineage-swine-h1n1-viruses-in-korean-swine-farms-using-a-newly-developed-one-step-multiplex-rt-qpcr

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Figure 2.docx available at https://authorea.com/users/623991/articles/646505-evidence-for-the-circulation-of-genotype-4-eurasian-avian-like-lineage-swine-h1n1-viruses-in-korean-swine-farms-using-a-newly-developed-one-step-multiplex-rt-qpcr