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Validation of two multiplex real-time PCR assays based on single nucleotide polymorphisms of the *HA1* gene of equine influenza A virus in order to differentiate between clade 1 and clade 2 Florida sublineage isolates

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Abstract. We validated 2 multiplex real-time PCR (rtPCR) assays based on single nucleotide polymorphisms (SNPs) of the hemagglutinin-1 (*HA1*) gene of H3N8 equine influenza A virus (EIV) to determine clade affiliation of prototype and field isolates. Initial validation of the 2 multiplex rtPCR assays (SNP1 and SNP2) was performed using nucleic acid from 14 EIV Florida sublineage clade 1 and 2 prototype strains. We included in our study previously banked EIV rtPCR-positive nasal secretions from 341 horses collected across the United States in 2012–2017 to determine their clade affiliation. All 14 EIV prototype strains were identified correctly as either Florida sublineage clade 1 or clade 2 using the 2 SNP target positions. Of 341 EIV rtPCR-positive samples, 337 (98.8%) and 4 (1.2%) isolates were classified as belonging to clade 1 and 2 Florida sublineage EIV, respectively. All clade 1 Florida sublineage EIV strains were detected in domestic horses, three clade 2 Florida sublineage EIV strains originated from horses recently imported into the United States, and one clade 2 Florida sublineage EIV strain originated from a healthy horse recently vaccinated with a modified-live intranasal EIV vaccine containing the American lineage strain A/eq/Kentucky/1991. EIV Florida sublineage clade differentiation using a fast and reliable multiplex rtPCR platform will help monitor the introduction of clade 2 Florida sublineage EIV strains into North America via international transportation.

Key words: Clade affiliation; equine influenza virus; horses; influenza A virus; multiplex rtPCR.

Contemporary equine influenza A virus (EIV; species *Influenza A virus*) strains circulating worldwide all belong to the H3N8 Florida sublineage (http://www.oie.int/our-scientific-expertise/specific-information-and-recommendations/equine-influenza). EIV surveillance data have shown that Florida sublineage viruses from both clade 1 and 2 circulate in Europe, whereas clade 1 viruses have been primarily reported from North America.[3,6,7,10,11] Although only clade 1 is considered endemic in North America, clade 2 EIV has been detected in horses imported into the United States.[1,12] Contemporary information on EIV clade affiliation in the United States is important not only from an epidemiologic standpoint but also to monitor and understand EIV outbreaks and update EIV vaccine strains in order to improve protection against clinical disease. In past years, clade affiliation of EIV strains has been monitored using conventional nucleic acid sequencing techniques, and more recently pyrosequencing, both of which are time- and labor-intensive.

Multiplex real-time PCR (rtPCR) assays for allelic discrimination have gained popularity in the equine infectious field to quickly and accurately discriminate between closely related viruses or genotypes of the same virus.[2,4,5,16,17] Multiplex rtPCR assays are fast, highly sensitive and specific, and do not require post-amplification steps. Sanger dideoxynucleotide sequencing is still considered the method of choice for determining whole genome sequences and performing phylogenetic analysis.[13] From an epidemiologic standpoint, it is important to determine

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Table 1. Primers and probes used to validate 2 equine influenza A virus Florida sublineage clade-specific multiplex real-time PCR assays (SNP1 and SNP2) based on 2 SNPs of the *HA1* gene.

Primers and probes	Sequence 5'–3'	Clade affiliation
SNP1-224 forward	GATGCAATGCTAGGAGACCCC	
SNP1-297 reverse	CTGCTTCTTTCTATAAAGAGGTCCCA	
SNP1-248 probe FAM	TGTGACGCCTTTCAGTAT	Clade 1
SNP1-248 probe VIC	TGTGACGTCTTTCAGTAT	Clade 2
SNP2-467 forward	AGCCGACTGAATTGGCTAACA	
SNP2-528 reverse	GGCATTGTCACATTCAATGTGG	
SNP2-490 probe FAM	ATCCGGAAGCTCTTA	Clade 1
SNP2-490 probe VIC	ATCCGGAAACTCTTA	Clade 2

Shaded nucleotides show the molecular differences between clade 1 and clade 2 equine influenza A virus. rtPCR = real-time PCR; SNP = single nucleotide polymorphism.

clade affiliation in real-time to monitor the introduction of clade 2 into North America via international transportation, monitor genetic drift of EIV, and monitor vaccine effectiveness. In an attempt to retain test accuracy, to further reduce turnaround time, and to monitor the occurrence of EIV clades in the United States, we established and validated a novel multiplex rtPCR assay targeting previously reported single nucleotide polymorphisms (SNPs) of the hemagglutinin-1 (*HA1*) gene of EIV.

