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Short Communication

Development and Evaluation of New Real-Time RT-PCR Assays for Identifying the Influenza A Virus Cluster IV H3N2 Variant

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SUMMARY: From 2005 to July 6, 2018, a total of 435 swine-origin influenza A H3N2 variant virus (H3N2v) infections in humans were reported in the USA. The largest H3N2v outbreak in the USA occurred in 2011–2012. This virus obtained the HA gene from the human seasonal H3N2 influenza A viruses (seasonal H3N2) via human-to-swine transmission in the mid-1990s and was classified as Cluster IV H3N2v. For early detection of public health threats associated with Cluster IV H3N2v distinct from seasonal H3N2, we developed highly specific and sensitive one-step real-time RT-PCR assays directly targeting the HA genes of Cluster IV H3N2v and seasonal H3N2. These assays are useful for the systematic surveillance and identification of Cluster IV H3N2v.

In the USA, 435 cases of human infection with swine-origin influenza A H3N2 viruses, designated influenza A H3N2 variant viruses (H3N2v), were reported between 2005 and July 6, 2018 (1). In 2011–2012, the largest H3N2v human outbreak occurred, involving 321 laboratory-confirmed cases; this raised concerns over a possible pandemic if efficient person-to-person transmission emerged. The majority of these H3N2v carried an HA gene obtained from the human seasonal influenza A H3N2 virus (seasonal H3N2), which was introduced in North American swine in the mid-1990s and classified as Cluster IV H3N2v (2–4). Human infections from swine carrying a novel H3N2v containing the HA gene from 2010–2011 human seasonal H3N2 strains, classified as human-like H3 viruses (human-like H3), were recently reported (5,6). Although hospitalization and death occur in some cases of H3N2v infection, the symptoms are usually mild to moderate, making H3N2v clinically indistinguishable from seasonal influenza. Consequently, for early detection of public health threats associated with H3N2v, systematic surveillance and discrimination between H3N2v and seasonal H3N2 are very important. Cluster IV H3N2v continue to circulate in swine in the USA, causing sporadic human infections (7–10). To distinguish Cluster IV H3N2v from seasonal H3N2, in this study, we developed real-time RT-PCR (rRT-PCR) assays directly targeting the HA genes of Cluster IV H3N2v and seasonal H3N2.

Primers and TaqMan MGB probes for the specific detection of the HA genes of Cluster IV H3N2v or seasonal H3N2 were designed from the conserved HA1 subunit regions (Table 1). Because our previously developed seasonal H3N2 rRT-PCR assay exhibited cross-reactivity with Cluster IV H3N2v (Table 2), new primer and probe set for distinguishing current seasonal H3N2 from Cluster IV H3N2v were also designed (11). To evaluate the sensitivity of both assays, 2 in vitro transcribed full-length H3 HA RNAs were synthesized from A/Indiana/09/2012 (Cluster IV H3N2v) (GISAID accession no. EPI378523) and A/Texas/50/2012 (seasonal H3N2) (EPI391247), as described previously (12). The Cluster IV H3N2v and seasonal H3N2 rRT-PCR assays were performed using AgPath-ID™ One-Step RT-PCR reagents (Thermo Fisher Scientific, Waltham, MA, USA) in 25-μL reaction mixtures containing 12.5 μL of 2 × RT-PCR Buffer, 1 μL of 25 × RT-PCR Enzyme Mix, 0.1 μL (20 U) of RNase Inhibitor (Thermo Fisher Scientific), forward and reverse primers (each 600 nM), probe (100 nM), and 5 μL of RNA template. The assays were carried out using a LightCycler® 480 II (Roche, Basel, Switzerland) under the following conditions: 50°C for 10 min, 95°C for 10 min, and 45 cycles of 15 s at 95°C, 30 s at 56°C, and 15 s at 72°C (13). Analytical sensitivity was assessed by testing 10-fold serial dilutions from 5.0×10^7 copies/reaction of in vitro transcribed full-length RNAs in 6 replicates. Standard curves for both assays are shown in Fig. 1. The detection limit of the Cluster IV H3N2v and seasonal H3N2 rRT-PCR assays was 6.0 and 3.0 copies per reaction by probit regression analysis, respectively, with a 95% probability endpoint. The R^2 value was 0.99 for both assays, and the standard curve slopes in the range of 5.0 to 5.0×10^7 copies per reaction were −3.26 and −3.22, respectively. Therefore, both assays exhibited good linearity and high sensitivity.

