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Rapid detection of equine influenza virus H3N8 subtype by insulated isothermal RT-PCR (iiRT-PCR) assay using the POCKIT™ Nucleic Acid Analyzer

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A B S T R A C T

Equine influenza (EI) is an acute, highly contagious viral respiratory disease of equids. Currently, equine influenza virus (EIV) subtype H3N8 continues to be the most important respiratory pathogen of horses in many countries around the world. The need to achieve a rapid diagnosis and to implement effective quarantine and movement restrictions is critical in controlling the spread of EIV. In this study, a novel, inexpensive and user-friendly assay based on an insulated isothermal RT-PCR (iiRT-PCR) method on the POCKIT™, a field-deployable device, was described and validated for point-of-need detection of EIV-H3N8 in clinical samples. The newly established iiRT-PCR assay targeting the EIV HA3 gene was evaluated for its sensitivity using *in vitro* transcribed (IVT) RNA, as well as ten-fold serial dilutions of RNA extracted from the prototype H3N8 strain A/equine/Miami/1/63. Inclusivity and exclusivity panels were tested for specificity evaluation. Published real-time RT-PCR (rRT-PCR) assays targeting the NP and HA3 genes were used as the reference standards for comparison of RNA extracted from field strains and from nasal swab samples collected from experimentally infected horses, respectively. Limit of detection with a 95% probability ($LoD_{95\%}$) was estimated to be 11 copies of IVT RNA. Clinical sensitivity analysis using RNA prepared from serial dilutions of a prototype EIV (Miami 1/63/H3N8) showed that the iiRT-PCR assay was about 100-fold more sensitive than the rRT-PCR assay targeting the NP gene of EIV subtype H3N8. The iiRT-PCR assay identified accurately fifteen EIV H3N8 strains and two canine influenza virus (CIV) H3N8 strains, and did not cross-react with H6N2, H7N7, H1N1 subtypes or any other equine respiratory viral pathogens. Finally, 100% agreement was found between the iiRT-PCR assay and the universal influenza virus type A rRT-PCR assay in detecting the EIV A/equine/Kentucky/7/07 strain in 56 nasal swab samples collected from experimentally inoculated horses. Therefore, the EIV H3N8 subtype specific iiRT-PCR assay along with the portable POCKIT™ Nucleic Acid Analyzer provides a highly reliable, sensitive and specific on-site detection system of both equine and canine influenza viruses.

1. Introduction

Equine influenza (EI) is an acute, highly contagious viral respiratory disease of horses, donkeys, and mules and is listed as a notifiable disease by the World Animal Health Organization (Office International des Epizooties [OIE]). Equine influenza virus (EIV) is considered enzootic in the USA, UK and many other European countries (Wilson, 1993). New Zealand and Iceland are the only countries that have remained continuously free of EIV. The first strain of EIV isolated in 1956 was of H7N7 configuration and designated influenza virus A/equine/Prague/56 (Sovinova et al., 1958; Webster, 1993). The last confirmed outbreak caused by an H7N7 subtype in horses was recorded in 1979 (van Maanen and Cullinane, 2002; Webster, 1993). Thus the H7N7 subtype is thought to be extinct or possibly still circulating at a very low level in nature (Ismail et al., 1990; Madic et al., 1996; Singh, 1994; Wright et al.,

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2007). A second EIV subtype, H3N8, was first isolated in 1963 and designated as influenza virus A/equine/Miami/1/63 (van Maanen and Cullinane, 2002; Waddell et al., 1963). This subtype has been associated with all confirmed outbreaks of EI since 1980. Extensive antigenic drift has been detected in this virus over the years (Brown et al., 1983; Bryant et al., 2009; Daniels et al., 1985; Klingeborn et al., 1980; Oxburgh et al., 1994; Ozaki et al., 2001; van Oirschot et al., 1981). This led to categorization of H3N8 EIV isolates from around the world into two lineages, American and Eurasian (Bryant et al., 2009; Daly et al., 1996; Lewis et al., 2011). Both subtypes belong to genus Influenza virus A in the family *Orthomyxoviridae* and possess a segmented (8 segments), single-stranded RNA genome of negative sense. In 2005, inter-species transmission of H3N8 EIV from horse to dog was reported for the first time without genetic reassortment and this virus has continued to evolve in dogs and is identified as CIV (Anderson et al., 2011; Crawford et al., 2005).