Published sequences from GenBank (NCBI) of the *HA1* gene of EIV clade 1 and 2 strains were subjected to BLAST analysis. These aligned sequences of clade 1 and 2 EIV strains were used to design primers and probes unique to each clade. Two SNPs along the *HA1* gene (SNP1 and SNP2) were identified, and assays were developed to allow discrimination between clade 1 and 2 Florida sublineage EIV strains (Table 1). The fluorophore used for clade 1 was FAM and for clade 2 was VIC. All rtPCR assays were designed using the primer express software (Thermo Fisher Scientific, Foster City, CA) following the guidelines proposed for designing multiplex rtPCR assays. The optimum primer temperature was 58°C, and the temperature of the rtPCR probes was 10°C higher to encourage the probe to remain bound. Real-time PCR probes and primers were synthesized by Life Technologies (Grand Island, NY). The homology of the primers and rtPCR probes was confirmed using a BLAST search against the nonredundant database of GenBank.

A total of 341 EIV rtPCR-positive samples (nasal secretions) collected in 2012–2017 (20 samples in 2012, 37 samples in 2013, 22 samples in 2014, 73 samples in 2015, 52 samples in 2016, and 137 samples in 2017) and kept frozen at −80°C were available for testing. Eighty-five EIV rtPCR-positive samples had been used previously to establish and validate a pyrosequencing assay able to differentiate between clade 1 and clade 2 Florida sublineage EIV strains.[1] Further, to assess the efficiency of multiplex rtPCR assays for the 2 EIV clades, analyses in triplicate were performed using Florida sublineage clade 1 (A/eq/Kentucky/1/2012, A/eq/New Hampshire/1/2013, A/eq/Ohio/1/2013, A/eq/Kentucky/1/2014, A/eq/Ohio/1/2014, A/eq/Kentucky/3/2015, A/eq/Minnesota/1/2015, A/eq/Illinois/1/2016, A/eq/New York/1/2016) and clade 2 (A/eq/Newmarket/5/2003, A/eq/Richmond/2007, A/eq/Shropshire/2010, A/eq/Devon/2011, A/eq/East Renfrewshire/2011), and one Eurasian lineage (A/eq/Aboyne/1/2005) EIV prototype strains.

All samples were processed for nucleic acid purification using an automated nucleic acid extraction system (CAS-1820 X-tractor Gene, Corbett Life Science, Mortlake, Australia) according to the manufacturer's recommendations. The QuantiTect reverse transcription kit (Qiagen, Valencia, CA) was used for complementary DNA (cDNA) synthesis following the manufacturer's directions with the following modifications. Ten microliters of RNA were digested with 1 µL of genomic DNA (gDNA) Wipe-Out buffer (Qiagen) by incubation at 42°C for 5 min and then briefly centrifuged. Genomic DNA contamination was tested by using 1 µL of digested RNA and running the rtPCR reference gene equine glyceraldehyde-3-phosphate dehydrogenase (*eGAPDH*). Then 0.5 µL of QuantiTect reverse transcriptase (Qiagen), 2 µL of QuantiTect RT buffer (Qiagen), 0.5 µL of RT primer mix (Qiagen), and 0.5 µL of 20 pmol random primers (Invitrogen, Carlsbad, CA) were added, brought up to a final volume of 20 µL, and incubated at 42°C for 40 min. The samples were inactivated at 95°C for 3 min, chilled, and 80 µL of water was added. Complementary DNA from nasal secretions was assayed for the presence of EIV using the 2 validated multiplex rtPCR assays.

The multiplex rtPCR primer–probe mixes were prepared by mixing 40 µL of the 100 pmol/µL forward primer, 40 µL of the 100 pmol/µL reverse primer, and 4 µL each of the two 100 pmol/µL probes in a final volume of 240 µL of water. The rtPCR reaction contained 0.42 µL of water, 0.58 µL of primer–probe mix (final concentration of 800 nM for each primer and 80 nM for the probe), 6 µL of commercial PCR master mix (Thermo Fisher Scientific), and 5 µL of the

Table 2. Validation results for 2 clade-specific multiplex rtPCR assays (SNP1 and SNP2) using selected EIV Florida sublineage clade 1 and 2 prototype strains.