Assay specificity was evaluated using RNAs extracted from 4 Cluster IV H3N2v and 6 seasonal H3N2 viruses along with 8 clinical nasal swab or aspirate specimens of seasonal H3N2 obtained between 2014 and 2017 from patients with respiratory symptoms. Viral RNA was extracted using a QIAamp® Viral RNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Crossing point (Cp) values of positive results for both assays are shown in Table 2. The Cluster IV H3N2v rRT-PCR assay was positive for all Cluster IV

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Table 1. Primers and probes used in the Cluster IV H3N2v and seasonal H3N2 rRT-PCR assays

Name	Sequence (5′–3′)[1)]	Position[2)]	Product size (bp)
NIID-Cluster IV H3 TMPrimer-F1	AGCCAACAAACTGTAATCCCGARTATCG	673–700	98
NIID-Cluster IV H3 TMPrimer-R1	TCTCCYGGTTTTACTATCGTCCA	748–770	
NIID-Cluster IV H3 Probe1	(FAM)TATGCTGGAKACACCCCTTAC(MGB)	715–735	
NIID-seasonal H3 TMPrimer-F1	GGGGGTTCACCACCCGGGTACRGAC	588–612	89
NIID-seasonal H3 TMPrimer-R1	GGCTTCTTTTGGTRGATACTGTGA	653–676	
NIID-seasonal H3 Probe1	(FAM)TTGAGCATACAGGAAGATTTGGTCC(MGB)	615–639	

1): Probe labeled with FAM at the 5′ end and MGB at the 3′ end.
2): Nucleotide numbering is based on the HA gene CDS of A/Indiana/09/2012 (Cluster IV H3N2v) for swine or A/Texas/50/2012 (seasonal H3N2) for human.

Table 2. Comparison of the established Cluster IV H3N2v and seasonal H3N2 rRT-PCR assays and existing TypeA rRT-PCR assay

	Samples	Subtype	Cluster	TypeA	Cluster IV H3N2v	seasonal H3N2	Former H3N2
Virus	A/Minnesota/11/2010 (X-203)	H3N2v	Cluster IV	17.46[1)]	17.31	ND	17.96
	A/Indiana/08/2011	H3N2v	Cluster IV	23.03	22.70	ND	22.99
	A/Indiana/10/2011 (X-213)	H3N2v	Cluster IV	18.17	17.22	ND	17.96
	A/Ohio/13/2012	H3N2v	Cluster IV	18.66	19.71	ND	20.30
	A/Uruguay/716/2007	H3N2	seasonal	17.09	ND	17.06	16.20
	A/Victoria/361/2011	H3N2	seasonal	13.94	ND	14.11	13.67
	A/New York/39/2012	H3N2	seasonal	15.83	ND	15.50	14.97
	A/Switzerland/9715293/2013	H3N2	seasonal	20.85	ND	20.53	20.18
	A/Singapore/INFIMH-16-0019/2016	H3N2	seasonal	19.35	ND	19.83	20.04
	A/Ibaraki/N12691/2017	H3N2	seasonal	16.61	ND	15.79	16.69
Clinical specimens	F14-53	H3N2	seasonal	22.45	ND	22.12	NT
	F15-7	H3N2	seasonal	22.23	ND	21.86	NT
	F16-17	H3N2	seasonal	21.92	ND	22.02	NT
	F16-93	H3N2	seasonal	19.21	ND	18.48	NT
	F16-96	H3N2	seasonal	20.47	ND	19.99	NT
	F16-99	H3N2	seasonal	18.69	ND	19.00	NT
	F17-14	H3N2	seasonal	25.00	ND	24.94	NT
	F17-26	H3N2	seasonal	21.31	ND	21.26	NT

1): Cp values were determined using the second derivative maximum method.
ND, not detected; NT, not tested.

