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Development and Evaluation of One-Step TaqMan Real-Time Reverse Transcription-PCR Assays Targeting Nucleoprotein, Matrix, and Hemagglutinin Genes of Equine Influenza Virus▽‡

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The objective of this study was to develop and evaluate new TaqMan real-time reverse transcription-PCR (rRT-PCR) assays by the use of the minor groove binding probe to detect a wide range of equine influenza virus (EIV) strains comprising both subtypes of the virus (H3N8 and H7N7). A total of eight rRT-PCR assays were developed, targeting the nucleoprotein (NP), matrix (M), and hemagglutinin (HA) genes of the two EIV subtypes. None of the eight assays cross-reacted with any of the other known equine respiratory viruses. Three rRT-PCR assays (EqFlu NP, M, and HA3) which can detect strains of the H3N8 subtype were evaluated using nasal swabs received for routine diagnosis and swabs collected from experimentally inoculated horses. All three rRT-PCR assays have greater specificity and sensitivity than virus isolation by egg inoculation (93%, 89%, and 87% sensitivity for EqFlu NP, EqFlu M, and EqFlu HA3 assays, respectively). These assays had analytical sensitivities of ≥10 EIV RNA molecules. Comparison of the sensitivities of rRT-PCR assays targeting the NP and M genes of both subtypes with egg inoculation and the Directigen Flu A test clearly shows that molecular assays provide the highest sensitivity. The EqFlu HA7 assay targeting the H7 HA gene is highly specific for the H7N7 subtype of EIV. It should enable highly reliable surveillance for the H7N7 subtype, which is thought to be extinct or possibly still circulating at a very low level in nature. The assays that we developed provide a fast and reliable means of EIV diagnosis and subtype identification of EIV subtypes.

Equine influenza (EI) is an acute, highly contagious viral respiratory disease of equids (horses, donkeys, mules, and zebras) caused by infection with type A influenza virus (27). Equine influenza virus (EIV) possesses a segmented (eight segments), single-stranded RNA genome of negative sense. The eight gene segments encode at least 10 polypeptides: two envelope glycoproteins (hemagglutinin [HA] and neuraminidase [NA]), two matrix proteins (M1 and M2), two nonstructural proteins (NS1 and NS2/nuclear export protein), three proteins that make up the viral RNA polymerase (PB1, PB2 and PA), and the nucleoprotein (NP). Some strains of EIV also express a recently discovered PB1-F2 mitochondrial protein (8, 45). The first strain of EIV isolated in 1956 was of H7N7 configuration and was designated influenza virus A/equine/Prague/56 (38, 43). The last confirmed outbreak caused by an H7N7 subtype in horses was recorded in 1979 (40, 43). A second EIV subtype, H3N8, was first isolated in 1963 and designated influenza virus A/equine/Miami/63 (40, 42). This subtype has been associated with all confirmed outbreaks of equine influenza since 1980. Extensive antigenic drift has been detected in this virus over the years (4, 5, 12, 21, 29, 31, 41). This led to the categorization of H3N8 EIV isolates from around the world into two lineages—American and Eurasian (5, 11). Currently, equine H3N8 influenza virus continues to be the most important equine respiratory pathogen of horses in many countries around the world. Equine influenza is considered endemic in the United States, the United Kingdom, and many other European countries (44). New Zealand and Iceland are the only countries that have remained continuously free of equine influenza. In 2005, interspecies transmission of H3N8 EIV from horse to dog was reported for the first time (10).

Influenza H3N8 virus spreads rapidly in susceptible horses and can result in very high morbidity within 24 to 48 h after exposure to the virus. Outbreaks of clinically mild forms of influenza or subclinical infections have been reported among vaccinated horses that are incompletely protected. Furthermore, many of the clinical signs of EI resemble those caused by other equine viral respiratory pathogens, such as equine herpesviruses 1 and 4 (EHV-1 and EHV-4), equine arteritis virus, equine rhinitis viruses A and B, and equine adenoviruses (32, 33). In light of its clinical similarity to other equine respiratory diseases, a provisional diagnosis of equine influenza must be confirmed by laboratory testing. The need to achieve a rapid diagnosis and to implement effective quarantine and movement restrictions is critical in controlling the spread of EI.

