Short communication

Translation of a laboratory-validated equine herpesvirus-1 specific real-time PCR assay into an insulated isothermal polymerase chain reaction (iiPCR) assay for point-of-need diagnosis using POCKIT[TM] nucleic acid analyzer

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ABSTRACT

Equine herpesvirus myeloencephalopathy (EHM), a major problem for the equine industry in the United States, is caused by equine herpesvirus-1 (EHV-1). In addition, EHV-1 is associated with upper respiratory disease, abortion, and chorioretinal lesions in horses. Here we describe the development and evaluation of an inexpensive, user-friendly insulated isothermal PCR (iiPCR) method targeting open reading 30 (*ORF30*) to detect both neuropathogenic and non-neuropathogenic strains on the field-deployable POCKIT[TM] device for point-of-need detection of EHV-1. The analytical sensitivity of the EHV-1 iiPCR assay was 13 genome equivalents per reaction. The assay did not cross react with ten non-target equine viral pathogens. Performance of the EHV-1 iiPCR assay was compared to two previously described real-time PCR (qPCR) assays in two laboratories by using 104 archived clinical samples. All 53 qPCR-positive and 46 of the 51 qPCR-negative samples tested positive and negative, respectively, by the iiPCR. The agreement between the two assays was 95.19% (confidence interval 90.48–99.90%) with a kappa value of 0.90. In conclusion, the newly developed EHV-1 iiPCR assay is robust to provide specificity and sensitivity comparable to qPCR assays for the detection of EHV-1 nucleic acid in clinical specimens.

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Equine herpesvirus-1 (EHV-1) is a double-stranded DNA virus that infects the vast majority of the world's equine populations (Allen et al., 2004). Depending on host and viral factors, exposure to EHV-1 can result in respiratory disease, abortion, neonatal deaths, and neurologic disease (equine herpesvirus myeloencephalopathy [EHM]) (Boehmer and Nimonkar, 2003; Borchers et al., 2006).

Recently, a link between EHV-1 infection and chorioretinal lesions in horses has been established (Hussey et al., 2013). Following natural infection, EHV-1 establishes life-long latent infection in the neurons within the trigeminal ganglia and/or lymphocytes in lymphoreticular tissues in a high percentage of infected horses (Allen et al., 2004). Reactivation of latent virus can lead to recrudescence of disease with associated viral shedding that may result in transmission of EHV-1 to susceptible in-contact horses (Allen and Breathnach, 2006; Allen et al., 2004). Although it appears that all EHV-1 strains can induce respiratory disease and abortion, only certain strains (neuropathogenic) have the potential to cause wide-scale outbreaks of EHM (Allen and Breathnach, 2006; Nugent et al., 2006). Within the past decade, a single nucleotide polymorphism (SNP) associated with the neuropathogenic or non-neuropathogenic phenotype of EHV-1 has been identified (Crabb

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et al., 1995; Nugent et al., 2006). The clinical signs of EHV-1-related respiratory disease can mimic those caused by other equine viral (e.g. equine influenza virus and equine arteritis virus) and bacterial (*Streptococcus pneumoniae*, *S. zooepidemicus*, *Actinobacillus* spp) respiratory pathogens, (Balasuriya et al., 2015; Powell, 1991; Wood et al., 2005). Similarly, EHV-1-induced neurologic disease must be differentiated from diseases caused by other infectious (equine protozoal myeloencephalitis, rabies, arboviral encephalitides, bacterial meningoencephalitis, etc.) and non-infectious causes (trauma, cervical compression myelopathy [wobbler syndrome], etc.). When confronted with a disease outbreak, confirmation of a provisional clinical diagnosis by means of a rapid, sensitive, and specific laboratory test(s) is essential to ensure that appropriate biosecurity and quarantine measures can be implemented without delay (Balasuriya et al., 2015).

Laboratory diagnosis of various clinical forms of EHV-1 infection is done either by direct detection of virus (virus isolation [VI]) and/or demonstration of viral antigen or viral nucleic acid detection or indirectly through serologic evidence of recent infection (Balasuriya et al., 2015). VI is labor intensive, time consuming, and can be subject to contamination. Thus, PCR based methods have largely superseded VI in most diagnostic laboratories which test specimens for EHV-1. Particularly, real-time PCR assays, have advantages of short turn-around time, and high specificity and sensitivity and have been accepted for routine EHV-1 detection and identification (Balasuriya et al., 2015). However, the requirement of sophisticated equipment and trained technicians has certainly prevented their application in resource-limited settings. Consequently, simple and rapid methods are needed to meet the needs of point-of-need EHV-1 detection. For this purpose, loop-mediated isothermal amplification (LAMP) assays aimed for field detection of EHV-1 have been reported (Nemoto et al., 2011; Nemoto et al., 2010). LAMP assay could be performed in a simple heating block. Nemoto et al. (2011) described a LAMP assay that could be performed without the nucleic acid extraction from the clinical specimens.