EIV H3N8 subtype spreads rapidly among susceptible horses and can result in very high morbidity within 24–48 h after exposure to the virus. Clinical signs of disease in horses include a sudden increase in body temperature (39–41 °C), depression, loss of appetite, labored breathing, muscle pains, stiffness, cough, and watery/mucopurulent nasal discharges. These signs are similar to those caused by other equine respiratory pathogens, such as equine rhinitis A and B viruses (ERAV and ERBV), equine arteritis virus (EAV), and equine herpesvirus (EHV)-1 and -4. Traditionally, laboratory diagnosis of EIV was achieved by virus isolation in embryonated hens' eggs or Madin–Darby canine kidney (MDCK) cells (van Maanen and Cullinane, 2002). EIV isolates from nasal and/or nasopharyngeal swabs are further confirmed by subtype-specific antisera using a hemagglutination-inhibition test. Being cumbersome and time-consuming, virus isolation does not allow rapid point-of-need detection of EIV, which could facilitate timely implementation of biosecurity measures, such as quarantine or movement restrictions, to help disease management and control. For an example, antigen detection immunoassays such as the Directigen Flu® A test kit (Becton-Dickinson, Sparks, MD) has been used, but this assay lacked sensitivity (Lu et al., 2009). Recently, various molecular assays have been developed to expedite the detection of EIV in clinical specimens. Specific detection of EIV/CIV H3N8 subtype nucleic acid in clinical specimens has been demonstrated with traditional reverse transcription polymerase chain reaction (RT-PCR), RT-nested PCR, rRT-PCR (Foord et al., 2009; Lu et al., 2009; Payungporn et al., 2008; Pecoraro et al., 2013; Spindel et al., 2007). These assays targeted the nucleocapsid (NP), hemagglutinin (HA), or matrix (M) gene of EIV. However, performance of molecular diagnostic assays requires trained technicians and sophisticated and expensive instruments and therefore these RT-PCR-based methods are not suitable for point-of-need EI diagnosis.

Recently, fluorescent probe hydrolysis-based insulated isothermal PCR (iiPCR) for amplification and detection of nucleic acid has been described (Tsai et al., 2012b). The iiPCR is highly sensitive and specific for the detection of both RNA and DNA and could be performed with a single heating source, hence does not require an expensive thermocycler (Chang et al., 2012; Tsai et al., 2012b). PCR mix in a capillary tube (R-tube™) is heated at the bottom and the natural thermal convection is induced by heat. Thermal convection drives fluid cycling through temperature gradients established by the Rayleigh–Bénard convection and the three PCR steps, namely denaturation, annealing, and extension, can be completed at different zones within the capillary tube. The feasibility of iiPCR was first shown in a simple thermally baffled device (Chang et al., 2012; Tsai et al., 2012b). A white spot syndrome virus iiRT-PCR assay was demonstrated to produce detectable amplicons in 30 min with notable sensitivity and specificity (Tsai et al., 2012a). However, detection of amplicons required gel analysis which renders the assay prone to amplicon contamination. Subsequent integration of hydrolysis probe technology into iiPCR and an optical detection module into the iiPCR device allowed automatic detection and interpretation of iiPCR results, reducing the risks of cross contamination greatly (Tsai et al., 2012b). Taking advantage of the fluorescent probe-based iiPCR methodology and a now commercially available iiRT-PCR instrument, POCKIT™ Nucleic Acid Analyzer (GeneReach USA, Lexington, MA, USA) (Chang et al., 2012; Tsai et al., 2012b), the iiRT-PCR assay was developed for the detection of EIV RNA in clinical specimens. The results of the assessment of analytical sensitivity, analytical specificity, repeatability, diagnostic accuracy, and proficiency of the newly established EIV specific iiRT-PCR assay is reported. Furthermore, the performance of the iiRT-PCR was compared to a previously described rRT-PCR assay targeting the NP gene of EIV (Lu et al., 2009, 2010).

