

Rapid Differential Detection of H1 and H3 Subtype Swine Influenza Viruses by a TaqMan-MGB Based Duplex One-Step Real Time RT-PCR Assay

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Abstract

Swine influenza is not only an economically important respiratory disease in swine, but also constantly poses a threat to human health. Hence, developing a rapid, sensitive and efficient detection method of swine influenza virus (SIV) is highly essential. By aligning the HA gene sequences of SIV circulating in China in recent 10 years, a H1 primer-probe set targeting both Eurasian avian-like H1N1 (EA H1N1) and Pandemic 2009 H1N1 (Pdm09 H1N1) lineages plus a H3 prime-probe set targeting the prevalent human-like H3N2 (HL H3N2) subtype were designed, respectively. Further, a TaqMan-MGB based duplex one-step real time RT-PCR (RRT-PCR) assay was established and evaluated. The duplex RRT-PCR possessed the detection limit of 5 copies/ μ L HA plasmid for each of the EA H1N1, Pdm09 H1N1 and HL H3N2 subtype SIVs, and matched an overall detection sensitivity of 100% and specificity of 91.67% with traditional virus isolation through chicken embryo inoculation using experimentally infected mice lung samples. Besides, the method showed high repeatability both within-run and between-runs, and no cross-reactivity against some commonly circulated porcine viruses in China. Furthermore, the duplex RRT-PCR method revealed a relatively higher prevalent rate of H1 than H3 subtype SIV in 166 nasal swabs from pigs in some slaughterhouse during October ~ December, 2019. This developed assay could be very helpful for rapid differential detection and routine surveillance of EA H1N1, Pdm09 H1N1 and HL H3N2 subtype SIVs in China.

1. Introduction

Swine influenza, normally caused by influenza A virus, is a highly contagious respiratory disease of swine [1]. Pigs independently infected with swine influenza virus (SIV) often exhibit typical respiratory symptoms, such as fever, coughing and difficulty breathing [2]. However, more severe clinical symptoms could be observed when other pathogens co-infected or secondarily-infected with SIV [3,4]. In addition, the fact that both avian and human influenza virus receptors are expressed in pig respiratory epithelial cells paves the way for influenza reassortants to spread across host barriers. Currently, SIV of H1N1, H1N2 and H3N2 subtypes are dominantly circulating in swine worldwide [5]. Especially in China, Eurasian avian-like H1N1 (EA H1N1), Pandemic 2009 H1N1 (Pdm09 H1N1) and human-like H3N2 (HL H3N2) are the three main prevalent lineages of H1 and H3 subtype SIV in the past 10 years [6,7].

Recently, EA H1N1 has been the overwhelmingly prevailing SIV in China and classified into six genotypes (G1-G6) according to its gene constellation [8]. Of extraordinary importance, G4 has been assessed to possess the highest possibility to transmit from swine to human and cause an influenza pandemic [8,9]. Actually, several human cases have been confirmed as directly infected with EA H1N1, such as that the 9-year-old boy suffering from fever and headache was tested SIV-RNA positive by the Tianjin Centers for Disease Control and Prevention in 2019 [9]. In addition, HL H3N2 SIV could also pose a threat to human health as the pathogen was once isolated from a 10-year-old girl in Guangdong province in 2018 [10]. Therefore, it is urgent to carry out systematical surveillance of SIV since swine could yet serve as “mixing vessel” for producing novel influenza reassortants from mammalian and avian viruses. And, to develop a sort of rapid, sensitive and specific method for SIV detection is of great significance not only for the prevention and control in swine, but also for the early warning of cross-host transmission in human.

The classical laboratory diagnostic methods of SIV infection include virus isolation through inoculation into specific-pathogen-free (SPF) embryonated chicken eggs or Madin-Darby canine kidney (MDCK) cells, and then followed by haemagglutinin (HA) and neuraminidase (NA) inhibition assays via defined antisera to subtype HA and NA, respectively [11,12]. However, this method is time-consuming and labor-intensive to conduct a large-scale survey of SIV detection [13,14]. Although some immunological assays like immunofluorescent staining and enzyme-linked immunosorbent assay that have been used to detect the viral nucleoprotein antigen provide results more quickly than viral isolation, the sensitivity is not stable while mainly depends on the affinity and compatibility of the antibodies used [14]. By contrast, the real-time RT-PCR (RRT-PCR) method for detection of PCR products in real time through fluorescent dyes or fluorescent labeled specific probes is endowed with all the combined qualities of high sensitivity, specificity and speediness. Especially, the probe with its 3' termini tagged of minor groove binder (MGB) instead of the traditional Tamra as fluorescence quencher has been widely

applied in the detection of various pathogens involving equine herpes virus 5, infectious bursal disease virus, bovine viral diarrhea virus and so on [15-17].

