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One-step multiplex RT-qPCR for the detection and subtyping of influenza A virus in swine in Brazil

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ABSTRACT

Pandemic H1N1, human-like H1N2 and H3N2 influenza A (IAV) viruses are co-circulating in swine herds in Brazil. The genetic analysis of the Brazilian IAVs has shown that they are genetically distinct from viruses found in swine in other countries; therefore, an update of the diagnostic assays for IAV detection and subtyping is needed. This study describes the development and validation of a TaqMan based – one-step multiplex RT-qPCR to discriminate the hemagglutinin and neuraminidase genes of the three major IAV subtypes circulating in pigs in Brazil. The RT-qPCR assays presented 100% (95.7–100, CI 95%) of diagnostic sensitivity in the analysis of 85 IAVs, previously characterized by sequencing. The limits of detection ranged from 5.09 × 101 to 5.09 × 103 viral RNA copies/μL. For the analytical specificity, 73 pig samples collected during 2017 and 2018 were analyzed, resulting in the identification of the subtype in 74.0% (62.9–82.7, CI 95%) of samples. From these, 46.3% were H3N2, 33.3% were H1N1, 11.1% were H1N2 and 3.7% were HxN1. Mixed viral infections (3.7%) and reassortant viruses (1.9%) were also detected by the test. This multiplex RT-qPCR assay provides a fast and specific diagnostic tool for identification of different subtypes and lineages of IAV in pigs, contributing to the monitoring of influenza in swine.

1. Introduction

Influenza A virus (IAV) causes an acute respiratory disease in swine, leading to significant economic losses for swine producers worldwide (Zhang et al., 2016). IAV belongs to the *Orthomyxoviridae* family and is characterized by a segmented genome consisting of eight single-stranded RNA molecules with negative polarity (Kawaoka and Neumann, 2012). The surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), are the most immunogenic virus proteins, and determination of the viral subtype is based on the antigenic properties of these two proteins (Nelson and Vincent, 2015). Although 16 antigenically different HAs and nine different NAs are described in the reservoir species (aquatic wild birds and shore birds), IAV subtypes detected in pigs are more restricted (Liu et al., 2009; Medina and García-Sastre, 2011; Neumann and Kawaoka, 2015).

Swine are considered candidates for the generation of pandemic IAVs because they are susceptible to infection with both human and avian IAVs, and allow the occurrence of reassortment events during co-infection of the host cell (Ito et al., 1998; Ma et al., 2009). The accumulation of mutations (antigenic drift), in addition to genomic reassortment (antigenic shift) on the virus genome increases influenza virus diversity over time (Medina and García-Sastre, 2011), leading to difficulties in the laboratory diagnosis, with further need for updates of the molecular-based diagnostic tests and vaccines for the novel circulating IAVs.

Various diagnostic methods have been used to detect IAV in pigs (Janke, 2014). Virus isolation (VI) in embryonated chicken eggs or on Madin-Darby canine kidney (MDCK) cells are considered the gold standard for the diagnosis of IAV infection (Zhang and Gauger, 2014). Additionally, the antigenic characterization of the isolated viruses can be performed by using the hemagglutination inhibition test (HI) (Kitikoon et al., 2014). Genomic sequencing is also employed and does provide more complete information on the origin of the virus, but its use is generally limited for economic reasons and is mostly restricted to research purposes. Although VI and HI tests are considered gold standards for IAV diagnosis, these techniques are time-consuming and labor-intensive. In contrast, molecular techniques, such as RT-qPCR, are fast, sensitive and specific for detection and characterization of IAVs

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(Zhang et al., 2016).

Influenza A viruses H1N1, H1N2 and H3N2 are the three major subtypes circulating in the swine populations around the world (Lewis et al., 2016; Nelson et al., 2015b). Although virus subtypes circulating in swine in different countries are similar, they are genetically and antigenically distinct (Kuntz-Simon and Madec, 2009; Vincent et al., 2014). Recently, Nelson et al. (2015a) have shown that influenza viruses isolated from swine in Brazil are genetically distinct from IAVs circulating in swine in other countries. Consequently, the development of diagnostic assays, able to identify locally adapted IAV strains, is important for the monitoring and control of influenza in swine. Additionally, one of the OIE/FAO Network of expertise on animal influenza (OFFLU) research priorities on influenza in swine is the development of molecular diagnostic tests using more specific primers for the identification of the major virus lineages (OFFLU Steering Committee, 2014, 2011).