2. Materials and methods

2.1. Viruses

Three H3N8 EIV prototype strains (A/equine/Alaska/91, A/equine/Kentucky/81, and A/equine/Miami/1/63) and one H7N7 EIV prototype strain (A/equine/Prague/56) were obtained from the National Veterinary Service Laboratories (NVSL), Ames, IA. Additionally, eleven EIV H3N8 strains, two canine H3N8 strains, two H1N1 strains (A/PR/8/34 and swine influenza strains), two avian H6N2 strains, and two equine H7N7 strains were obtained from the World Organization for Animal Health (OIE) reference laboratory for EI at the Gluck Equine Research Center, University of Kentucky, Lexington, KY and the Animal Health Diagnostic Center, New York State College of Veterinary Medicine, Cornell University, Ithaca, NY to be included in the study. In order to determine the specificity of the iiRT-PCR assay, other equine viral pathogens were also included in the study: the reference Bucyrus strain of EAV, EHV-1, EHV-2, EHV-3, EHV-4, EHV-5, ERAV, ERBV, equine adenoviruses 1 and 2 (EAdV-1 and EAdV-2), and Salem virus (Table 1).

2.2. Clinical samples

A total of 56 archived nasal swab samples collected from 14 horses that were experimentally challenged with the A/equine/Kentucky/7/07 strain of EIV were included in the study. Samples were collected prior to experimental inoculation (day 0) and 2–4 days post experimental inoculation with the virus. The nasal swabs were placed in 7 ml of virus transport medium (VTM; Hanks' Balanced Salt Solution, 25 mM HEPES Buffer, 0.5% bovine serum albumin fraction V and antibiotics) and transported to the laboratory on ice for processing. The experimental inoculation of horses and collection of clinical samples were performed according to a protocol approved by the Institutional Animal Care and Use Committee at the University of Kentucky, Lexington, KY.

2.3. Nucleic acid extraction

For clinical sensitivity analysis, allantoic fluids collected from chicken embryos infected with prototype virus EIV (A/equine/Miami/63, H3N8) were 10-fold serially diluted (10^0–10^7) in MEM medium and subjected to RNA extraction. Nucleic acids were extracted from 50 μl of each virus dilution with Ambion® MagMAX™ Total Nucleic Acid Isolation Kit (Applied Biosystems, Foster City, CA, USA) as described in the user manual. Similarly, 50 μl of nasal swab sample in VTM was used for the nucleic acid extraction from experimentally inoculated horses. RNA was eluted from the beads with 50 μl of Elution Buffer and stored at −80 °C.

Table 1
Influenza and other equine viruses included in this study.

Sample ID	Virus subtype	iiPCR result
A/Equine/Miami/1/63	H3N8	+
A/Equine/Ohio/1/03	H3N8	+
A/Equine/Kentucky/5/02	H3N8	+
A/Equine/Lex/63	H3N8	+
A/Equine/Kentucky/1/94	H3N8	+
A/Equine/VA/131054-3	H3N8	+
A/Equine/NY VLS #951	H3N8	+
A/Equine/110600	H3N8	+
A/Equine/239936-99	H3N8	+
A/Equine/Kentucky/1/81	H3N8	+
A/Equine/M2 63	H3N8	+
A/Equine/HLC 91	H3N8	+
A/Equine/MT/9233/07	H3N8	+
A/Equine/TX/117793/05	H3N8	+
A/Equine/Ohio/113461-2/05	H3N8	+
A/Canine/VLS #882	H3N8	+
A/Canine/NY/4982, 3-06	H3N8	+
A/PR/8/34	H1N1	–
Swine H1N1 RNA	H1N1	–
Avian vRNA-M72-d7	H6N2	–
Avian vRNA-M76-d7	H6N2	–
A/equine/NewYork/49/73	H7N7	–
A/equine/Prague/56	H7N7	–
EAV KY84	NA	–
E9 EAdV (NVSL[a])	NA	–
EHV-1 T262	NA	–
EHV-2 HESKA	NA	–
EHV-3 VR352	NA	–
EHV-4 VR2230	NA	–
EQ HERPES Virus 4	NA	–
EHV-5 KD-05	NA	–
ERAV (NVSL)	NA	–
ERBV (NVSL)	NA	–
Salem virus	NA	–
Water sample	NA	–
Positive control	NA	+
Negative control	NA	–