In this study, based on the Taqman-MGB probes targeting conserved HA gene regions, an one-step duplex RRT-PCR method for rapid detection and differentiation of H1 and H3 subtype SIV was successfully established. The sensitivity, specificity, repeatability and the primary clinical applications of the developed assay was also determined.

2. Material And Methods

Primers and probes design

By retrieving the database of Global Initiative on Sharing All Influenza Data (GISAID), a total of 254 swine H1N1 viruses during 2009-2019 and all the 60 swine H3N2 viruses in China (up to 1 Oct, 2019) were downloaded for sequence alignment via MEGA (version 6.06) software. Next, suitable H1 (Fig.1 A) and H3 (Fig.1 B) primer-probe sets targeting the conserved HA gene regions were designed and validated by Primer Express 3.0 software, respectively. The primers and probes used in the study were as follows: H1 forward primer, GGCTAYCATGCBAACAATTC; H1 reverse primer, TGGGTTGCCMAGGATCCA; H1 probe, FAM-ACAGACACTGTMGACACA-MGB; H3 forward primer, GAAATGGGAAAAGCTCAATAATGA; H3 reverse primer, ATTTCTCATYCCTGTTGCCAA; H3 probe, HEX-CCAAATGGAAGCATT-MGB. The amplification length of the H1 and H3 primer-probe sets were 179 bp and 184 bp, respectively.

Reaction composition and thermal cycling

HiScript II U+ One Step qRT-PCR Probe Kit (Vazyme Co., Nanjing, China) was used in our H1 and H3 duplex RRT-PCR assay to achieve the effect of reverse transcription and quantitation in one reaction tube. The H1 and H3 primers and probes were all synthesized by Tsingke Biological Technology Corporation (Nanjing, China). The RRT-PCR assay was operated in a 20 μ L reaction mixture which contains 2x One Step PCR Mix (Vazyme) 10 μ L, Enzyme Mix (Vazyme) 1 μ L, both H1 and H3 probes (10 μ M) each 0.4 μ L, both H1 and H3 forward and reverse primers (10 μ M) each 0.8 μ L, RNA sample 4 μ L, 50x ROX 0.4 μ L and RNase-free water 0.6 μ L. Thermal cycling of the RRT-PCR was performed in an ABI 7500 FAST real-time PCR machine (Applied biosystems). The temperature control steps were as follows. Step 1: 55°C for 15min of reverse transcription, step 2: 95°C for 30s, step 3: 95°C for 30s and then 60°C for 38s with quantitation to maintain 40 cycles.

Standard plasmid for RRT-PCR sensitivity and repeatability determination

Three SIVs of F15, JS38 and SZ14 respectively from EA H1N1, Pdm09 H1N1 and HL H3N2 lineages were used to amplify the HA genes. Each amplified HA gene was then inserted into the pHW2000 vector to construct the plasmid for standard curve plotting and repeatability test of the RRT-PCR assay. In particular, the plasmids mixture was divided into two different sets to represent the duplex detection of either EA H1N1 or Pdm09 H1N1 with HL H3N2, one set contained F15-pHW2000 and SZ14-pHW2000 while the other included JS38-pHW2000 and SZ14-pHW2000. Finally, two plasmid sets were serially diluted to 500000, 50000, 5000, 500, 50, 5 copies/ μ L to perform duplex detection for drawing standard curves.

For testing repeatability, 10-fold concentrations from 50000~50 copies/ μ L of F15-pHW2000 and SZ14-pHW2000 plasmid mixture were selected to conduct inter- and intra-comparison. Specifically, triplicates of the 4 dilutions of plasmids were performed on the same plate within a run while each of the reactions were respectively repeated on 3 separate days between runs of the duplex RRT-PCR assay.