In addition, an inexpensive, user-friendly, and portable PCR system for animal point-of-need/point-of-care (PON/POC) testing has been available recently (POCKIT[TM] Nucleic Acid Analyzer, GeneReach USA, Lexington, MA, USA; approximately US$8000 per system) (Chang et al., 2012; Tsai et al., 2012). This system relies on fluorescent probe hydrolysis-insulated isothermal PCR (iiPCR) driven by natural liquid convection in a capillary tube heated at the bottom (Chang et al., 2012; Tsai et al., 2014; Tsai et al., 2012). The convection helps cycle the reaction components sequentially through different temperature zones to achieve the 3 stages (denaturation, annealing, and extension) of the PCR. The iiPCR reactions and data processing are completed without the needs of post-amplification processing to generate qualitative positive or negative results in less than one hour. Several assays based on this system have been demonstrated to provide sensitivity and specificity equivalent to those of reference real-time PCR (qPCR) or nested PCR for the detection of a significant number of animal and human viral pathogens including equine influenza virus and equine arteritis virus (Ambagala et al., 2015; Balasuriya et al., 2014; Carossino et al., 2016; Chua et al., 2016; Go et al., 2016; Kuo et al., 2016; Lung et al., 2015; Soltan et al., 2016; Tsai et al., 2014; Tsen et al., 2013; Wilkes et al., 2015a; Wilkes et al., 2015b; Wilkes et al., 2014; Zhang et al., 2016).

The primary objective of this study was to incorporate primers and probe targeting a conserved sequence in the open reading frame 30 (ORF30, DNA polymerase gene) from a previously described EHV-1 qPCR assay (Leutenegger et al., 2008) into the iiPCR system to establish a PON/POC assay for the detection of both EHV-1 genotypes (neuropathogenic and non-neuropathogenic) in clinical samples using the field-deployable POCKIT[TM] device. The SNP associated with the neuropathogenic and non-neuropathogenic genotypes could be differentiated by the two probes in the qPCR assay. However, preliminary test results (not shown) showed that this could not be achieved in iiPCR, likely due to that the annealing step of PCR is carried out in a range of temperatures in convective PCR. Consequently, the EHV-1 iiPCR contained only one probe and yet could detect all EHV-1 strains. Here we would like to stress that the 56-bp amplicon was detected by a probe labeled with 6-carboxyfluorescein (FAM) at the 5′ end, and a minor groove binding group (MGB) with a non-fluorescent quencher (NFQ) at the 3′ end (Applied Biosystems, Foster City, CA, USA). The final assay reaction contained $0.25\,\mu M$ forward primer, $0.5\,\mu M$ reverse primer, $0.1\,\mu M$ probe, and 120 U Taq DNA polymerase (BioMi, Taichung, Taiwan). The components were lyophilized and reconstituted right before the reaction with $50\,\mu L$ of Premix Buffer B (GeneReach USA). After mixing $5\,\mu L$ of the nucleic acid sample with $50\,\mu L$ of the reconstituted iiPCR solution, $50\,\mu L$ of the final mixture was transferred to an R-tube[TM] (GeneReach USA). The tube was spun briefly in a cubee[TM] mini centrifuge (GeneReach USA) and placed into the POCKIT[TM] device to complete iiPCR amplification and detection.