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Traditionally, the gold-standard laboratory test for the diagnosis of EI was attempted virus isolation (VI) from nasal swabs/washings in embryonated hens' eggs (28). Following isolation, the virus was subtyped by means of the hemagglutination-inhibition test, using sera specific for the H3N8 or H7N7 subtype. These methods are time-consuming and cumbersome. In the past decade, antigen detection immunoassays, such as the Directigen Flu A test kit (Becton-Dickinson, Sparks, MD) and nucleic acid amplification-based assays (standard reverse transcription-PCR [RT-PCR] or real-time RT-PCR [rRT-PCR]), were developed and evaluated by various groups (7, 13, 15, 16, 26, 30, 34, 35, 46). The antigen detection immunoassay kits are designed to detect the NP of both influenza A and B viruses (7, 40). Of the commercially available antigen detection immunoassays, the Directigen Flu A test has been used for some considerable time to detect EIV in nasal swabs by certain laboratories (7, 46). While this assay has been found to be most useful as an initial screening test to confirm a diagnosis of EI during an outbreak, its limited sensitivity does not make it an ideal method for the diagnosis of EIV infection on an individual animal basis (34). There have been several reports of the use of RT-PCR assays for the detection of influenza virus in clinical specimens (13–15, 30); however, such assays were not widely used for the routine diagnosis of this disease. This changed, however, following the introduction of EI into Australia in 2007, when an rRT-PCR developed to detect the avian influenza virus matrix gene was used as the molecular diagnostic method of choice for EI (15, 39). That country now requires RT-PCR testing for EIV as part of both the preentry and postentry systems of quarantine and testing of horses from countries where EIV is endemic (6). The objective of the current study was to develop several TaqMan rRT-PCR assays capable of detecting a wide range of EIV strains comprising both subtypes of EIV without the inherent problems associated with the current laboratory diagnosis of EI. The approach taken was to develop new rRT-PCR assays by the use of a TaqMan minor groove binding (MGB) probe targeting the NP, M, H3, and H7 HA genes of the virus. MGB rRT-PCR assays targeting the NP and M genes of EIV have not been previously reported. The assays were developed using the subtype prototype strains of EIV and then evaluated using archived strains of EIV and clinical specimens. The overall goal was to identify which of these assays would be of greatest value for confirmation of a diagnosis of this infection.

MATERIALS AND METHODS

Viruses. EIV strains A/equine/Prague/56, A/equine/Alaska/91, A/equine/Kentucky/81, and A/equine/Miami/63 were obtained from the National Veterinary Service Laboratories (NVSL), Ames, IA (see Table S1 in the supplemental material). Six EIV isolates, A/equine/New York/73, A/equine/Kentucky/02, A/equine/Ohio/03, A/equine/Newmarket/2/93, A/equine/Aboyne/05, and A/equine/Richmond/07, were obtained from the World Organization for Animal Health (OIE) reference laboratory for EI at the Gluck Equine Research Center, University of Kentucky. In addition, 13 previously confirmed EIV isolates from the Livestock Disease Diagnostic Center, University of Kentucky, and the Animal Health Diagnostic Center, New York State College of Veterinary Medicine, Cornell University, were also included in the study. In order to determine the specificity of the rRT-PCR assays, other equine viral pathogens were also included in the study: the reference Bucyrus strain of equine arteritis virus, EHV-1, EHV-2, EHV-3, EHV-4, EHV-5, equine rhinitis viruses A and B, equine adenoviruses 1 and 2, and Salem virus.

Clinical samples. A total of 211 archived nasal swabs from horses experimentally inoculated with the EIV A/equine/Kentucky/02 strain and 149 archived nasal swabs (field samples) submitted to the OIE reference laboratory for routine EI diagnostic testing were included in the study. The 149 field samples included 48 nasal swab samples collected from horses in preexport quarantine, and the remaining 101 samples were collected from horses with evidence of respiratory disease where EI was suspected. Field samples were submitted as swabs and transported at 4°C to the OIE reference laboratory for EI at the Gluck Equine Research Center. Each nasal swab from the field was resuspended in 2.5 ml of phosphate-buffered saline (pH 7.5) and stored at 4°C. The 211 archived nasal samples were collected from 25 horses that were experimentally challenged with the A/equine/Kentucky/02 strain of EIV. The samples were collected from days 1 to 8 postexposure. The remaining 11 samples were collected from 11 horses 3 days prior to experimental exposure to the virus. The nasal swabs from experimentally challenged horses were placed in 5 ml of transport medium (phosphate-buffered saline containing 10% glycerol, 1 mg/ml gentamicin together with 8 IU/ml of penicillin, 8 μg/ml of streptomycin, and 0.02 IU/ml of amphotericin B [Invitrogen, Carlsbad, CA]) and stored at 4°C.

VI. All the samples submitted for attempted VI were processed within 24 h of collection at the OIE reference laboratory for EI at the Gluck Equine Research Center. The 149 nasal swabs collected in the field between 2007 and 2009 and the 211 nasal swabs from an experimental horse challenge study were inoculated into the allantoic cavity of embryonated hens' eggs and harvested as described previously (28).