Virus strain for sensitivity and specificity assay

9 strains of SIV isolated by our laboratory during 2010~2019, including 4 EA H1N1, 3 Pdm09 H1N1 and 2 HL H3N2 were used in the study (Table.1) [18,19]. The viruses were all propagated in 9-day-old SPF chicken eggs and then the infected allantoic fluid was harvested to serve as viral seeds. And the value of 50% embryo infectious dose (EID₅₀) was determined for each SIV to express the detection limit of the RRT-PCR method.

Some commonly circulated porcine viruses in China containing classical swine fever virus (CSFV), pseudorabies virus (PRV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus 2 (PCV2), porcine epidemic diarrhea virus (PEDV) and porcine parvovirus (PPV) were also applied to measure the specificity of the RRT-PCR method in clinical application.

Animal experiment

Three groups of 20 six-week-old female BALB/c mice (Yangzhou University Laboratory Animal center, China) were intranasally inoculated with 10^{5.0}EID₅₀/50μL of SG03(EA H1N1), JS285(Pdm09 H1N1) and SZ14 (HL H3N2), respectively. 5 infected mice were humanely euthanized on 1, 3, 5 and 7 days post inoculation (dpi), and the lungs were collected to serve as experimental samples for verifying the detection accuracy of the duplex RRT-PCR method as compared with traditional virus isolation.

Application of RRT-PCR in SIV epidemiological surveillance

A total of 166 swine nasal swabs collecting from one slaughter-house in Yangzhou city of Jiangsu province monthly from October to December, 2019 were treated as the clinical specimen to test the application of the duplex RRT-PCR method. Since the slaughter-house aperiodically harbored pigs from different areas in neighbourhood, we got 54 samples from Yancheng city of Jiangsu province in October, 56 samples from Suzhou city of Anhui province in November and 56 samples from Huaian city of Jiangsu province in December, respectively. These swabs immersed in Dulbecco's modified Eagle's medium (DMEM) were packaged appropriately on ice and transported to the laboratory as soon as possible.

Virus isolation

Homogenated mice lung tissues and filtered swine nasal swabs were inoculated into the allantoic cavities of 9-day-old SPF embryonated chicken eggs. After incubation of 96h, the allantoic fluid was collected for hemagglutinin (HA) assay to determine the potential presence of SIV. And the subtype of the HA positive samples were defined by sequencing of the PCR amplicons with primers according to Hoffman et al [20].

RNA extraction

An EASY spin tissue/cell RNA rapid extraction kit (Yuanpinghao, Tianjing, China) was used to extract RNA. According to the manufacturer's instructions, RNA was prepared from 350μL virus cultures or specimens, and eluted with 30μL DEPC treated water.

3. Results

3.1 Standard curves and amplification plots of RRT-PCR

The amplification plots and standard curves for the two reaction sets including plasmids of EA H1N1 and HL H3N2 (Fig. S1, Panel A) or plasmids of Pdm09 H1N1 and HL H3N2 (Fig.S1, Panel B) were determined under the circumstance of serially

diluting each plasmid into 500000, 50000, 5000, 500, 50 and 5 copies/ μ L, respectively. As shown from the amplification plots in Fig. S1, the detection limit for either duplex detection of EA or Pdm09 H1N1 and HL H3N2 plasmids was 5 copies/ μ L. And the standard curves indicated that the linear correlation for EA H1N1 and HL H3N2 group were $y=-3.38x+36.106$ (Efficiency 97.640%, $R^2=0.999$) and $y=-3.335x+37.406$ (Efficiency 99.470%, $R^2=0.999$) while for Pdm09 H1N1 and HL H3N2 group were $y=-3.301x+35.933$ (Efficiency 100.889%, $R^2=0.997$) and $y=-3.301x+35.977$ (Efficiency 100.885%, $R^2=0.996$), respectively.

3.2 Repeatability evaluation of RRT-PCR

For evaluating the repeatability of the RRT-PCR assay, the plasmid mixture of F15-PHW2000 and SZ14-PHW2000 was serially diluted to 50000, 5000, 500, 50 copies/ μ L and then used to perform the intra- and inter-comparison. As shown in Table 2, the coefficient values (CV) for the duplex detection of H1 and H3 subtype SIV were both < 3% between runs or with a run, which suggested a high repeatability of the method.