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2.4. In vitro transcribed (IVT) RNA

Standard RNA was generated from a plasmid containing a fragment of the HA gene of A/equine/Kentucky/1/94 (H3N8) strain of EIV (nucleotide numbers 198–1200; GenBank accession No. L39914.1) by *in vitro* transcription using the MAXIscript T7 kit (Ambion, Austin, TX, USA). Residual DNA was removed using the Ambion Turbo DNA-free kit (Applied Biosystems). Concentration of RNA was measured by a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Houston, TX, USA). Single use aliquots were stored at −80 °C. Serial dilutions (100, 50, and 10 copies/reaction) of IVT RNA made in 20 ng/μl yeast tRNA were used to evaluate analytical sensitivity of the iiRT-PCR assay.

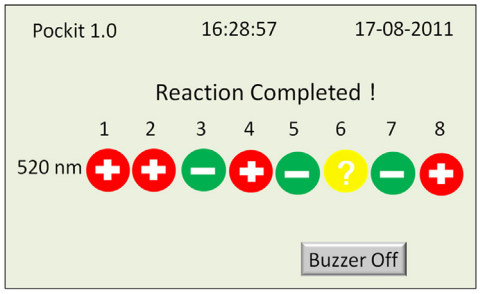
2.5. EIV H3N8 subtype specific iiRT-PCR assay

The EIV H3N8 subtype specific iiRT-PCR assay was designed on the basis of the probe hydrolysis-based POCKIT™ method described previously (Tsai et al., 2012b). One hundred and thirty five influenza A virus HA3 gene nucleotide sequences from North and South America available in the GenBank database were aligned to identify the conserved regions to design subtype specific primers and probes to be used in the iiRT-PCR assays. EIV H3N8 HA3 gene specific primer and probe pair was designed with Primer Express software (Applied Biosystems) according to the recommended principles for iiPCR (Table 2) (http://www.iipcr.com/eweb/uploadfile/20130522114104277.pdf). Annealing temperatures of the probe were approximately 10 °C higher than those of the iiPCR primers. No major secondary structures were found in the amplicon, based on prediction made by the MFold program (http://mfold.rna.albany.edu/?q=mfold). The EIV H3N8 HA3 gene specific primer and probe pair was assembled into a kit (POCKIT™ Influenza H3N8 Detection Kit (GeneReach USA; Supplementary Table 1)) and used in this study, according to the manufacturer's instructions. Briefly, the R-tube(s)™ provided with the reagents were labeled with the sample identification numbers (one tube per test sample). One Premix for each sample was prepared by adding 50 μl of Premix Buffer B to each Premix tube. Subsequently, 5 μl of test nucleic acid extract (or positive/negative control standard) was added into each Premix tube. Each reaction was mixed well by pipetting up and down several times. A 50 μl volume of Premix/sample mixture was transferred into a labeled R-tube (the final assay reaction mix consisted of Premix Buffer B, 0.5 μM forward and reverse primer each, 0.04 μM probe, 4 U MMLV Reverse Transcriptase [BioMi, Taichung, Taiwan], 2 U Ribonuclease Inhibitor [Takara, Shiga, Japan], and 120 U Taq DNA polymerase [BioMi]). Each R-tube™ was sealed with a cap, spun briefly in a microcentrifuge (Cubee™, GeneReach USA), and placed into the POCKIT™ Nucleic Acid Analyzer. Default program of the POCKIT™ device includes an RT step at 50 °C for 10 min and an iiPCR step at 95 °C for about 30 min. The reaction completes in less than 1 h. Similar to the optical detection module described in the original iiPCR device (Chang et al., 2012; Tsai et al., 2012b), POCKIT™ collects optical signals through an integrated circuits controller-regulated (CMOS) sensor. Signal-to-noise (S/N) ratios were calculated by dividing light signals collected after iiPCR by those from before iiPCR (Tsai et al., 2012b). According to default S/N thresholds, results are converted automatically to "+", "−", or "?", which are shown on the display screen at the end of the program