Viral nucleic acid isolation. Viral nucleic acid was isolated from archived nasal swabs (stored at −80°C), allantoic fluid (AF) of EIV isolates, or tissue culture fluid containing other equine respiratory viruses by the use of a commercial kit (Macherey-Nagel NucleoSpin 8 virus kit; Bethlehem, PA) and an XTR-1820 automatic nucleic acid extraction machine (Qiagen Inc., Santa Clara, CA). The rRT-PCRs were set up with a CAS-1200 machine (Qiagen Inc., Santa Clara, CA). Briefly, 140 μl of each sample was used for nucleic acid extraction according to the manufacturer's instructions. The viral nucleic acid was eluted in 60 μl of nuclease-free water and stored at −80°C.

Primers and probes. The primers and probes used in this study were designed using Primer Express software v3.0 (Applied Biosystems, Forest City, CA). Three primer and TaqMan MGB probe sets (EqFlu NP, EqFlu M, and EqFlu HA3) targeting conserved regions of the NP, M, and H3 HA genes were designed after aligning 17, 21, and 79 sequences of the H3N8 subtype of EIV, respectively, from GenBank (Table 1). The EqFlu HA3-Mia was designed to target the H3 HA gene, particularly that of the A/equine/Miami/63 strain. Three primer and probe sets (EqFlu NP-Pra, EqFlu M-Pra and EqFlu HA7-Pra) were designed solely based on the NP, M, and H7 HA genes of the A/equine/Prague/56 strain, respectively. In addition, the EqFlu HA7 primer and probe set was designed to target a highly conserved region after aligning 12 H7 HA gene sequences available in GenBank.

One-step real-time RT-PCR. A one-tube TaqMan rRT-PCR assay was performed using the TaqMan one-step RT-PCR master mix in a 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA) as described previously (23). Each rRT-PCR run included a control without RNA (containing the reaction mixture with 5 μl of water [no template control]) and positive controls containing in vitro-transcribed (IVT) RNA.

IVT RNA synthesis and determination of analytical sensitivity of rRT-PCR assays. The viral nucleic acid extracted from the H3N8 strains A/equine/Miami/63 and A/equine/Kentucky/02 was used as the template for amplification of the NP, M, and H3 HA genes for IVT RNA generation. Briefly, these genes were RT-PCR amplified using forward and reverse primers flanking the target gene sequence in rRT-PCR assays (see Table S2 in the supplemental material). The RT-PCR products were gel purified and cloned into the pDrive cloning vector according to the manufacturer's instructions (Qiagen PCR cloning kit; Qiagen Inc., Santa Clara, CA). The plasmids were purified using a commercial kit (QIAamp miniprep kit; Qiagen Inc., Santa Clara, CA). The authenticity of each RT-PCR product was confirmed by sequencing both strands of DNA. Following sequencing, the recombinant plasmids with the NP, M, and H3 HA genes from two different EIV H3N8 strains (A/equine/Miami/63 and A/equine/Kentucky/02) were used to generate IVT RNA. Runoff RNA transcripts were generated from BamHI-linearized recombinant plasmids (Sp6 orientation) or XhoI-linearized recombinant plasmids (T7 orientation) according to a previously described protocol (1). The concentration of the IVT RNA molecules per microliter was calculated according to the following formula as described before (23): number of IVT RNA molecules/μl = [Avogadro number × IVT RNA concentration (g/μl)]/IVT RNA molecular weight (g), where the Avogadro number is 6.022×10^{23}.