3.3 Detection limit of different virus strains

By using different HA subtypes or lineages of SIV strains, the detection limit of the duplex RRT-PCR was also calculated as expressed in EID₅₀/mL. As displayed in Table 1 and Fig. S2, the lowest detection concentrations ranged from 2.1 to 42 EID₅₀/mL for the 4 EA H1N1 viruses (F15, SG03, ZG14, ZG16) and from 21 to 32 EID₅₀/mL for the 3 Pdm09 H1N1 viruses (JS38, JS48, JS285), respectively. Whereas for the 2 HL H3N2 viruses (SZ14, YC44), the positive threshold were both 2.4 EID₅₀/mL. In addition, we also found that 10 times more HL H3N2 viruses than the Pdm09 H1N1 viruses could be detected via the RRT-PCR. However, 10 times more HL H3N2 viruses than the Pdm09 H1N1 viruses could be detected via the RRT-PCR, which might due to the different degree of base degeneracy between the H1 and H3 primer-probe sets.

3.4 Specificity of the primer-probe sets of RRT-PCR

For evaluating the degree of conservation of the designed primer-probe sets, the forward primer, reverse primer and probe for detection of H1 and H3 subtype SIV were initially analyzed via the BLAST tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), respectively. When searching BLAST with the parameter of Max target sequences set to 5000 and the other parameters as default, a total of 2777, 1108 and 4920 H1NX plus 4825,4363 and 4340 H3NX influenza sequences were well matched with the forward primer, reverse primer and probe of H1 and H3, respectively. As shown in Table S1, the H1 primer-probe set matched mainly H1N1 and secondly H1N2 and then some H1N3-N6 influenza viruses while the H3 primer-probe set matched predominantly H3N2 and some H3N1 or H3N2 viruses, respectively. Evidently, no HA subtypes other than H1 or H3 were matched by BLAST tool with the specific primers and probes.

Subsequently, the specificity of the primer-probe sets was further measured with some other commonly-existed swine pathogens in clinical in China, including CSFV, PRV, PRRSV, PCV2, PEDV and PPV. As obviously shown in Fig.S3, just the positive control of H1 and H3 SIVs displayed amplification curves in their individual fluorescence channel (FAM and HEX), whereas the other 6 swine viruses were all detected as negative.

3.5 Detection accuracy of the RRT-PCR in experimental samples

To compare the detection accuracy of the duplex RRT-PCR method with classical virus isolation, lungs from SG03(EA H1N1), JS285(Pdm09 H1N1) and SZ14(HL H3N2) infected mice were treated for RRT-PCR detection and embryo inoculation, respectively. During the 7-day trial period, 5 lung specimens from each group were collected every other day since 1 dpi except that only 3 samples from JS285(Pdm09 H1N1) group were obtained on 7 dpi due to the death of 2 infected mice.

Thus, a total of 58 specimens were used for the test. As shown in Table 3, nearly all the lung samples got consistent results between RRT-PCR and virus isolation but with an exception in the detection of JS285(Pdm09 H1N1) infected mice. Specifically, each of the 5 specimens in the JS285(Pdm09 H1N1) group on 1 dpi were tested positive via RRT-PCR, while just 4 were verified by chicken embryo inoculation with 2 at the first inoculation and the other 2 after another consecutive blind passage. In addition, 3 and 5 samples respectively from SG03(EA H1N1) and SZ14(HL H3N2) infected mice on 7dpi were uniformly determined negative through both the two methods. It was also noteworthy that all the 20 lung tissues collected in either the SG03(EA H1N1) or the SZ14(HL H3N2) group matched an identical positive rate from the RRT-PCR, as compared with the traditional virus isolation. Collectively, the detection accuracies between RRT-PCR and virus isolation for SG03(EA H1N1), JS285(Pdm09 H1N1) and SZ14(HL H3N2) were 100% (20/20), 94.44% (17/18) and 100% (20/20), respectively. And the RRT-PCR method possessed an overall detection sensitivity of 100% and specificity of 91.67% (Table 3).

3.6 Application of the RRT-PCR in SIV surveillance

A total of 166 swine nasal swabs collecting monthly in October (n=54), November (n=56) and December (n=56), 2019 were used for SIV surveillance by applying the RRT-PCR method. As shown in Table 4, both H1 and H3 subtype SIVs were detected in each month. Specifically, 5, 16 and 5 samples were H1 positive while 7, 7 and 2 samples were H3 positive in October, November and December, respectively. Besides, 1, 5 and 0 samples were both H1 and H3 positive in October, November and December, respectively. Thus, the corresponding positive percentages were 9.26%, 28.57%, 8.92% for H1 and 12.96%, 12.50%, 3.57% for H3, and both H1 and H3 positive percentages were 1.85%, 8.93% and 0%. In addition, the overall prevalent rate of H1 (15.66%) was relatively higher than that of H3 (9.64%) subtype SIV.