Performance of the EHV-1 iiPCR was compared with two different qPCR assays (Table 1) used at the Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY (GERC-UK) (Smith et al., 2012) and the Animal Disease Diagnostic Laboratory, Ohio Department of Agriculture, Reynoldsburg, OH (ADDL-ODA) (Leutenegger et al., 2008). The assays were run independently in the two laboratories in parallel with the iiPCR. Both reference qPCR assays could discriminate between the EHV-1 neuropathogenic and non-neuropathogenic genotype based on the *ORF30* A_{2254} and G_{2254} polymorphisms by using target-specific TaqMan probes (Table 1). The qPCR at GERC-UK was performed as previously described by Smith et al. (2012). Briefly, for a 25-μl reaction, $5\,\mu L$ of DNA template was combined with $20\,\mu L$ of the master mix composed of the following: $1.25\,\mu L$ of the A_{2254} primer/probe mix (400 nM of each primer and 200 nM of the probe), $1.25\,\mu L$ of the G_{2254} primer/probe mix (400 nM of each primer and 175 nM of the probe), $12.5\,\mu L$ of QuantiTect Multiplex PCR Master Mix (Qiagen, Valencia, CA, USA), and $5\,\mu L$ of nuclease-free water. Using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems), the following thermocycling conditions were used with the Fast 7500 mode: initial denaturation at $95°$ C for 15 min, followed by 40 cycles at $95°$ C for 15 s and $58°$ C for 1 min. For the qPCR at ADDL-ODA laboratory, the 12-μL reaction contained $5\,\mu L$ DNA template, 400 nm of each primer, 80 nM TaqMan probe, and $6\,\mu L$ of Universal TaqMan Mastermix with AmpErase UNG (Applied Biosystems) (Leutenegger et al., 2008). Reactions were performed in a 7500 Fast Real-Time PCR System (Applied Biosystems) using the thermal cycling program including 2 min at $50°$ C, 10 min at $95°$ C, and 40 cycles of 15 s at $95°$ C and 60 s at $60°$ C. The new EHV-1 iiPCR described here also targeted the same region in the *ORF30* of EHV-1 as the qPCR assays used for comparison (Table 1).

The specificity of the EHV-1 iiPCR was determined by using 11 archived frozen ($-70°$C) tissue culture fluid (TCF) samples containing either neuropathogenic (G_{2254}; n = 8) or non-neuropathogenic ($A_{2254\,[wild\,type]}$; n = 3) strains of EHV-1 and one EHV-1 vaccine strain (modified live virus vaccine [Rhinomune[®], Boehringer Ingelheim Vetmedica, St. Joseph, MO, USA]) (Table 2). The prototype non-neuropathogenic T262 and neuropathogenic T953 (Findlay) strains were included. The exclusivity panel of viruses included TCF containing other members of the family *Herpesviridae* known to infect horses (EHV-2, EHV-3 [ATCC V*R*-352] [ATCC, Manassas, MA, USA], EHV-4 [ATCC VR-2230], and EHV-5 [Bell et al., 2006]). In addition, 16 samples representing ten other common equine pathogens, including equine rhinitis virus A (NVSL-0600EDV8501) and B

Table 1
Primers and probes used in the qPCR and iiPCR assays.

Assay	Target gene	Name	Location (nt)[a]	Sequence (5′–3′)	Comments
EHV-1 E_1 allelic-discrimination qPCR assay (Smith et al., 2012)	ORF30	E1Fwd	2229 – 2245	TCTGGCCGGGCTTCAAC	Forward primer
		E1Rev	2284 – 2266	TTTGGTCACCCACCTCGAA	Reverse primer
		$E1Pr_{A2254}$	2247 – 2262	5HEX-ATCCGTCAACTACTCG-BHQ2a	Probe for non-neuropathogenic strain
		$E1Pr_{G2254}$	2247 – 2262	6FAM-ATCCGTCGACTACTCG-BHQ1	Probe for neuropathogenic strain
EHV-1 allelic-discrimination qPCR assay (Leutenegger et al., 2008)	ORF30	EHV1-29f	2228–2245	ATCTGGCCGGGCTTCAAC	Forward primer
		EHV1-82r	2281–2263	GGTCACCCACCTCGAACGT	Reverse primer
		EHV1N NP	2247–2262	VIC-ATCCGTCAACTACTCG	Probe for non-neuropathogenic strain
		EHV1N P	2247–2262	6FAM-ATCCGTCGACTACTCG	Probe for neuropathogenic strain
EHV−1 iiPCR[b]	ORF30	EHV1 iiF	2228–2245	ATCTGGCCGGGCTTCAAC	Forward primer
		EHV1 iiR	2281–2263	GGTCACCCACCTCGAACGT	Reverse primer
		EHV1 iiP	2247–2262	6FAM-ATCCGTCGACTACTCG-MGB-NFQ	Probe

[a] Nucleotides were numbered based on GenBank accession number KC924819.
[b] The primers and probe are identical to Leutenegger et al. (2008).

Table 2
Specificity analysis of EHV-1 specific iiPCR.