EIV prototype strain	rtPCR result from SNP1	rtPCR result from SNP2
A/eq/Kentucky/1/2012 (clade 1)	Clade 1	Clade 1
A/eq/New Hampshire/1/2013 (clade 1)	Clade 1	Clade 1
A/eq/Ohio/1/2013 (clade 1)	Clade 1	Clade 1
A/eq/Kentucky/1/2014 (clade 1)	Clade 1	Clade 1
A/eq/Ohio/1/2014 (clade 1)	Clade 1	Clade 1
A/eq/Kentucky/3/2015 (clade 1)	Clade 1	Clade 1
A/eq/Minnesota/1/2015 (clade 1)	Clade 1	Clade 1
A/eq/Illinois/1/2016 (clade 1)	Clade 1	Clade 1
A/eq/New York/1/2016 (clade 1)	Clade 1	Clade 1
A/eq/Newmarket/5/2003 (clade 2)	Clade 2	Clade 2
A/eq/Richmond/2007 (clade 2)	Clade 2	Clade 2
A/eq/Shropshire/2010 (clade 2)	Clade 2	Clade 2
A/eq/Devon/2011 (clade 2)	Clade 2	Clade 2
A/eq/East Renfrewshire/2011 (clade 2)	Clade 2	Clade 2
A/eq/Aboyne/1/2005 (Eurasian)	Negative	Clade 2

EIV = equine influenza A virus; rtPCR = real-time PCR; SNP = single nucleotide polymorphism.

HA1 nucleotide positions	281/282
SNP1 clade 1	GATGCAATGCTAGGAGACCCCCACTGTGACGCCTTTCAGTATGAGAATTGGGACCTCTTTATAGAAAGAAGCAG
SNP1 clade 2	GATGCAATGCTAGGAGACCCCCACTGTGACGTCTTTCAGTATGAGAATTGGGACCTCTTTATAGAAAGAAGCAG
A/eq/Aboyne/1/2005	GATGCAATGCTAGGAGACCCCCATTGTGATGATTTTCAGTATGAGAGTTGGGACCTCTTCATAGAAAGAAGCAG
A/eq/Kentucky/91	GATGCAATGCTAGGAGACCCCCACTGTGACGTCTTTCAGTATGAGAATTGGGACCTCTTTATAGAAAGAAGCAG
HA1 nucleotide positions	**524/525**
SNP2 clade 1	AGCCGACTGAATTGGCTAACAAAATCCGGAAGCTCTTACCCCACATTGAATGTGACAATGCC
SNP2 clade 2	AGCCGACTGAATTGGCTAACAAAATCCGGAAACTCTTATCCCACATTGAATGTGACAATGCC
A/eq/Aboyne/1/2005	AGCAGACTGAACTGGCTAACAAAATCTGGAAATTCTTACCCCACATTAAATGTGACAATGCC
A/eq/Kentucky/91	AGCCGACTGAATTGGCTAACAAAATCCGGAAACTCTTATCCCACATTGAATGTGACAATGCC

Figure 1. Nucleotide alignment of primers and probes for single nucleotide polymorphism (SNP)1 and SNP2 multiplex real-time PCR assays and partial nucleotide sequence of A/eq/Aboyne/1/2005 and A/eq/Kentucky/1991. Forward primer is highlighted in yellow, reverse primer in green, and probes are in blue. Nucleotide differences at positions 281/282 and 524/525 are in boldface.

cDNA in a final volume of 12 µL. The samples were placed in a 384-well plate and amplified (7900HT Fast real-time PCR system, Thermo Fisher Scientific) using the manufacturer's standard amplification conditions (2 min at 50°C, 10 min at 95°C, then 40 cycles of 15 s at 95°C and 60 s at 60°C). Fluorescent signals were collected during the annealing temperature, and the cycle threshold (Ct) was calculated and exported with a threshold of 0.1 and a baseline of 3–12. The Ct was defined as the cycle in which there was a significant increase in reporter signal, above the threshold. The 384-well plate was also analyzed using allelic discrimination with a 2-time point change in fluorescence. An X-Y graph was plotted with each allele on an axis. All contemporary EIV Florida sublineage clade 1 and 2 prototype strains were correctly identified using both multiplex rtPCR assays (Table 2). The Eurasian lineage prototype A/eq/Aboyne/1/2005 could not be definitively classified based on the 2 rtPCR multiplex assays. The multiplex rtPCR assay for SNP1 yielded a negative result; the multiplex rtPCR assay for SNP2 yielded a clade 2 result for A/eq/Aboyne/1/2005. Nucleotide sequence alignment of A/eq/Aboyne/1/2005 with the primers and probes of SNP1 and SNP2 multiplex rtPCR assays showed 2 nucleotide mismatches at position 281/282 and 524/525 of the *HA1* gene, respectively (Fig. 1).