4. Discussion

In this study, an one-step duplex RRT-PCR with high sensitivity, specificity and repeatability was successfully established and evaluated for detection of H1 and H3 subtype SIVs. As revealed by relevant epidemiological investigations and the recorded items in influenza virus databases, EA H1N1 and Pdm09 H1N1 were the main circulating lineages of H1 subtype SIV in the recent decade [6,19,21,22]. Therefore, we desired to detect either the two H1 lineages in our developed RRT-PCR assay by design of a single primer-probe set targeting to the highly conserved regions of both EA H1N1 and Pdm09 H1N1. Although the dominantly prevalent status of EA H1N1 in China has been more and more evidently since 2011, part or even the whole set of internal genes of Pdm09 H1N1 were yet found to be embedded in epidemic strains of H1 and H3 subtype SIVs [8,23]. Hence, the possibility of the risk that Pdm09 H1N1 virus may revival again in pig herds should not be ignored, and the suitable detection methods are indispensably required.

Before applying the duplex RRT-PCR method in detection of clinical swab samples, we initially evaluated the test accuracy in experimental samples. The 48 samples out of 58 lung tissues of experimentally infected mice were detected positive by RRT-PCR, while the virus isolation succeeded in 47 samples with just 1 specimen missed. Therefore, the RRT-PCR exhibited good sensitivity, especially that lung tissues from SG03(EA H1N1) group and SZ14(HL H3N2) infected mice both obtained 100% detection consistency between the two methods. Of note, in the JS285(Pdm09 H1N1) group, 2 specimens on 1 dpi failed to be detected as HA positive until a second blind-passage in SPF chicken embryos, which alternatively indicated that the RRT-PCR might be more sensitive than virus isolation. Subsequently, the duplex RRT-PCR method was further applied for SIV surveillance in swine nasal swabs from some slaughterhouse in China in 2019. And the three-month data from October to December revealed that H1 and H3 subtype SIVs co-circulated in each month in the investigated area. Particularly, H1 possessed an obviously higher overall positive rate than H3 subtype SIV, which was generally consistent with the results reported by some other research groups in China [6,8].

As possessing higher sensitivity and shorter testing time over traditional virus isolation, more and more RRT-PCR methods have been developed for SIV detection [24,25]. For example, Slomka et al [11] had reported two independent RRT-PCR assays targeting the M gene or the HA gene to detect established SIVs or Pdm09 H1N1 in European pig populations, respectively.

Additionally, to differentiate the Pdm09 H1N1 from other influenza viruses containing classical swine H1, EA H1, HL H1/H3 and avian H9N2 in swine, Hiromoto et al [26] had designed six pairs of HA gene primer-probe sets that were respectively applied in each singleplex RRT-PCR assay. Besides, Nagarajan et al established two tetraplex RRT-PCR methods for the simultaneous detection of common M, H1, H3 SIV genes and N2 SIV genes or H5 genes from Eurasian highly pathogenic avian influenza viruses [27]. Although those RRT-PCR for SIV detection are documented with different advantages, they could hardly differentiate the prevalent lineages of EA H1N1, Pdm09 H1N1 and HL H3N2 in China within a single multiplex reaction.

Our duplex RRT-PCR employed two HA specific primer-probe sets to detect the H1 and H3 subtype SIVs at the same time, especially that the H1 primers and fluorescent probe could simultaneously match the EA H1N1 and Pdm09 H1N1. Besides, the primer-probe sets displayed no mismatch with other HA subtypes influenza viruses via the Primer-BLAST search and no cross reaction with other swine viruses that commonly existed in China. The detection limit of the method could reach 5 copies/ μ L nucleic acids of HA gene or 2.1~42 EID₅₀/mL of SIV, and the CV was lower than 3% in both intra- and inter-assays which indicated good repeatability. Moreover, the RRT-PCR obtained an average sensitivity of 100% and specificity of 91.67% in detection of experimentally infected mice lungs, especially with totally identical results to virus isolation in EA H1N1 and HL H3N2 groups. Therefore, this duplex RRT-PCR for H1 and H3 subtype SIV detection not merely presented high specificity, sensitivity and repeatability but also showed high detection precision rate in the comparison with virus isolation, which indicated a bright application prospect in serving the method as an effective tool for SIV surveillance and offering instructive information for SIV isolation.