The analytical sensitivity of the EqFlu NP, EqFlu M, EqFlu HA3, and EqFlu

TABLE 1. Primers and probes used in the rRT-PCR assays

rRT-PCR assay name	EIV subtype	Primer or probe[a]	Sequence (5′ to 3′) (nt location)	GenBank accession no.
EqFlu NP	H3N8	EqFlu NP F	GAAGGGCGGCTGATTCAGA (157-175)	DQ124184
		EqFlu NP R	TTCGTCGAATGCCGAAAGTAC (199-219)	
		EqFlu NP Pr	[b]CAGCATAACAATAGAAAGGA[c] (177-196)	
EqFlu M		EqFlu M F	ACCGAGGTCGAAACGTACGT (38-57)	DQ124188
		EqFlu M R	CGCGATCTCGGCTTTGA (84-100)	
		EqFlu M Pr	[b]CTCTCTATCGTACCATCAGG[c] (59-78)	
EqFlu HA3		EqFlu HA3 F	TCACATGGACAGGTGTCACTCA (448-469)	L39914
		EqFlu HA3 R	GGCTGATCCCCTTTTGCA (485-506)	
		EqFlu HA3 Pr	[b]AACGGAAGAAGTGGAGC[c] (471-487)	
EqFlu HA3-Mia		EqFlu HA3-Mia F	GCAGTGCTTTCAGCAATTGC (346-365)	M29257
		EqFlu HA3-Mia R	AGAGACCGGAGCGATGCA (389-406)	
		EqFlu HA3-Mia Pr	[d]CCATATGACGTCCCTGACT[c] (369-387)	
EqFlu NP-Pra	H7N7	EqFlu NP-Pra F	GGCGTCTCAAGGCACCAA (48-65)	M63748
		EqFlu NP-Pra R	TCTGGCGTTCTCCACCAGTT (87-106)	
		EqFlu NP-Pra Pr	[b]CGACCTTATGAACAAATG[c] (67-84)	
EqFlu M-Pra		EqFlu M-Pra F	CGCGCAGAGACTTGAGAATG (97-116)	CY005801
		EqFlu M-Pra R	CATTCCATGAGAGCCTCAAGATCT (136-159)	
		EqFlu M-Pra Pr	[b]TTTGCAGGGAAAAATA[c] (119-134)	
EqFlu HA7-Pra		EqFlu HA7-Pra F	CAATGGAGAGACTAGCGCATGT (441-462)	X62552
		EqFlu HA7-Pra R	AGAAGCCATTTCATCTCTGCATAA (483-506)	
		EqFlu HA7-Pra Pr	[e]AAGGTCAAGATCTTCC[c] (465-480)	
EqFlu HA7		EqFlu HA7 F	TCCTCTGTGTACGTGCAGATAAAATC (59-84)	X62556
		EqFlu HA7 R	GGGTGTCTACTTTGGTTCCATTAGA (106-130)	
		EqFlu HA7 Pr	[b]CCTAGGACGTCATGCTG[c] (87-103)	

[a] F, forward primer; R, reverse primer; Pr, probe.
[b] Reporter dye (6-carboxyfluorescein)-labeled nucleotide.
[c] Nonfluorescent quencher dye (MGB)-labeled nucleotide.
[d] Reporter dye (VIC)-labeled nucleotide.
[e] Reporter dye (NED)-labeled nucleotide.

HA3-Mia rRT-PCR assays was determined using the specific IVT RNA generated from the recombinant plasmids.

Determination of detection limits of rRT-PCR, Directigen Flu A test, and egg inoculation. Using serial decimal dilutions (10^{-1} to 10^{-10}) of the H7N7 (A/equine/Prague/56; 4.65×10^5 50% egg infectious doses [EID_{50}]/ml) and H3N8 (A/equine/Kentucky/02; 10^7 EID_{50}/ml) subtype strains, the detection limits of the rRT-PCR assays, the Directigen Flu A test (Becton-Dickinson, Sparks, MD), and egg inoculation were evaluated. To avoid interassay variation, equal aliquots of each dilution were used in all three assays. Briefly, 5 μl of RNA isolated from 140 μl of each decimal dilution were used in rRT-PCR assays as described above. One hundred twenty-five microliters of each specimen was tested with the Directigen Flu A test according to the manufacturer's recommendations. For egg inoculation, an inoculum of 100 μl of each specimen was used. A total of four eggs were inoculated with each sample (28).

RT-PCR amplification and sequencing of NP and M genes. The full-length NP gene sequence (nucleotides [nt] 1 to 1565) and partial M gene sequence (nt 1 to 830) of the A/equine/New York/73 strain were RT-PCR amplified using a standard laboratory protocol. Briefly, viral nucleic acid was isolated from AF by using a Qiagen viral RNA extraction kit (Qiagen Inc., Santa Clara, CA) according to the manufacturer's instructions. Viral RNA was reverse transcribed with UNI-12 primer (see Table S2 in the supplemental material), using the AccuScript high-fidelity RT-PCR system (Stratagene, La Jolla, CA) (18). Each cDNA was then amplified by PCR using Accuprime Pfx DNA polymerase (Invitrogen, Carlsbad, CA) with NP- and M-specific forward and reverse primers (see Table S2 in the supplemental material). Both PCR products were gel purified, and the full-length NP (1,565 bp) and partial M (1,027 bp) genes of the A/equine/New York/73 strain were determined by sequencing both strands of DNA by using gene-specific primers (see Table S2 in the supplemental material). The NP sequence of the A/equine/New York/73 strain was compared to the published sequences of A/equine/Miami/63 and A/equine/Prague/56 (GenBank accession numbers M22575 and M63748, respectively). Similarly, the partial M sequence of A/equine/New York/73 was compared to those of the same prototype strains (GenBank accession numbers AF001674 and CY005801, respectively).

Statistical analysis. Statistical evaluation of the performance of the three EIV primer and probe sets (EqFlu NP, EqFlu M, and EqFlu HA3) for the detection of EIV nucleic acids was carried out to determine the respective sensitivities and specificities of each assay and to compare these values with those of VI by egg inoculation. Sensitivities for the three H3N8 subtype-specific rRT-PCR assays and VI were estimated by Clopper-Pearson exact binomial methods using experimental samples, with statistical comparisons made by employing exact binomial paired tests (9).