Of the 341 EIV rtPCR-positive samples, 337 (98.8%) were classified as Florida sublineage clade 1 EIV strains; 4 (1.2%) were classified as Florida sublineage clade 2 EIV strains (Table 3). All EIV clade 1 Florida sublineage strains originated from domestic horses; 3 clade 2 Florida sublineage strains were from horses imported from Europe (2 horses from Germany and 1 horse from France). Two of the 3 horses originated from the same location in southern California and were diagnosed with equine influenza in March of 2012, whereas the third horse was located in Virginia at the time it was diagnosed with EIV (October 2014). One additional healthy horse, vaccinated with a modified-live virus (MLV) intranasal EIV vaccine (Flu Avert I.N., Merck

Table 3. Equine influenza A virus Florida sublineage clade affiliation of 307 nasal secretions collected from 2012 to 2017.

Year	No. of samples	Clade 1	Clade 2
2012	20	18	2
2013	37	36	0
2014	22	22	1
2015	73	73	0
2016	52	52	0
2017	137	136	1
Total	341	337 (98.8%)	4 (1.2%)

Animal Health, Omaha, NE) 24 h prior to collection of nasal secretions tested positive by rtPCR for Florida sublineage clade 2 EIV. This horse was part of a clade 1 Florida sublineage EIV outbreak in December 2017 and sampled because of biosecurity protocols (i.e., healthy horses were sampled in order to monitor potential exposure to the outbreak EIV strain).

Using a panel of contemporary isolated and characterized clade 1 and clade 2 Florida sublineage EIV strains, the 2 multiplex rtPCR assays yielded 3 outcomes: clade 1, clade 2, and discrepant results between SNP1 and SNP2 assays. Fourteen of 15 (93%) isolates were properly classified as clade 1 or clade 2 Florida sublineage EIV. Only one EIV Eurasian lineage isolate (A/eq/Aboyne/1/2005) gave equivocal results, prompting further sequence comparisons. The inability of the SNP1 multiplex rtPCR assay to detect A/eq/Aboyne/1/2005 was related to nucleotide mismatches at position 281/282 of the *HA1* gene, preventing the binding of either of the 2 PCR probes. For SNP2, the single nucleotide mismatch at position 525 of the *HA1* gene still allowed the annealing of 1 of the 2 PCR probes, and classified this strain as a clade 2 Florida sublineage EIV. Although the 2 multiplex rtPCR assays were initially designed to reliably classify clade affiliation, it appears that this technology will also be able to monitor EIV strains with discrepant results between the 2 SNP multiplex assays. The OIE expert surveillance panel on EIV reported that all viruses isolated and characterized in 2017 were from clades 1 and 2 of the Florida sublineage, and that Eurasian lineage and Kentucky and South America sublineage EIV isolates were presumed not to circulate any longer. However, one must keep in mind that global surveillance of equine influenza is not 100%, and other viruses not belonging to the Florida sublineage clade 1 or 2 may still be circulating undetected. Genetic characterization of contemporary EIV isolates is key to establishing vaccine strain recommendations, and it is not the goal of the multiplex SNP assays to replace in-depth genetic characterization of EIV strains. Discrepant results should always prompt the laboratory to further investigate the genetic makeup of the EIV strain through sequencing of the *HA1* gene, thereby monitoring genetic drift.