The aim of this study was to develop and validate a one-step multiplex RT-qPCR assay for the rapid identification of different subtypes and lineages of IAVs circulating in swine in Brazil, therefore contributing to the surveillance of IAV in pigs.

2. Material and methods

2.1. Primers and probes design

Nucleotide sequences of the HA and NA genes, obtained from IAVs isolated from swine samples in Brazil, were selected from the Influenza Virus Sequence Database (https://www.ncbi.nlm.nih.gov/genomes/FLU). A total of 46 H1 sequences from pandemic/2009 virus (H1pdm), 21 H1 sequences from seasonal human influenza virus origin (H1hu), 13 H3 sequences, 50 N1 sequences from pandemic/2009 virus (N1pdm), two N1 sequences from seasonal human influenza virus origin (N1hu) and 36 N2 sequences were selected. The target regions were determined for each gene segment (H1pdm, H1hu, H3, N1pdm, N1hu and N2) using BLASTn (Altschul et al., 1990) to find conserved regions within each gene segment. Primers and probes for each gene segment were designed by Primer Express software (Applied Biosystems). The sequences of primers and probes are listed in Table 1.

2.2. Production of standard RNAs for absolute quantification

Viral RNA was extracted from IAVs isolated from swine lung using MagMAX Viral RNA Isolation Kit (AMB18365, Ambion) and RT-PCR products were generated for each target gene (HA – H1pdm, H1hu, H3; and NA – N1pdm, N1hu, N2) using the primers sets described in Table 2. The amplicons were cloned into pCR2.1 TOPO vector (TOPO TA Cloning Kit, 450641, Invitrogen) and plasmid DNAs were transformed into One Shot TOP10 Chemically Competent *Escherichia coli* cells (C404003, Invitrogen), following the manufacturer's recommendations. Plasmid DNAs were purified by PureYield Plasmid Miniprep System Kit (A1222, Promega) and positive clones, containing the targeted sequences, were confirmed by PCR and subsequent DNA sequencing using M13 primers (M13 Forward (-20), N52002, Invitrogen; M13 Reverse, N53002, Invitrogen). Plasmids containing the target genes were linearized and used in the *in vitro* transcription (MEGAscript T7 High Yield Transcription Kit, AM1333, Ambion). The obtained RNAs were quantified (Qubit RNA HS Assay Kit, Q32852, Invitrogen), the number of viral RNA copies were calculated (Fey et al., 2004) and tenfold serial dilutions of each standard were prepared.

2.3. Standardization and performance evaluation of the one-step multiplex RT-qPCR

The RT-qPCR assay was performed in two distinct reactions; one reaction was focused on the HA gene (H1pdm, H1hu and H3) and the other reaction was focused on the NA gene (N1pdm, N1hu and N2). The standard RNAs for each gene segment were used in the standardization of both assays by using the AgPath-ID One-Step RT-PCR Kit (4387391, Ambion). The RT-qPCR assays were performed in a reaction volume of 25 µL. For the standardization of the HA and NA reactions, different concentrations of buffer solution (0.5, 0.75 and 1X), forward and reverse primers (80, 120, 160, 200, 240, 280, 320, 360, 400 and 440 nM)

Table 1
Sets of primers and probes sequences for the one-step multiplex RT-qPCR (HA and NA gene segments).