The analysis of data from testing field specimens was conducted using a Bayesian model developed by Branscum et al. (3) that enables estimation of the sensitivity and specificity of a test when true infection status is unknown. Separate models were used for comparing VI with each rRT-PCR assay under the assumption of conditional independence of tests. Independent beta prior distributions for the four sensitivities were constructed using information derived from the horse challenge study. Specifically, average sensitivities on days 2 to 8 were used as prior modes for the sensitivity on each of the three rRT-PCR assays, with the smallest values among the lower endpoints of days 2 to 8 used as prior fifth percentiles. The modes and lower percentiles were used to identify an appropriate beta prior distribution. For the VI test, the prior 95th percentile for sensitivity was set at 0.93, with a mode of 0.51. Based on testing for possible cross-reaction with other viruses, the priors for the specificities of each of the three rRT-PCRs had a mode of 0.95 and a fifth percentile of 0.80. The specificity of VI was set equal to 1. An empirical Bayes approach was used to place a prior on the prevalence that had a mode of 0.32 and a 99th percentile of 0.45. Gibbs sampling was used to simulate from posterior distributions. Five chains were run with separated starting values (there was no indication of lack of convergence), a

TABLE 2. Comparison of the specificity of H3N8 and H7N7 subtype-specific rRT-PCR assays

EIV subtype	EIV prototype strain and recent isolate	Virus titer (EID_{50}/ml)	Detection of indicated subtype by rRT-PCR[a]							
			H3N8				H7N7			
			EqFlu NP	EqFlu M	EqFlu HA3	EqFlu HA3-Mia	EqFlu NP-Pra	EqFlu M-Pra	EqFlu HA7-Pra	EqFlu HA7
H3N8	A/equine/Miami/63	4.65×10^5	+	+	−	+	−	−	−	−
	A/equine/Alaska/91[b]	2.14×10^9	+	+	+	−	−	−	−	−
	A/equine/Kentucky/81[b]	3.16×10^8	+	+	+	−	−	−	−	−
	A/equine/Kentucky/02[b]	2.15×10^7	+	+	+	−	−	−	−	−
	A/equine/Ohio/03[b]	3.16×10^7	+	+	+	−	−	−	−	−
	A/equine/Newmarket/2/93[c]	1×10^8	+	+	+	−	−	−	−	−
	A/equine/Aboyne/05[c]	6.81×10^3	+	+	+	−	−	−	−	−
	A/equine/Richmond/07[c]	4.65×10^5	+	+	+	−	−	−	−	−
H7N7	A/equine/Prague/56	4.65×10^5	−	−	−	−	+	+	+	+
	A/equine/New York/73	4.7×10^7	+	−	−	−	−	+	+	+

[a] +, Nucleic acid was detectable by the rRT-PCR; −, nucleic acid was undetectable by the rRT-PCR.
[b] American lineage.
[c] Eurasian lineage.

burn-in of 10,000 iterates was used, and inferences were based on 100,000 iterates. Data analysis was implemented in R 2.7 and WinBUGS 1.4.3 (24, 36).

RESULTS

Selection of rRT-PCR assays targeting NP, M, and HA genes of H3N8 and H7N7 subtypes of equine influenza. A total of eight rRT-PCR assays were developed to target the NP, M, and HA genes of H7N7 and H3N8 EIV subtypes (Table 1). The assays were evaluated using prototype strains of each EIV subtype as well as recent virus isolates representing both American and Eurasian lineages (Table 2). The EqFlu NP, EqFlu M, EqFlu HA3, and EqFlu HA3-Mia assays were designed to detect the EIV H3N8 subtype. The EqFlu NP assay detected not only all tested H3N8 strains but also one H7N7 strain (A/equine/New York/73). The EqFlu M assay successfully distinguished all H3N8 strains and did not cross-react with either of the two H7N7 strains tested. The EqFlu HA3 assay was able to detect all H3N8 subtype strains except for the prototype virus (A/equine/Miami/63). On the other hand, the EqFlu HA3-Mia assay which was designed solely based on the A/equine/Miami/63 sequence could only detect the prototype virus (Table 2). Similarly, the other four (EqFlu NP-Pra, EqFlu M-Pra, EqFlu HA7-Pra, and EqFlu HA7) assays were designed to detect the EIV H7N7 subtype. Both H7N7 subtype viruses tested gave positive results with EqFlu M-Pra, EqFlu HA7-Pra, and EqFlu HA7 assays (Table 2). However, the EqFlu NP-Pra assay could detect only the prototype strain (A/equine/Prague/56) and not the A/equine/New York/73 strain. None of these assays specific for the H7N7 subtype cross-reacted with any viruses of the H3N8 subtype. All eight rRT-PCR assays were highly specific in that none of them detected any of the other common equine respiratory viruses tested.