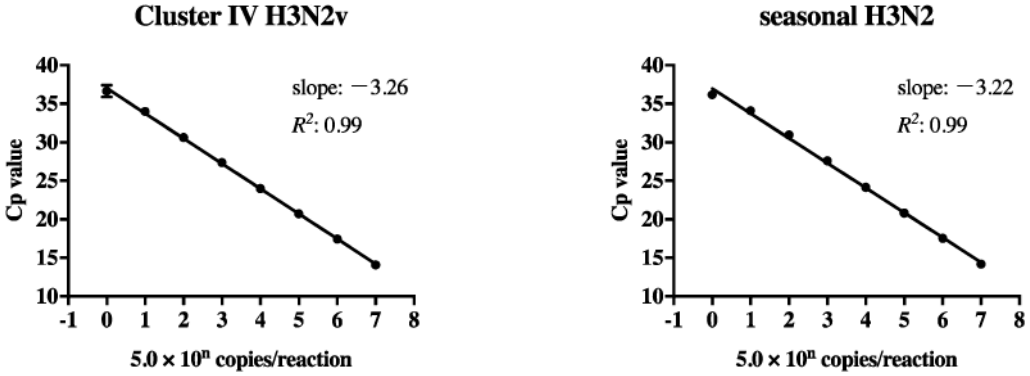

Fig 1. Dynamic range of the Cluster IV H3N2v and seasonal H3N2 rRT-PCR assays. Standard curves (Cp value vs. $\log_{10}$ concentration) for serial dilutions of in vitro transcribed HA gene RNAs generated using average Cp values from 6 replicates. The correlation coefficient (R^2) and slope are shown. A/Indiana/09/2012 (Cluster IV H3N2v) and A/Texas/50/2012 (seasonal H3N2) were used as templates for the Cluster IV H3N2v and seasonal H3N2 rRT-PCR assays, respectively.

H3N2v RNAs but for none of the seasonal H3N2 RNAs or the clinical specimen RNAs. The seasonal H3N2 rRT-PCR assay was positive for all seasonal H3N2 RNAs and clinical specimen RNAs but for none of the Cluster IV H3N2v RNAs. The TypeA rRT-PCR assay targeting the highly conserved matrix gene common to all influenza A viruses was used as a comparative control (11). The differences in Cp values between the Cluster IV H3N2v and TypeA and seasonal H3N2 and TypeA rRT-PCR assays were < 1.05 and < 0.73, respectively. Both

assays exhibited high sensitivity similar to the TypeA rRT-PCR assay. The sequences of the HA gene of the human-like H3 including primers and probe regions, were very similar to those of the current seasonal H3N2. The developed seasonal H3N2 assay system detected HA genes of both seasonal H3N2 and human-like H3, whereas the Cluster IV H3N2v assay system detected only the Cluster IV H3N2v HA gene.

Both rRT-PCR assays did not react to any of the RNAs extracted from 25 representative subtypes (H1–H15, except for H3) of influenza A viruses, 3 influenza B viruses, 6 clinical specimens including H1N1pdm09 or influenza B viruses (data not shown). Moreover, these assays did not react to any of the RNAs extracted from 19 other viral respiratory pathogens (respiratory syncytial virus A and B; human parainfluenza virus type 1, 2, 3, 4a, and 4b; human rhinovirus type A and B; human metapneumovirus type A1 and B2; human coronavirus OC43, 229E, NL63, and HKU1; human bocavirus; human enterovirus; and human adenovirus 2 and 4) (data not shown). Consequently, both rRT-PCR assays exhibited high specificity against the respective HA genes of the Cluster IV H3N2v and seasonal H3N2.

Although human-like H3 has become the predominant H3N2 among swine in the USA, Cluster IV H3N2v is still maintained; that is, the threat to public health associated with Cluster IV H3N2v remains (10). Our previously developed rRT-PCR assays for discriminating influenza virus types, subtypes, and lineages can be performed under the same conditions as the assays developed in this study (11,13–15). Hence, by combining the previous and newly developed assays, Cluster IV H3N2v can be directly identified with high sensitivity and specificity for diagnosis and monitoring without sequencing the H3 HA gene.

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Conflict of interest None to declare.

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