Specific primers and probes	Primers and probes sequences (5' → 3')	Position	Fragment (bp)
H1pdm[a]_F[e]	CACAAAWTTGAGACTGGYMACA	1007 – 1028	101
H1pdm_R[f]	CTGTCCAYCCYCCTTCAAT	1107 – 1089	
H1pdm_Probe	FAM-CCTATTTGGRGCCATTGCYGGTT-QSY	1064 – 1086	
H1hu[b,c]_F	GGTTTGTTTGGWGCCATTGC	1062 – 1081	106
H1hu_R	CAGCATAVCCAGAYCCTTGC	1167 – 1148	
H1hu_Probe	VIC-TTCATTGAAGGRGGDTGGACTGGAAT-QSY	1086 – 1111	
H3[d]_F	GTTGGTAYGGTTTCAGGCATC	1115 – 1135	93
H3_R	TCCCAYTGATTTGGTCRATTG	1207 – 1187	
H3_Probe	NED-CAAGCWGCAGAYCTTAAAAGYACTCAAGCA-QSY	1156 – 1185	
N1pdm[a]_F	GAGGARTGYTCYTGYTATCCTGA	849 – 871	89
N1pdm_R	AAAGACACCCAHGGYCGRTT	937 – 918	
N1pdm_Probe	FAM-ATGTGTRTGCAGGGATAACTGGCATGG-QSY	887 – 913	
N1hu[c]_F	CCGATGGCCCGAGTAATG	748 – 765	97
N1hu_R	TGGAAATTGGGTGCATTTAACTC	844 – 822	
N1hu_Probe	NED-CCGCCTCGTACAAGATCTTCAAGATCGA-QSY	769 – 796	
N2[b,d]_F	GGGTRTYCCRTTTCAYTTGGGAA	508 – 530	123
N2_R	CTGGCRGTTGCATTTTYATCATG	630 – 608	
N2_Probe	VIC-CAAGTGTGYATDGCATGGTCCAGYTCAA-QSY	536 – 563	
SPUD[g]_F	AACTTGGCTTTAATGGACCTCCA	449 – 471	
SPUD_R	ACATTCATCCTTACATGGCACCA	549 – 527	101
SPUD_Probe	Cy5-TGCACAAGCTATGGAACACCACGT-BHQ2	482 – 505	

[a] Pandemic H1N1.
[b] Human-like H1N2.
[c] Human-like H1N1.
[d] Human-like H3N2.
[e] Forward primer.
[f] Reverse primer.
[g] Described by Nolan et al., 2006.

Table 2
Sets of primers sequences for the production of standard RNAs (H1pdm, H1hu, H3, N1pdm, N1hu and N2) for absolute quantification.

Specific primers	Primers sequences (5′ → 3′)	Position	PCR products (bp)	Reference strain
H1pdm[a]_F[e]	ATGCTGGATCTGGTATTATCATT	859 – 881	411	A/swine/Brazil/25-15/2015 (H1N1) MH559931
H1pdm_R[f]	ACTCTTTGCCTACTGCTGTG	1269 – 1250		
H1hu[b,c]_F	AACAGCAGTCTTCCTTTCCA	939 – 958	295	A/swine/Brazil/223-15-1/2015 (H1N2) MH560035
H1hu_R	TCTCAATTACAGAATTCACCTTGTT	1233 – 1209		
H3[d]_F	ACAGGGATGCGGAATGTACC	1021 – 1040	344	A/swine/Brazil/28-15-8/2015 (H3N2) MH559963
H3_R	TCCAGGGCAACAAGAAGCTC	1364 – 1345		
N1pdm[a]_F	TAATGACCGATGGACCAAGT	742 – 761	364	A/swine/Brazil/25-15/2015 (H1N1) MH559933
N1pdm_R	GTTCTCCCTATCCAAACACCA	1105 – 1085		
N1hu[c]_F	ACAAGAGTCTGAATGTGTCTGC	698 – 719	295	A/swine/Brazil/103-14-2/2014 (H1N1) MG572190
N1hu_R	ATCACCGAATACTCCACTGC	992 – 973		
N2[b,d]_F	ACAACAGGCATTCAAATAACACA	441 – 463	389	A/swine/Brazil/28-15-8/2015 (H3N2) MH559965
N2_R	CCCTGACAGTTGGCTAATATGGA	829 – 807		

[a] Pandemic H1N1.
[b] Human-like H1N2.
[c] Human-like H1N1.
[d] Human-like H3N2.
[e] Forward primer.
[f] Reverse primer.

Table 3
Standardized protocol for the one-step multiplex RT-qPCR (HA and NA).