Declarations

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This study was performed in strict concordance with the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of People's Republic of China. The animal experiment protocol was approved by Jiangsu Province Administrative Committee for Laboratory Animals (approved NO. SYXK-SU-2017-0044), and complied with the guidelines of Jiangsu laboratory animal welfare and ethics of Jiangsu Administrative Committee of Laboratory Animals.

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Tables

Table 1 The basic information of SIVs used in the study

Virus strain	Abbreviation	Subtype	Lineage	EID ₅₀ /mL	Detection limit (EID ₅₀ /mL)
A/swine/Jiangsu/zg14/2011	ZG14	H1N1	Eurasian avian-like (EA)	10 ^{7.625}	42
A/swine/Jiangsu/zg16/2011	ZG16	H1N1	Eurasian avian-like (EA)	10 ^{7.783}	6.1
A/swine/Hebei/F15ZJK/2017	F15	H1N1	Eurasian avian-like (EA)	10 ^{7.5}	32
A/swine/Shandong/SG03/2019	SG03	H1N1	Eurasian avian-like (EA)	10 ^{6.682}	2.1
A/swine/Jiangsu/38/2010	JS38	H1N1	Pandemic 2009 (Pdm09)	10 ^{7.5}	21
A/swine/Jiangsu/48/2010	JS48	H1N1	Pandemic 2009 (Pdm09)	10 ^{7.5}	32
A/swine/Jiangsu/285/2010	JS285	H1N1	Pandemic 2009 (Pdm09)	10 ^{7.33}	21
A/Swine/Anhui/SZ1014/2019	SZ14	H3N2	Human-like (HL)	10 ^{6.375}	2.4
A/Swine/Jiangsu/YC1044/2019	YC44	H3N2	Human-like (HL)	10 ^{6.318}	2.4

Table 2 Intra-assay and inter-assay comparisons of the H1 and H3 duplex RRT-PCR

Plasmid (copies/ μL)	HA gene	Intra-assay						Inter-assay					
		Ct			Mean	SD	CV (%)	Ct			Mean	SD	CV (%)
		1	2	3				1	2	3			
5x10 ⁴	H1	20.61	20.99	20.74	20.78	0.19	0.93	20.15	20.05	19.98	20.06	0.09	0.43
	H3	22.68	22.82	22.36	22.62	0.24	1.04	20.96	20.39	20.29	20.55	0.36	1.76
5x10 ³	H1	24.15	24.12	24.29	24.19	0.09	0.38	23.54	23.66	23.7	23.63	0.08	0.35
	H3	25.86	25.78	25.84	25.83	0.04	0.16	24.47	23.98	24.09	24.18	0.26	1.06
5x10 ²	H1	27.45	27.50	27.37	27.44	0.07	0.24	26.97	27.56	27.53	27.35	0.33	1.21
	H3	29.24	29.02	28.73	29.00	0.26	0.88	27.93	28.05	27.98	27.99	0.06	0.22
5x10 ¹	H1	30.97	31.06	30.98	31.00	0.05	0.16	30.71	31.24	31.77	31.24	0.53	1.70
	H3	32.67	32.55	32.67	32.63	0.07	0.21	31.97	31.94	31.88	31.93	0.05	0.14

Table 3 Sensitivity and Specificity of the H1 and H3 duplex RRT-PCR as compared with virus isolation in embryonated chicken eggs[†]

Virus	Sample NO.	Detection method			
		RRT-PCR		Virus isolation [‡]	
		Positive	Negative	Positive	Negative
SG03(EA H1N1)	20	17	3	17	3
JS285(Pdm09 H1N1)	18	18	0	15+2 [‡]	1
SZ14(HL H3N2)	20	13	7	13	7
Total	58	48	10	47	11
Sensitivity = 47/(47+0)*100% = 100%					
Specificity = 11/(11+(48-47)) *100% = 91.67 %					

[†] Lung tissues from experimentally challenged BALB/c mice were collected to serve as the testing samples.