Table 2
Primers and probe sequences used in H3N8 iiRT-PCR, H3N8 *NP* gene specific rRT-PCR assay, and universal influenza virus rRT-PCR assay.

Assay name	Primer/probe name	Sequence (5′–3′)	Nucleotide location	Target gene	Reference
H3N8 iiRT-PCR assay	H3N8-F (Forward Primer)	GTCACTCAAAACGGAAGAAGTGGA	462–485[a]	HA	This paper
	H3N8-R (Reverse Primer)	GTTAGGCATTGTTACATTCAATGTG	561–584		
	H3N8-P (Probe)	FAM-TCGGCTAAAGAAACTATCG-MGB	506–524		
H3N8 *NP* gene specific rRT-PCR assay	EqFlu NP F	GAAGGGCGGCTGATTCAGA	157–175[b]	NP	Lu et al. (2009)
	EqFlu NP R	TTCGTCGAATGCCGAAAGTAC	199–219		
	EqFlu NP Pr	FAM-CAGCATAACAATAGAAAGGA-MGB	177–196		
Universal influenza virus rRT-PCR assay	InfA Forward	GACC**R**ATCCTGTCACCTCTGACTGAC	171–192[c]	M	http://www.who.int/csr/resources/publications/swineflu/CDCRealtimeRTPCR_SwineH1Assay-2009_20090430.pdf
	InfA Reverse	AGGGCATT**Y**TGGACAAA**K**CGTCTA	253–276		
	InfA Probe	FAM-TGCAGTCCTCGCTCACTGGGCACG-MGB	226–249		

[a] Based on GenBank Accession number L39914.1.
[b] Based on GenBank Accession number DQ124184.
[c] Based on GenBank Accession number DQ124188 (degenerated primers: R = G or T, Y = C or T and K = G or T).



520nm	Interpretation
⊕	Positive
⊖	Negative
?	Repeat reaction with freshly-prepared nucleic acid or perform rRT-PCR along with iiRT-PCR

Fig. 1. An example of results displayed on the monitor of the POCKIT™ Nucleic Acid Analyzer is shown above.

(Fig. 1). A "?" result indicates that the signals are ambiguous and the sample should be tested again.

2.6. EIV H3N8 subtype specific rRT-PCR assay

EIV H3N8 *NP* gene specific rRT-PCR was performed using the TaqMan one-step RT-PCR master mix (Applied Biosystems) in a 7500 Fast Real-Time PCR system (Applied Biosystems) as described previously (Lu et al., 2009). 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 IVT RNA.

2.7. Universal influenza virus rRT-PCR assay

The nasal swab samples from horses that were inoculated experimentally with the EIV H3N8 subtype (A/equine/Kentucky/7/07) were tested by the previously described universal Influenza A rRT-PCR assay (InfA primer probe set) according to a published protocol from the Centers for Disease Control (CDC): (http://www.who.int/csr/resources/publications/swineflu/CDCrealtimeRTPCRprotocol_20090428.pdf).

2.8. Statistical analysis

Statistical probit analysis, a non-linear regression model, was performed using commercial software SPSS 14.0 (SPSS Inc., Chicago, IL, USA) to determine $LoD_{95\%}$.