One-step multiplex RT-qPCR for HA	
Component	**Concentration in the reaction**
Nuclease-free water	1.36μL
2X RT-PCR buffer*	0.75X
Detection enhancer	1.67μL
Forward primer for H1pdm	200nM
Reverse primer for H1pdm	200nM
H1pdm probe	48nM
Forward primer for H1hu	160nM
Reverse primer for H1hu	160nM
H1hu probe	48nM
Forward primer for H3	200nM
Reverse primer for H3	200nM
H3 probe	60nM
Forward primer for SPUD	200nM
Reverse primer for SPUD	200nM
SPUD probe	60nM
25X RT-PCR enzyme mix	1X
SPUD DNA	1.0μL
RNA sample	5.0μL
Total volume per reaction	**25.0μL**
One-step multiplex RT-qPCR for NA	
Component	**Concentration in the reaction**
Nuclease-free water	0.06μL
2X RT-PCR buffer*	0.75X
Detection enhancer	1.67μL
Forward primer for N1pdm	400nM
Reverse primer for N1pdm	400nM
N1pdm probe	132nM
Forward primer for N1hu	200nM
Reverse primer for N1hu	200nM
N1hu probe	60nM
Forward primer for N2	200nM
Reverse Primer for N2	200nM
N2 probe	48nM
Forward primer for SPUD	120nM
Reverse primer for SPUD	120nM
SPUD probe	36nM
25X RT-PCR enzyme mix	1X
SPUD DNA	1.0μL
RNA sample	5.0μL
Total volume per reaction	**25.0μL**

* Buffer solution contains ROX passive reference dye.

and probes (24, 36, 48, 60, 72, 84, 96, 108, 120, 132 and 144 nM) were evaluated. Additionally, an internal control (SPUD) (Nolan et al., 2006) was included in the assays, but the probe was modified with the label Cy5 on 5′ and BHQ-2 on 3′ (Table 1). The runs were executed on an ABI 7500 Real-Time PCR System (Applied Biosystems).

To determine the limit of detection (LOD) for both RT-qPCR assays, tenfold dilutions of each standard RNA, containing 5.09×10^8 down to $5.09 \times 10^{\circ}$ viral RNA copies per microliter, were also analyzed. The standard curves were determined by plotting the logarithm of the standard RNA copies number against the measured quantification cycle (Cq) values.

The repeatability (intra-assay variance) and reproducibility (inter-assay variance) of the assays were assessed using tenfold dilutions of the standard RNAs for HA (H1pdm, H1hu and H3) and for NA (N1pdm, N1hu and N2). The assays were performed in triplicate and in three different runs to evaluate the coefficients of variation (CVs) of both assays. Intra- and inter-assay CVs based on the Cq values were calculated by Statistical Analysis System software (SAS, 2012).

2.4. Analytical and diagnostic specificity of the one-step multiplex RT-qPCR

For the evaluation of the analytical specificity for both assays, 85 IAV strains isolated from swine from 2009 to 2016, and previously characterized by genomic sequencing, were selected for testing. In addition, to verify the diagnostic specificity of the assays, 50 clinical samples collected from pigs and considered negative for IAV by RT-PCR (matrix (M) gene) (Fouchier et al., 2000) and positive for other swine pathogens as porcine circovirus type 2, porcine circovirus type 3, porcine parvovirus type 1, senecavirus A, *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica*, *Haemophilus parasuis*, *Mycoplasma hyopneumoniae*, *Pasteurella multocida* and Streptococcus *suis* were also selected to be tested by the one-step multiplex RT-qPCR.

2.5. Evaluation of clinical samples by the one-step multiplex RT-qPCR

Seventy-three clinical samples (34 nasal swabs and 39 lung tissues samples) collected from pigs during 2017 and 2018, and previously diagnosed as positive for IAV by RT-PCR (M gene) (Fouchier et al., 2000), were selected. Viral RNA was extracted using MagMAX Viral RNA Isolation Kit (AMB18365, Ambion) according to the manufacturer's recommendations, and the samples were run on the one-step multiplex RT-qPCR assays developed in this study.

Table 4
Intra- and inter-assay variability of the one-step multiplex RT-qPCR for HA and for NA gene segments.