Target		Sample information	iiPCR	qPCR (C^T)		qPCR(CT)
				G_{2254}	A_{2254}	
Equine herpes virus-1	G_{2254}	EHV-1 T953	+	33.55		GERC-UK
		EHM isolate nEHV-1	+	22.7	–	ADDL-ODA
		EHM isolate nEHV-1	+	22.5	–	ADDL-ODA
		EHM isolate nEHV-1	+	33.4	–	ADDL-ODA
		EHM isolate nEHV-1	+	20.8	–	ADDL-ODA
		(2258 subset[a]) Abortion isolate nEHV-1	+	28.4	–	ADDL-ODA
		(2258 subset[a]) Abortion isolate nEHV-1	+	21	–	ADDL-ODA
		EHV-1 PEL	+	15.55	–	ADDL-ODA
	A_{2254}	EHV-1 T262	+	–	18.51	GERC-UK
		Abortion isolate wild type EHV-1	+	28.7	28.9	ADDL-ODA
		Abortion isolate wild type EHV-1	+	21.8	21.9	ADDL-ODA
		EHV−1 MLV vaccine strain[a]	+	25.00	23.58	ADDL-ODA
Equine herpes virus-2	EHV-2 isolate		–	–	–	ADDL-ODA
	EHV-2 HESKA		–	–	–	GERC-UK
Equine herpes virus-3		EHV-3 VR352	–	–	–	GERC-UK
Equine herpes virus-4	EHV-4 isolate		–	–	–	ADDL-ODA
	EHV-4 VR2230		–	–	–	GERC-UK
	EHV-4 isolate		–	–	–	GERC-UK
Equine herpes virus-5	EHV-5 isolate		–	–	–	ADDL-ODA
	EHV-5 KD-05		–	–	–	GERC-UK
Equine rhinitis virus A		ERAV (NVSL)	–	–	–	GERC-UK
Equine rhinitis virus B		ERBV (NVSL)	–	–	–	GERC-UK
Salem virus		Salem Virus	–	–	–	GERC-UK
Equine arteritis virus		EAV KY84	–	–	–	GERC-UK
Equine adenovirus		E9 EAdV (NVSL)	–	–	–	GERC-UK
Equine influenza virus	EIV Ohio (H3N8)		–	–	–	GERC-UK
	EIV KY2 EP-3 (H3N8)		–	–	–	GERC-UK
	EIV NY (H3N8)		–	–	–	GERC-UK

iiPCR, insulated isothermal PCR; qPCR, real-time PCR; nEHV-1, neuropathogenic EHV-1; and GERC-UK, Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY; ADDL-ODA, Animal Disease Diagnostic Laboratory, Ohio Department of Agriculture, Reynoldsburg, OH.

[a] EHV-1 vaccine strain (modified live virus vaccine [Rhinomune®, Boehringer Ingelheim Vetmedica, St. Joseph, MO].

(NVSL-0610EDV85010); equine adenovirus 1 (NVSL-001EDV8401) and 2; equine influenza virus type A1 (equine-1/Prague/1/56 [H7N7; ATCC VR-297) and A2 (equine/Miami1/63/[H3N8; NVSL-060IDV0501]; equine/Kentucky/81 [H3N8; NVSL-040IDV0001]; equine/Alaska/29759/91 [H3N8; NVSL-020IDV9101); equine arteritis virus [EAV, KY84], and Salem virus, a novel paramyxovirus of horses (Glaser et al., 2002), were tested (Table 2). Nucleic acids from TCF were extracted using the Ambion® MagMAX™ Total Nucleic Acid Isolation Kit (Applied Biosystems) as previously described (Smith et al., 2012) and stored at −80°C until further use. With the EHV-1 iiPCR and both qPCR assays, the 11 EHV-1 samples tested positive and all samples in the exclusivity panel gave negative results (Table 2), demonstrating that the established reaction was highly specific for EHV-1.

Analytical sensitivity of the EHV-1 iiPCR was determined initially with 10-fold serial dilutions of a plasmid DNA containing the target sequence in ORF30 (nt2731 – 53131, GenBank accession number AY665713). Copy numbers of the plasmid were determined by a spectrophotometer (NanoDrop 2000, Thermo Fisher, Suwanee, GA, USA). Positive results were obtained in the iiPCR reactions containing 1000 (7/7, 100%), 100 (21/21, 100%), 50 (21/21, 100%), and 10 (16/21, 76.19%) copies of the plasmid DNA. The ten no-template controls were negative by the EHV-1 iiPCR. Probit analysis (95% confidence interval) determined the analytical sensitivity of the assay to be 13 copies per reaction. Analytical sensitivity of the EHV-1 iiPCR in detecting a neuropathogenic EHV-1 T953 (Findlay, G_{2254}) and a non-neuropathogenic T262 (A_{2254}) strain was further evaluated by comparison with that of the two reference qPCR assays at the GERC-UK (Smith et al., 2012) and the ADDL-ODA laboratories (Leutenegger et al., 2008). Nucleic acid extracts were prepared from 10-fold serial dilutions of TCF using the Ambion® MagMAX™ Total Nucleic Acid Isolation Kit (Applied Biosystems) and tested in triplicate by all three assays side-by-side to compare their detection limit. The lowest dilution with positive results in