3. Results

3.1. Analytical sensitivity of EIV subtype H3N8 virus iiRT-PCR assay

EIV subtype H3N8 specific iiRT-PCR assay targeting a highly conserved region of the HA gene generated a 118-bp amplicon that is detected by the fluorescent label probe. The reaction conditions, such as concentrations of primers and probe, Taq DNA polymerase, and reverse transcriptase, were tested systematically to obtain the highest sensitivity and specificity (data not shown). Following optimization of the assay conditions, the reagents including primers and probes, were lyophilized and used in this study as described in the materials and methods section. Analytical sensitivity of the established assay was determined by using 10-fold serial dilutions of IVT RNA. Analysis of 20 replicates of 100, 50 and 10 copies of standard RNA showed that 100%, 100% and 90%, respectively, of the reactions produced positive signals (Table 3). The $LoD_{95\%}$ estimated by probit regression analysis was 11 copies/reaction with standard RNA.

Table 3
Analytical sensitivity of H3N8 iiRT-PCR assay.

Copies/reaction	Total test	Positive	Rate (%)	Avg.	SD
NTC[a]	12	0	0	1.00	0.03
100	20	20	100	3.67	0.19
50	20	20	100	3.61	0.36
10	20	18	90	3.50	0.81
PROBIT	0.920	10.207	–	–	
	0.930	10.360	–	–	
	0.940	10.530	–	–	
	0.950[b]	10.725	–	–	
	0.960	10.953	–	–	
	0.970	11.234	–	–	
	0.980	11.607	–	–	
	0.990	12.195	–	–	

[a] NTC, no template control.
[b] 95% hit rate is 10.725 by Probit analysis ($L0D_{95\%}$ is 11 copies/reaction).

The sensitivity of the EIV H3N8 subtype specific iiRT-PCR assay was further evaluated by comparing detection endpoints of a previously described rRT-PCR assay (Lu et al., 2009) targeting the NP gene using EIV RNA extracted from 10-fold serial dilutions (10^0–10^{-7}) containing the prototype EIV strain (A/equine/Miami/1/63, H3N8). Detection endpoints were observed at 10^{-3} and 10^{-5} dilutions for rRT-PCR and iiRT-PCR assays, respectively (Table 4), indicating that the iiRT-PCR assay is about 100-fold more sensitive than rRT-PCR in detecting EIV.

3.2. Specificity of EIV subtype H3N8 virus iiRT-PCR assay

The specificity of the iiRT-PCR assay was determined with 17 H3N8 strains, including 2 CIV isolates (Table 1). The newly

Table 4
Comparison of analytical sensitivity between validated H3N8 *NP* gene specific rRT-PCR and iiRT-PCR using viral RNA from A/Miami/1/63, egg P-2 HA titer 1:128.

Sample dilution	EIV *NP* rRT-PCR (Ct)			iiRT-PCR result[a]
NTC[b]	–			–
Positive amplification control	20.27	ND[c]	ND	+
Undiluted	26.44	26.48	26.54	2/2
10^{-1}	29.83	30.03	30.13	2/2
10^{-2}	33.81	34	34.22	2/2
10^{-3}	36.73	37.32	37.3	2/2
10^{-4}	–	38.98	–	2/2
10^{-5}	–	–	–	2/2
10^{-6}	–	–	–	0/2
10^{-7}	–	–	–	0/2
10^{-8}	–	–	–	NA
10^{-9}	–	–	–	NA
10^{-10}	–	–	–	NA

[a] Performed in duplicate.
[b] NTC, no template control.
[c] ND, not done.

Table 5
Sensitivity and specificity of iiRT-PCR as compared to H3N8 *NP* gene specific rRT-PCR.

		rRT-PCR		
		Positive	Negative	Total
iiRT-PCR	Positive	17	0	17
	Negative	0	23	23
	Total	17	23	40

established iiRT-PCR assay generated positive signals with all the EIV and CIV strains that were tested. Furthermore, there was no cross reactivity with any of the viruses included in the exclusivity test panel. These viruses included avian (H6N2, and 4 H7N7) and swine (H1N1) influenza virus subtypes and other common equine viral pathogens (EHV-1, -2, -3, -4, and -5, EAV, ERAV, ERBV, Salem virus, and EAdV-1 (Tables 1 and 5).