Gene segment	Viral RNA copies/μL	Mean Cq	Intra-assay		Inter-assay	
			CV (%)	SD	CV (%)	SD
H1pdm	5.09×10^8	6.96	2.08	0.14	10.12	0.70
	5.09×10^7	11.78	2.72	0.32	6.86	0.80
	5.09×10^6	16.18	1.92	0.31	5.39	0.87
	5.09×10^5	20.56	1.21	0.24	2.79	0.57
	5.09×10^4	25.28	2.24	0.56	4.74	1.19
	5.09×10^3	31.11	1.84	0.57	5.30	1.65
	5.09×10^2	37.15	0.63	0.23	1.67	0.62
H1hu	5.09×10^8	12.64	2.00	0.25	1.78	0.22
	5.09×10^7	16.21	2.81	0.45	2.23	0.36
	5.09×10^6	21.10	1.35	0.28	1.86	0.39
	5.09×10^5	25.13	1.02	0.25	1.04	0.26
	5.09×10^4	30.70	0.55	0.16	3.11	0.95
	5.09×10^3	35.86	0.63	0.22	1.87	0.67
H3	5.09×10^8	8.18	2.28	0.18	1.98	0.16
	5.09×10^7	12.20	1.91	0.23	2.55	0.31
	5.09×10^6	16.41	1.84	0.30	1.16	0.19
	5.09×10^5	20.60	1.72	0.35	1.09	0.22
	5.09×10^4	24.88	1.33	0.33	1.90	0.47
	5.09×10^3	30.21	1.19	0.36	4.32	1.30
	5.09×10^2	36.43	0.71	0.25	1.24	0.45
N1pdm	5.09×10^8	5.79	2.94	0.17	2.82	0.16
	5.09×10^7	10.25	2.27	0.23	9.01	0.92
	5.09×10^6	14.55	2.00	0.29	1.59	0.23
	5.09×10^5	18.57	1.50	0.27	1.31	0.24
	5.09×10^4	22.71	0.87	0.19	3.12	0.70
	5.09×10^3	28.08	2.44	0.68	4.53	1.27
	5.09×10^2	36.19	1.41	0.51	3.77	1.36
N1hu	5.09×10^8	7.97	0.42	0.03	0.28	0.02
	5.09×10^7	11.76	0.64	0.07	0.58	0.06
	5.09×10^6	15.41	0.34	0.05	0.72	0.11
	5.09×10^5	18.66	0.17	0.03	0.51	0.09
	5.09×10^4	22.24	0.27	0.06	0.10	0.02
	5.09×10^3	25.32	0.19	0.04	0.38	0.09
	5.09×10^2	29.04	0.31	0.09	1.02	0.29
	5.09×10^1	36.53	1.43	0.52	0.27	0.10
N2	5.09×10^8	8.31	2.57	0.21	3.66	0.30
	5.09×10^7	12.76	1.60	0.20	0.90	0.11
	5.09×10^6	17.03	0.64	0.10	3.29	0.56
	5.09×10^5	20.65	1.01	0.20	1.47	0.30
	5.09×10^4	25.43	2.04	0.51	2.06	0.52
	5.09×10^3	29.51	0.32	0.09	3.06	0.90
	5.09×10^2	34.94	0.57	0.19	2.67	0.93

Cq = quantification cycle.
CV = coefficient of variation.
SD = standard deviation.

3. Results

3.1. Standardized protocol for the one-step multiplex RT-qPCR

The standardized protocol for the amplification of HA and NA gene segments is shown in Table 3. The detection enhancer was included in the reactions to minimize the formation of secondary structures. Cycling parameters for both reactions (HA and NA) were established as follows: reverse transcription at 45 °C for 10 min, followed by initial denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 30 s. Collection of fluorescence signal was during the annealing step and the threshold was automatically set.

3.2. Performance of the one-step multiplex RT-qPCR

The Cq values for both HA and NA segments are shown in Table 4. For the HA segment, the Cq values ranged from 6.96 to 37.15 cycles with a linear correlation (R^2) of 0.99 between the Cq value and the logarithm of the standard RNA copies number. For the NA segment, the Cq values ranged from 5.79 to 36.53 cycles with a R^2 of 0.99. The internal control (SPUD) was consistently detected with a Cq of ˜25.