In summary, both EqFlu NP and EqFlu M primers and probe sets were able to detect all eight H3N8 strains representing both American and Eurasian lineages that were included in this study. With the exception of the first A/equine/Miami/63 isolate, the EqFlu HA3 primers and probe set detected most recent H3N8 isolates. Therefore, three out of four rRT-PCR assays specific for the H3N8 subtype, EqFlu NP, EqFlu M, and EqFlu HA3, were selected for further evaluation with clinical specimens.

Analytical sensitivity of rRT-PCR assays targeting NP, M, and H3 HA genes of H3N8 subtype. In order to determine the analytical sensitivity of the rRT-PCR assays targeting the NP, M, and H3 HA genes of EIV nucleic acid, serial decimal molecule dilutions (10^0 to 10^{10}) of IVT RNA containing these genes derived from A/equine/Miami/63 and A/equine/Kentucky/02 strains of EIV were tested with the EqFlu NP, EqFlu M, EqFlu HA3, and EqFlu HA3-Mia rRT-PCR assays. The assays were independently repeated three times. The IVT NP, M, or H3 HA RNA from each strain was calculated based on the molecular weight and concentration of the IVT RNA. Regression analysis confirmed linearity in all six assays (R^2 was 0.9968 for A/equine/Miami/63 and 0.9976 for A/equine/Kentucky/02, using EqFlu NP rRT-PCR; 0.996 for A/equine/Miami/63 and 0.995 for A/equine/Kentucky/02, using EqFlu M rRT-PCR; 0.9987 for A/equine/Kentucky/02, using EqFlu HA3 rRT-PCR; and 0.9994 for A/equine/Miami/63, using EqFlu HA3-Mia rRT-PCR). The EqFlu NP rRT-PCR detected a minimum of 10 RNA molecules from both the A/equine/Kentucky/02 and A/equine/Miami/63 strains, and the cycle threshold (C_T) value for A/equine/Kentucky/02 was at least four cycles lower than that for A/equine/Miami/63 over all the IVT RNA dilutions tested (see Fig. S1 in the supplemental material). The ranges of magnitude using the EqFlu M rRT-PCR for the A/equine/Miami/63 and A/equine/Kentucky/02 strains were 1 and 10 molecules, respectively (see Fig. S2 in the supplemental material). Both EqFlu HA3 and EqFlu HA3-Mia rRT-PCR assays detected a minimum of 10 RNA molecules (see Fig. S3 in the supplemental material). These data clearly indicate that each of these assays could detect as few as 10 RNA molecules.

Comparison of sensitivities of the rRT-PCR assays targeting NP and M genes, the Directigen Flu A test and egg inoculation. Using serial decimal dilutions of A/equine/Kentucky/02 (H3N8 subtype) and A/equine/Prague/56 (H7N7 subtype), the detection limits of the EqFlu NP, EqFlu M,

TABLE 3. Comparison of subtype-specific rRT-PCR assays targeting NP and M genes with egg inoculation and Directigen Flu A test[a]

EIV prototype strain	Subtype	Virus titer (EID_{50}/ml)	Result for:					
			Directigen Flu A test	Egg inoculation	rRT-PCR assay[b]			
					EqFlu NP	EqFlu M	EqFlu NP-Pra	EqFlu M-Pra
A/equine/Kentucky/02	H3N8	10^7	10^{-2}	10^{-6}	10^{-7}	10^{-7}	NA	NA
A/equine/Prague/56	H7N7	4.65×10^5	10^{-2}	10^{-5}	NA	NA	10^{-6}	10^{-7}

[a] Serial decimal dilutions of EIV strains were tested in the comparison study by egg inoculation, Directigen Flu A test, and rRT-PCR assays. Numbers shown in the table represent the serial decimal dilution factors.
[b] NA, not applicable.

EqFlu NP-Pra, and EqFlu M-Pra rRT-PCR assays were compared to those of the Directigen Flu A test and egg inoculation (Table 3). The highest dilutions that the Directigen Flu A test and egg inoculation could detect were the 10^{-2} and 10^{-6} virus dilutions, respectively. In contrast, the M- and NP-specific rRT-PCR assays targeting both subtypes of the virus were at least more than 10^4 times more sensitive than the Directigen Flu A test. Similarly, the assays had sensitivity at least 1 log higher than that of egg inoculation.

Evaluation of H3N8 subtype-specific rRT-PCR assays for the detection of EIV in clinical specimens. Three rRT-PCR assays (EqFlu NP, EqFlu M, and EqFlu HA3 assays) targeting the NP, M, and H3 HA genes were further evaluated using a range of clinical specimens. Of the 211 archived nasal swab samples collected from horses that were challenged with the A/equine/Kentucky/02 strain, 164, 166, and 153 tested positive using the rRT-PCR assays targeting NP, M, and HA genes, respectively (Table 4). Of the 149 archived nasal swabs from field cases of respiratory disease, 41, 25, and 27 tested positive with the NP, M, and HA assays, respectively. Of the 149 samples, 48 nasal swabs were from horses that were scheduled for international shipment, and all these samples tested negative for EIV nucleic acid by the use of each of these three rRT-PCR assays.