3.3. Reproducibility test of EIV subtype H3N8 iiRT-PCR assay

Reproducibility of the H3N8 EIV subtype specific iiRT-PCR assay was assessed by testing independently (three experimental runs) 6 replicates of the nucleic acid extract of 10^{-3} dilution of allantoic fluid (A/equine/Miami/1/63, H3N8). All 18 reactions yielded positive POCKIT™ results, indicating excellent intra- and inter-assay reproducibility of the established assay (Table 6).

3.4. Analysis of nasal swab samples from experimentally infected horses using EIV H3N8 subtype specific iiRT-PCR assay

EIV H3N8 subtype specific iiRT-PCR was compared with a influenza A universal rRT-PCR assay developed by Centers for Disease Control and Prevention (CDC) for its diagnostic accuracy in detecting EIV RNA isolated from randomized equine clinical samples. Viral RNA extracted from 56 nasal swab samples collected from horses that were infected experimentally with EIV (A/equine/Kentucky/7/07, H3N8) were tested with both assays in parallel. Both pre- and post-inoculation samples were included in the study. Of these samples, 42 tested positive for EIV virus RNA, whereas 14 samples that were collected prior to experimental inoculation were negative by both assays (Supplementary Table 2 and Table 7). These data suggest clearly that the iiRT-PCR assay has the same sensitivity and specificity as the universal rRT-PCR in detecting EIV in equine swab samples.

Table 7
Clinical sensitivity and specificity of iiRT-PCR as compared to previously validated universal rRT-PCR assay by CDC.

		rRT-PCR		
		Positive	Negative	Total
iiRT-PCR	Positive	42	0	42
	Negative	0	14	14
	Total	42	14	56

Table 8
Comparison of H3N8 iiRT-PCR Reagents in ABI 7500 and POCKIT™ System using A/equine/Miami/1/63 viral RNA.

Dilution factor	ABI 7500 (Ct)		POCKIT™ results
Undiluted	20.5	21.6	2/2
10^{-1}	23.3	23.0	2/2
10^{-2}	26.8	26.7	2/2
10^{-3}	29.8	29.6	2/2
10^{-4}	33.8	34.1	2/2
10^{-5}	36.0	37.9	2/2
10^{-6}	39.2	–	0/2
10^{-7}	–	–	0/2

3.5. Testing H3N8 iiRT-PCR reagents in ABI 7500 and POCKIT™ systems

The RNA extracted from 10-fold serial dilutions (undiluted to 10^7) of allantoic fluid containing the prototype virus EIV (A/equine/Miami/1/63, H3N8) was tested with iiRT-PCR reagents on both ABI 7500 Fast and POCKIT™ systems. Interestingly, iiRT-PCR reagents on ABI 7500 Fast instruments provided sensitivity equivalent to that of the POCKIT™ system (Table 8).

4. Discussion

The H3N8 subtype specific iiRT-PCR assay showed a high analytical sensitivity with $LoD_{95\%}$ of 11 copies of HA3 RNA molecules

Table 6
Reproducibility assays (intra- and inter-assay variability of iiRT-PCR).

1st run	A/Equine/Miami/63[b]						Negative	Positive
Result	(+)	(+)	(+)	(+)	(+)	(+)	(−)	(+)
s/n[a]	3.73	3.59	4.23	3.68	3.60	3.83	0.96	3.57
2nd run	A/Equine/Miami/63[b]						Negative	Positive
Result	(+)	(+)	(+)	(+)	(+)	(+)	(−)	(+)
s/n	3.86	3.67	3.83	3.66	3.54	3.40	0.94	3.41
3rd run	A/Equine/Miami/63[b]						Negative	Positive
Result	(+)	(+)	(+)	(+)	(+)	(+)	(−)	(+)
s/n	3.54	3.66	3.38	3.68	3.65	3.59	0.97	3.72