The intra- and inter-assay CVs of both one-step multiplex RT-qPCR assays are shown in Table 4. The LOD for the HA segment was as follows: 5.09×10^2 viral RNA copies/μL for H1pdm, 5.09×10^3 for H1hu, and 5.09×10^2 for H3. In addition, the LOD for the NA segment was as follows: 5.09×10^2 viral RNA copies/μL for N1pdm, 5.09×10^1 for N1hu and 5.09×10^2 for N2.

Table 5
Diagnostic sensitivity and analytical specificity of sequenced/isolated strains and clinical field samples, respectively, by one-step multiplex RT-qPCR.

Subtype/lineage	Sequenced/isolated strains	Clinical field samples
pandemic H1N1	45	18
human-like H1N2	17	6
human-like H3N2	11	25
H1pdmN2[r]	3	1
human-like H1N1[r]	3	0
pandemic H1N1 + human-like H3N2	4	0
human-like H1N2 + human-like H3N2	1	0
human-like H3N2 + H1pdmN2[r]	1	0
pandemic H1N1 + human-like H1N2	0	1
pandemic H1N1 + H1pdmN2[r]	0	1
HxN1pdm	0	2
Not subtyped	**0**	**19**
Total	**85**	**73**

pdm = pandemic.
[r] Reassortant virus.

3.3. Analytical and diagnostic specificity of the one-step multiplex RT-qPCR on viral isolates

A high analytical specificity (100%; 95.7–100.0, CI 95%), with the correct assignment of IAV subtypes and viral lineages, was demonstrated by using the RT-qPCR assays in the analysis of 85 IAV isolates previously characterized by genomic sequencing. The Cq values ranged from 12.54 to 25.47 cycles. From these 85 IAV isolates, 53.0% were classified as pandemic H1N1, 20.0% as human-like H1N2, 13.0% as human-like H3N2, 3.5% as human-like H1N1, 3.5% as H1pdmN2, and, co-infection with two virus subtypes was detected in 7.0% of the samples (Table 5).

The diagnostic specificity of the RT-qPCR assays was of 100% (92.9–100.0, CI 95%), resulting in the absence of fluorescence signal for other viral and bacterial swine pathogens.

3.4. Evaluation of clinical samples by the one-step multiplex RT-qPCR

The viral subtype and the lineage of origin were determined in 54 out of 73 samples (74.0%; 62.9–82.7, CI 95%), as follows: 46.3% were derived from the seasonal human influenza H3N2 virus, 33.3% were pandemic H1N1, 11.1% were derived from the seasonal human influenza H1N2 virus and 1.9% were a reassortant H1pdmN2. Co-infection with two virus subtypes was detected in two out of 54 (3.7%) samples. In two (3.7%) samples, only the NA gene was identified (HxN1pdm) (Table 5). In general, the Cq values ranged from 16.22 to 39.98 cycles. For 26.0% (17.3–37.1, CI 95%) of clinical samples, the virus subtype was not determined.

4. Discussion

A high genetic diversity of IAVs circulating in swine in most pork producing countries has been described (Lewis et al., 2016; Vincent et al., 2014). Since the genetic characteristics of IAVs in swine are related to their geographic distribution (Nelson et al., 2015b; Simon et al.,

2014), specific diagnostic methods to detect locally adapted strains are necessary to monitor the circulation of influenza viruses in pigs. Currently, there is no commercially available diagnostic test in Brazil to differentiate the IAV subtypes circulating in pigs. The only test available in diagnostic laboratories for IAV detection is a RT-PCR targeting the influenza virus matrix (M) gene. However, such RT-PCR is not able to differentiate the viral subtypes and lineages or to detect mixed infections or neither to infer if IAVs are reassortants. In this sense, the determination of the virus subtype and probable origin of HA and NA genes may complement the diagnosis and is extremely important to trace back the spread of such viruses in the swine population. In this study, we evaluated two one-step multiplex RT-qPCR assays to differentiate the main IAV subtypes circulating in swine in Brazil *i.e.* pandemic H1N1, human-like H1N2 and H3N2 (Nelson et al., 2015a) and potential reassortants that may be originated from these viruses. Besides, a recently isolated H1N1 virus, derived from a seasonal human influenza virus, was also detected by using the RT-qPCR developed here (unpublished data). The RT-qPCR assays provided good repeatability and reproducibility, represented by the low values of CVs of intra- and inter-assays at every viral RNA concentration of the standard curves, indicating a good linearity. In addition, there was an excellent level of agreement (100%; 95.7–100.0, CI 95%) between the results provided by the multiplex RT-qPCR and genomic sequencing, in relation to detection of IAV subtypes and the virus lineage designation. In our study, cross-reactivity with other swine pathogens was not observed, confirming a high (100%; 92.9–100.0, CI 95%) diagnostic specificity of the assays.