Our results on clade affiliation for EIV strains collected from 341 horses with equine influenza showed that clade 1 has predominated in the United States during the last 6 y. This is in agreement with 2016 and 2018 studies,[1,14] and also with the 2017 annual report of the OIE expert surveillance panel (http://www.oie.int/our-scientific-expertise/specific-information-and-recommendations/equine-influenza). A 2014 study has shown that the A/eq/Kentucky/91 cold-adapted intranasal EIV vaccine strain can be detected via rtPCR for up to 5 d in recently vaccinated horses.[9] Although the A/eq/Kentucky/91 strain is an American lineage strain, the nucleotide sequence at the level of both SNPs classified this strain as clade 2 Florida sublineage EIV. This means that the 2 assays able to detect Florida sublineage clade 2 EIV strains will also detect other American lineage viruses of the non-Florida sublineage. Although clade 2 Florida sublineage EIV strains do not appear to have yet become endemic in the United States, it is important to consider vaccination (especially in the face of recent use of a MLV intranasal vaccine) and travel history in any horse testing rtPCR-positive for Florida sublineage clade 2 EIV. Given that international transportation of horses has previously been shown to represent a major risk for influenza epidemics,[8,15] it is important that resident horses be protected against the eventual introduction of contemporary EIV strains. The OIE expert surveillance panel on equine influenza vaccines supports the inclusion of both Florida sublineage clade 1 and clade 2 EIV isolates to help prevent failure of vaccine efficacy (http://www.oie.int/our-scientific-expertise/specific-information-and-recommendations/equine-influenza). Fortunately, various EIV vaccines containing both clade 1 and clade 2 Florida sublineage EIV strains are commercially available in the United States.

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Declaration of conflicting interests

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References

1. Bernardino PN, et al. Pyrosequencing as a fast and reliable tool to determine clade affiliation for equine influenza A virus. J Vet Diagn Invest 2016;28:323–326.

2. Brault AC, et al. Multiplex qRT-PCR for the detection of Western equine encephalomyelitis, St. Louis encephalitis, and West Nile viral RNA in mosquito pools (Diptera: *Culicidae*). J Med Entomol 2015;52:491–499.
3. Bryant NA, et al. Isolation and characterisation of equine influenza viruses (H3N8) from Europe and North America from 2008 to 2009. Vet Microbiol 2011;147:19–27.
4. Cook RF, et al. Development of a multiplex real-time reverse transcriptase-polymerase chain reaction for equine infectious anemia virus (EIAV). J Virol Methods 2002;105:171–179.
5. Diallo IS, et al. Multiplex real-time PCR for the detection and differentiation of equid herpesvirus 1 (EHV-1) and equid herpesvirus 4 (EHV-4). Vet Microbiol 2007;123:93–103.
6. Fougerolle S, et al. Genetic evolution of equine influenza virus strains (H3N8) isolated in France from 1967 to 2015 and the implications of several potential pathogenic factors. Virology 2017;505:210–217.
7. Gildea S, et al. The molecular epidemiology of equine influenza in Ireland from 2007–2010 and its international significance. Equine Vet J 2012;44:387–389.
8. Guthrie AJ, et al. The circumstances surrounding the outbreak and spread of equine influenza in South Africa. Rev Sci Tech 1999;18:179–185.
9. Harms C, et al. Detection of modified-live equine intranasal vaccine pathogens in adult horses using quantitative PCR. Vet Rec 2014;175:510.
10. Legrand LJ, et al. Genetic evolution of equine influenza strains isolated in France from 2005 to 2010. Equine Vet J 2015;47:207–211.
11. Olguin Perglione C, et al. Molecular epidemiology and spatio-temporal dynamics of the H3N8 equine influenza virus in South America. Pathogens 2016;5:4.
12. Pusterla N, et al. Detection of clade 2 equine influenza virus in an adult horse recently imported to the USA. Equine Vet Educ 2014;26:453–455.
13. Rash A, et al. Evolution and divergence of H3N8 equine influenza viruses circulating in the United Kingdom from 2013 to 2017. Pathogens 2017;6:6.
14. Sreenivasan CC, et al. Phylogenetic analysis and characterization of a sporadic isolate of equine influenza A H3N8 from an unvaccinated horse in 2018. Viruses 2018;10:31.
15. Watson J, et al. The 2007 outbreak of equine influenza in Australia: lessons learned for international trade in horses. Rev Sci Tech 2011;30:87–93.
16. Weyer CT, et al. Development of three triplex real-time reverse transcription PCR assays for the qualitative molecular typing of the nine serotypes of African horse sickness virus. J Virol Methods 2015;223:69–74.
17. Wilson WC, et al. Field evaluation of a multiplex real-time reverse transcription polymerase chain reaction assay for detection of vesicular stomatitis virus. J Vet Diagn Invest 2009;21:179–186.