[‡] Numbers right of “+” indicated that the samples were initially detected negative at the first inoculation but confirmed positive within 3 consecutive blind passage in embryonated chicken eggs.

Table 4 RRT-PCR detection of swine nasal swabs in one slaughterhouse collected from October to December, 2019

Collection Date	Number	Source of pig herds	H1 positive		H3 positive		H1&H3 positive	
			Number	Rate (%)	Number	Rate (%)	Number	Rate (%)
October 30	54	Yancheng city, Jiangsu province	5	9.26	7	12.96	1	1.85
November 30	56	Suzhou city, Anhui province	16	28.57	7	12.50	5	8.93
December 24	56	Huaian city, Jiangsu province	5	8.92	2	3.57	0	0

Figures

A			Forward primer (5' -3')	Probe(5' -3')	Reverse primer(3' -5')
	Lineage	Genotype	GGCTAYCATGCBAAACAATTC	ACAGACACTGTMGACACA	TGGATCCTKGGCAACCCA
A/swine/Henan/08/2012 (H1N1)	EA	G1
A/swine/Henan/97/2011 (H1N1)	EA	G1
A/swine/Tianjin/184/2011 (H1N1)	EA	G2
A/swine/Tianjin/42/2011 (H1N1)	EA	G2
A/swine/Guangdong/SS1/2012 (H1N1)	EA	G3
A/swine/Guangdong/104/2013 (H1N1)	EA	G3	•• T •..... T •..... • C •.....
A/swine/Jiangsu/J006/2018 (H1N1)	EA	G4
A/swine/Shandong/0337/2017 (H1N1)	EA	G4
A/swine/Guangxi/NNLX/2014 (H1N1)	EA	G4
A/swine/Guangxi/BB1/2013 (H1N1)	EA	G5 • C •.....
A/swine/Guangxi/QZ5/2014 (H1N1)	EA	G5
A/swine/Tianjin/158/2015 (H1N1)	EA	G6
A/swine/Tianjin/78/2015 (H1N1)	EA	G6
A/swine/Jiangsu/48/2010 (H1N1)	Pdm09		•• T •..... • A •• T ••
A/swine/Jiangsu/285/2010 (H1N1)	Pdm09		•• T •..... • A •• T ••

B			Forward primer(5' -3')	Probe (5' -3')	Reverse primer(3' -5')
	Lineage		G A A A T G G G A A A A G C T C A A T A A T G A	C C A A A T G G A A A C A T T	T T G G C A A C A G G R A T G A G A A A T
A/swine/Guangxi/NS2783/2010 (H3N2)	HL		A •• G •.....
A/swine/Hong_Kong/NS2439/2011 (H3N2)	HL		A •• G •.....
A/swine/Hong_Kong/2503/2011 (H3N2)	HL		A •• G •.....
A/swine/HuNan/01/2008 (H3N2)	HL		•• G •..... • C • G ••
A/swine/Eastern_China/S17/2003 (H3N2)	HL		•• G •..... • C • G ••
A/swine/Henan/1/2010 (H3N2)	HL		•• G •..... • C • G ••
A/swine/Guangdong/L5/2010 (H3N2)	HL		•• G •..... • C • G ••
A/swine/Guangdong/L22/2010 (H3N2)	HL		•• G •..... • C • G ••
A/swine/Guangdong/L23/2010 (H3N2)	HL		• C • C •..... • C • G ••
A/swine/Guangxi/NNXD/2013 (H3N2)	HL		A •.....
A/swine/Guangxi/JGB4/2013 (H3N2)	HL		A •.....
A/swine/Jiangsu/P3589/2016 (H3N2)	HL		A • G •.....
A/swine/Jiangsu/POS3593/2016 (H3N2)	HL		A • G •.....
A/swine/Guangxi/JG1/2014 (H3N2)	HL		A •.....

Figure 1

Sequence alignment of the H1 and H3 primer-probe sets with HA genes of representative SIV strains in recent years. (A) Sequence alignment of H1 primer-probe set. (B) Sequence alignment of H3 primer-probe set. The HA genes used for comparison were all downloaded from GISAID or GenBank, including SIV of EA H1N1, Pdm09 H1N1 and HL H3N2 lineages. Additionally, distinct genotypes of EA H1N1 reference viruses were also labelled as G1-G5 according to Sun et al [8]. Nucleotides identical with the primers or probes were replaced by black dots, while the different ones were shown by capital letters.

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