The assay performance was evaluated in the analysis of 73 samples collected from pigs during 2017 and 2018, and previously considered positive to IAV by RT-PCR (M gene) (Fouchier et al., 2000). The virus subtype was determined in 74.0% (62.9–82.7, CI 95%) of samples. Although the limits of detection of the assays were high (ranging from 5.09×10^1 to 5.09×10^3 viral RNA copies per microliter), the subtype could not be determined in 26.0% (17.3–37.1, CI 95%) clinical samples. However, for 3.7% samples, only the NA segment was determined by the NA assay. This could be explained by the higher limit of detection for the NA segment compared to the limit of detection for the HA segment. The lack of ability to determine the virus subtype in 19 out of 73 clinical samples is probably due to the presence of low viral loads (below the limits of detection of the assays). In addition, despite the primers sets and probes have being designed for a conserved region of HA and NA genes, high mutation rate is observed on those segments, which could also lead to difficulties in the subtype determination of these viruses. Also, even in rare events, other IAV subtypes than H1N1, H1N2 and H3N2 can infect pigs (Landolt and Olsen, 2007; Zell et al., 2013). Such situation could also hinder virus subtyping, and, for this reason, a periodic update of those reagents is necessary in order to detect new emerging viruses. Previous reports on the molecular detection and IAVs subtyping have been published, with different sensitivities and specificities. Chiapponi et al. (2012) tested 27 clinical samples from pigs, with unsuccessful virus isolation, and determined the viral subtype in 56% of the cases.

Distinct influenza virus lineages circulate in different geographic regions. Therefore, tests based on the virus lineages are available for subtyping of IAVs in swine in North America (Choi et al., 2002; Nagarajan et al., 2010; Richt et al., 2004), Asia (Fu et al., 2010; Lee et al., 2008) and Europe (Bonin et al., 2018; Chiapponi et al., 2012; Henritzi et al., 2016). Although various multiplex RT-PCRs have been described, it is difficult to compare the results due to the particularities displayed on the experimental design and steps applied for the standardization of the assay. In this study, we used previously sequenced IAVs and negative IAV samples for the assay validation and afterwards, the assay was tested on clinical samples. Usually, RT-PCR assays described in the literature identify only the virus subtype which is present in the sample, however, the assays developed in this study also provide the identification of the HA and NA virus lineage (pandemic H1N1, human-like H1N1, H1N2 or H3N2). Additionally, the test was able to detect mixed viral infections and reassortant viruses, even before performing genomic sequencing, providing the basis for an improved early detection of novel viruses in swine. Subsequently, the genomic sequencing confirmed the presence of reassortant viruses, as well as it confirmed the virus lineages present in the samples (data not shown).

The emergence of pandemic H1N1 in 2009, followed by reassortment events among H1N1pdm and enzootic IAVs circulating in the swine population has increased the genetic diversity of IAVs (Bonin et al., 2018). Due to the possibility of transmission of IAVs from pigs to humans, the monitoring and surveillance of circulating viruses in swine herds is highly recommended, as a way to quickly identify emerging viruses (Schmidt et al., 2016; Vincent et al., 2014). An accurate and rapid diagnosis and characterization of IAVs in swine is important to control pig-to-pig and pig-to-human transmission (Zhang and Harmon, 2014). In this sense, the RT-qPCR developed and validated in this study showed to be a highly sensitive and specific method to discriminate the viral subtypes circulating in Brazilian swine herds and once implemented in diagnostic laboratories, it will provide more information on the prevalence of IAV subtypes in swine herds.

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Competing interests

The authors declare that they have no competing interests.

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