[a] Signal-to-noise ratios.
[b] 10^{-3} dilution sample analyzed in Table 4.

per reaction. Interestingly, the iiRT-PCR had two logs higher sensitivity than the validated rRT-PCR assay targeting the NP gene to detect EIV in clinical specimens. Similarly the iiRT-PCR reagents could detect at least two logs less virus when run on the ABI 7500 Fast machine. Taken together these data demonstrated clearly that iiRT-PCR reagents could detect at least two logs less virus present in clinical specimens than the rRT-PCR targeting the NP gene of EIV H3N8. Greater sensitivity widens the window of detection because virus shedding as detected by RT-PCR of nasal or nasopharyngeal swabs often declines by 1–2 orders of magnitude within a few days of its peak early after appearance of clinical signs (Paillot et al., 2013). Furthermore, the iiRT-PCR assay was highly specific for EIV and CIV H3N8 strains, as it did not amplify the HA genes from other influenza subtypes (H1N1, H6N2, and H7N7; Table 4) and did not cross react with other common equine viral pathogens (EHV-1–5, ERAV, ERBV, Salem virus, and EAdV-1). Analysis of clinical samples showed that the H3N8 iiRT-PCR assay was in 100% agreement with a universal rRT-PCR using nasal swab samples collected from experimentally infected horses.

One of the major advantages of iiRT-PCR is its simple protocol. Assembly of the reaction involves only three simple steps, namely rehydration of the lyophilized reagents, addition of sample nucleic acids, and transfer of reaction mixture into reaction tubes and placing on the POCKIT™ Nucleic Acid Analyzer. The reaction can be completed in 1 h in a portable POCKIT™ device. In combination with a quick nucleic acid extraction kit from GeneReach, it takes less than 90 min to generate results for a sample. The template RNA could be prepared either by column or magnetic bead based purification methods. The cubee™ Mini-Centrifuge along with RNA extraction reagents are included in the POCKIT™ Xpress™ Portable PCR Platform (GeneReach USA; http://genereach-us.com) for on-site testing of clinical specimens. Another advantage of the POCKIT Xpress™ Portable PCR Platform is that it can detect the target-derived amplicon without post amplification manipulations. Recently, an RT-LAMP assay has also been reported for the detection of EIV H3N8 subtype RNA in clinical specimens (Nemoto et al., 2011). Although the LAMP assays could be performed in a simple incubator under isothermal conditions, the PCR products are detected by turbidity using a real-time turbidimeter. However, turbidity signals derived from non-specific amplicons are likely to raise risks of false-positive results. In contrast, signals are derived from amplicon-specific probe hydrolysis in the H3N8 iiRT-PCR assay, reducing the risks of detecting non-specifically amplified products significantly. Furthermore, the POCKIT™ Nucleic Acid Analyzer, approved by OIE, is capable of converting results automatically to "+", "−", or "?", which are shown on the display screen at the end of the program.

A diagnostic assay allowing point-of-need detection of EIV would aid in efficient and timely management of the EI outbreaks. Keeping the reaction tubes closed during the whole process reduces the risks of carry-over contamination with the iiPCR significantly. The simple automatic detection module of POCKIT™ also makes the method user friendly. In addition, iiPCR assays prepared in lyophilized format could be shipped at ambient temperatures and stored for at least 2 years, reducing shipping and storage costs greatly for veterinary clinics and diagnostic laboratories. The POCKIT Xpress™ Portable PCR Platform along with lyophilized H3N8 iiPCR reagents (GeneReach USA) provides very high specificity and higher sensitivity than the H3N8 rRT-PCR assay and has the potential to be used by farms, race tracks, veterinary clinics, and veterinary diagnostic laboratories for routine point-of-need diagnosis of EIV infection in horses. Furthermore, the H3N8 iiRT-PCR assay can be used to detect CIV in clinical specimens. In summary, it provides a simple, rapid, highly sensitive, and specific point-of-need diagnostic assay for the detection of both EIV and CIV nucleic acids in clinical specimens.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2014.06.016.

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