In the case of the experimental horse challenge study, estimated sensitivities of each rRT-PCR assay and *P* values for a possible difference in sensitivity with VI by egg inoculation on days 1 to 8 postinfection were calculated based on the assumption that every swab tested was truly positive for EIV (see Table S3 in the supplemental material). The results indicate that the rRT-PCRs targeting the NP and M genes were significantly more sensitive than VI by egg inoculation on day 1 postinfection; also, these two assays had higher sensitivities between days 3 and 5. At later time points in the horse inoculation experiment (days 6 to 8), all three rRT-PCRs were significantly more sensitive than VI ($P \leq 0.02$). The data demonstrate that the rRT-PCR assays had comparable or greater sensitivity in detecting nucleic acid than did VI by egg inoculation at each time point from days 1 to 8 postinfection (see Table S3 in the supplemental material).

The estimated sensitivity and specificity of each rRT-PCR assay and the difference in sensitivity between rRT-PCR and VI by egg inoculation were calculated using a Bayesian analysis of the 149 field samples (see Table S4 in the supplemental material). Seven horses that tested VI positive were classified as EIV positive. A total of 48 horses that tested negative on all three rRT-PCRs and were VI negative were classified as EIV negative. The unknown EIV status of the remaining 94 horses was imputed in the Bayesian analysis. All three rRT-PCR assays had higher sensitivity (with posterior probability of 1) than did VI by egg inoculation. The PCR assay targeting the NP gene had the highest sensitivity (93%; 95% credible interval, 77% to 99%) and had the largest increase in sensitivity over VI (58%; 95% credible interval, 37% to 76%). The other two rRT-PCR assays (EqFlu M and EqFlu HA3) had similar high sensitivities (89% and 87%) with smaller increases in sensitivity over VI (39% and 35%), respectively. All three assays had estimated specificities that were ≥96%. The higher sensitivity of the rRT-PCR assay targeting the NP gene of the H3N8 virus compared to the other two assays targeting the M and H3 HA genes is consistent with the sequence conservation seen in the NP gene among various EIV strains.

Cross-reaction of EqFlu NP rRT-PCR assay with A/equine/New York/73 (H7N7). Because the A/equine/New York/73 strain (H7N7 subtype) was detected by the EqFlu NP assay that was originally designed to detect H3N8 strains but not by the EqFlu NP-Pra assay, the NP and partial M genes of this virus were sequenced (GenBank accession numbers FJ499496 and FJ666099, respectively). The M gene of A/equine/New York/73 had 97.7% identity with A/equine/Prague/56 strain, as

TABLE 4. Comparison of sensitivities of VI in eggs and rRT-PCR assays for detection of EIV using archived clinical specimens

Clinical specimen tested	No. of specimens tested by:							
	VI		rRT-PCR assay					
			EqFlu NP		EqFlu M		EqFlu HA3	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
211 Nasal swabs from a horse challenge study	98	113	164	47	166	45	153	58
149 Field nasal swabs	7	142	41	108	25	124	27	122

expected. In contrast, the NP gene of that strain had higher sequence identity with the H3N8 prototype A/equine/Miami/63 than with the H7N7 prototype A/equine/Prague/56 strain (96.3% and 83.3%, respectively). These data clearly indicate that despite being an H7N7 virus, the NP gene of the A/equine/New York/73 strain is very similar to that of the H3N8 virus. The forward primer of the EqFlu NP assay had a 100% match with the A/equine/New York/73 NP gene, while there was only a single nucleotide mismatch in both reverse primer and probe binding regions. These two changes did not compromise the detection capability of the rRT-PCR assay. In contrast, the reverse primer and the probe of the EqFlu NP-Pra assay had two and three nucleotide mismatches, respectively, compared with the A/equine/New York/73 strain, which compromised the sensitivity of the assay.

DISCUSSION

Eight new MGB probe-based rRT-PCR assays targeting the NP, M, H3, and H7 HA genes of two EIV subtypes were developed and evaluated in this study. Four of the primer sets targeted the NP, M, and H3 HA genes of H3N8 subtype and the remaining four targeted the respective genes of H7N7 subtype. The assays were based on MGB probes which provide several advantages over other real-time PCR chemistries. The MGB probes are shorter in length (12 to 18 bases) than are conventional TaqMan probes. Since such probes are less liable to sequence mismatches, this results in the increased specificity of the rRT-PCR assays (47). The 3′-end nonfluorescent quencher dye dramatically reduces the background fluorescence of the reaction, hence a smaller chance of false-positive results (22). For the primer and probe design, the conserved regions of these four genes were determined by alignment of sequences of the H3N8 and H7N7 strains available in GenBank. The EqFlu NP and EqFlu M assays were able to detect all the prototype H3N8 strains. The EqFlu HA3 assay did not give a positive reaction with the A/equine/Miami/63 (H3N8), which may be the result of antigenic drift. Sequence comparison with a recent isolate revealed that the H3 HA gene of A/equine/Miami/63 has only 91% sequence homology with that of A/equine/Kentucky/02 (data not shown). There are one and three nucleotide mismatches in the reverse primer and probe region of the EqFlu HA3 assay, respectively, compared to the H3 HA sequence of the A/equine/Miami/63 virus, thus reducing the efficiency of the assay. Sequence analysis of A/equine/New York/73 (H7N7) clearly indicates that its NP gene was derived from the H3N8 subtype, providing confirmation of reassortment between H3N8 and H7N7 strains of EIV in the field since the 1970s, as previously reported (2, 17, 20). Since the EqFlu HA7 assay appears to be highly specific for H7N7 EIV, it should enable highly reliable surveillance for the H7N7 subtype, which is thought to be extinct or possibly still circulating at a very low level in nature (19, 25, 37, 45). Regrettably, the EqFlu HA7 assay could not be adequately evaluated because of the very limited number of isolates of this EIV subtype available for testing in this study. None of these assays specific for both EIV subtypes gave a positive result with other common equine respiratory viral pathogens, such as equine herpes viruses and equine rhinitis viruses, confirming 100% specificity for EIV. In summary, the primer and probe sets designed and evaluated in this study allow the identification of both equine influenza subtypes. It would appear that they likely can also detect any reassortments of these two EIV subtypes that may currently be in circulation in nature. Furthermore, H3N8 subtype-specific assays were able to detect both Eurasian and American lineage strains of EIV.

Three rRT-PCR assays (EqFlu NP, EqFlu M, and EqFlu HA3 assays) targeting the NP, M, and H3 HA genes of the H3N8 EIV subtype were further evaluated by using two sets of clinical samples: nasal swabs from an experimental challenge study and nasal swabs (field samples) submitted to the OIE reference laboratory for EI for routine diagnostic testing. Using nucleic acid extracted from the samples collected from a group of experimentally inoculated horses, both the EqFlu NP and EqFlu M assays were shown to have significantly higher respective sensitivities than did egg inoculation during the time course of the study (days 1 to 8). Furthermore, with few exceptions, all three assays were able to detect EI nucleic acid from day 1 postchallenge before clinical signs of disease were observed. The samples that were positive only by rRT-PCR and not by VI were confirmed to be truly EIV positive by a previously published standard RT-PCR assay (data not shown) (16). The minimal analytical sensitivity of these assays can reach up to 10 IVT RNA molecules. As such, these assays provide valuable tools for distinguishing EI from clinically similar diseases on an individual animal basis. These newly developed rRT-PCR assays performed exceedingly well not only in virus detection but also in distinguishing the different subtypes of EIV based on the various samples evaluated in this study. Such assays can provide a fast and reliable means of EIV diagnosis and are especially useful in screening samples during a suspected outbreak of EI.

Comparison of the sensitivities of the four rRT-PCR assays targeting the NP and M genes with egg inoculation and the Directigen Flu A test clearly shows that the molecular assays provide the highest sensitivity. Previous studies have compared relative sensitivities of VI in embryonated eggs, antigen detection (Directigen Flu A test), and nucleic acid amplification (nested RT-PCR targeting the NP gene and standard RT-PCR targeting the M gene) for detection of EIV (34). The authors have shown that RT-PCR assays using M primers and VI in embryonated eggs proved to be the most sensitive methods for virus detection. The Directigen Flu A test was the least sensitive method for detection of EIV. In a similar study, Yamanaka et al. (46) demonstrated that VI in embryonated eggs is more sensitive than the five rapid antigen detection kits evaluated. Furthermore, analysis of nasal swabs from a limited number of experimentally inoculated horses also showed that VI by egg inoculation and the standard RT-PCR had comparable sensitivities in detecting EIV (34). Data from this study also demonstrated that the Directigen Flu A test had the lowest sensitivity, followed by VI, in embryonated eggs. Furthermore, evaluation of clinical samples and serial dilutions of EIV prototype strains has confirmed that rRT-PCR assays have higher sensitivity than does egg inoculation.

In conclusion, newly developed rRT-PCR assays targeting NP, M, and HA genes were found to be highly sensitive and specific compared to the Directigen Flu A test and VI in embryonated eggs. The assays provided a fast and reliable means of virus detection and disease surveillance, with, it

would appear, the additional advantage of being able to identify antigenic shift between the two subtypes of EIV.

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