Table 3

Analytical sensitivity comparison between EHV-1 ii PCR and two allelic discrimination qPCR assays using viral genomic DNA of non-neuropathogenic and neuropathogenic strains.

Lab	Log10 (Dilutions)	EHV-1 T953 (Findlay, G_{2254})						EHV-1 T262 (A_{2254})					
		iiPCR			qPCR[a] (C^T)			iiPCR			qPCR[b] (C^T)		
GERC-UK	3	+	+	+	33.55	33.45	33.54	+	+	+	30.06	30.06	30.07
	4	**+**	**+**	**+**	**36.61**	**36.98**	**36.46**	+	+	+	33.45	33.18	33.28
	5	–	–	+	–	–	39.45	**+**	**+**	**+**	**36.53**	**37.17**	**36.35**
	6	–	–	–	–	–	–	+	–	–	–	39.61	–
	7	–	–	–	–	–	–	–	–	–	–	–	–
ADDL-ODA	3	**+**	**+**	**+**	**33.69**	**33.81**	**33.82**	+	+	+	28.91	28.99	29.11
	4	–	–	+	36.37	–	35.35	**+**	**+**	**+**	**32.30**	**32.25**	**32.62**
	5	–	–	–	36.78	–	–	–	–	–	–	38.07	35.53
	6	ND	ND	ND	–	–	35.64	–	–	–	38.12	–	39.12

iiPCR, insulated isothermal PCR; qPCR, real-time PCR; GERC-UK, Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY; ADDL-ODA, Animal Disease Diagnostic Laboratory, Ohio Department of Agriculture, Reynoldsburg, OH.

+, positive.

–, negative.

ND, not determined.

[a] neuropathogenic EHV-1 signal (G_{2254}, FAM).

[b] non-neuropathogenic EHV-1 signal (A_{2254}, VIC).

all three repeats was considered as the detection limit. Compared to the GERC-UK qPCR, the EHV-1 iiPCR had equivalent sensitivity with the T953 (10^{-4} dilution) and T262 (10^{-5} dilution) strains (Table 3). Similarly, the iiPCR had detection limits comparable to that of the ADDL-ODA qPCR in detecting both T953 (10^{-3} dilution) and T262 (10^{-4} dilution) strains (Table 3). Therefore, the EHV-1 iiPCR had analytical sensitivity equivalent to those of the two previously described qPCR assays (Leutenegger et al., 2008; Smith et al., 2012).

Finally, we evaluated the performance of the newly established iiPCR for the detection of EHV-1 nucleic acids in 104 archived clinical samples. These were comprised of 71 nasal swabs, 4 buffy coat samples, 13 tissues samples from abortions (4 placental and 9 fetal tissues), and archived DNA from 16 confirmed EHV-1 abortions and were tested by the iiPCR along with the qPCR analysis at GERC-UK (Smith et al., 2012). Nucleic acids were extracted using the Ambion® MagMAX^TM Total Nucleic Acid Isolation Kit (Applied Biosystems). All 53 of the qPCR-positive samples were also positive by iiPCR; however, only 46 of the 51 samples negative by qPCR were negative by iiPCR (Table 4; Supplemental Table). Allelic discrimination qPCR showed that the 53 qPCR positive clinical samples were all non-neuropathogenic (A_{2254}) (C^T = 17.09–38.30) subtypes. Overall, compared to the qPCR method, the EHV-1 iiPCR had sensitivity of 100% (confidence interval [CI] 95%: 95.14–100%) and specificity of 90.20% (CI 95%: 81.08-99.31%). The agreement between the two methods was 95.19% (CI 95%: 90.48-99.90%) with an index of agreement (kappa value) of 0.90. The five discrepant nasal swab samples (sample identification numbers 36, 41, 68, 90, and 97) were retested in single reaction by the qPCR (Smith et al., 2012) and in triplicate by the iiPCR. All the samples were negative by qPCR, while one out of three was positive by iiPCR (Table 5). Thus, both assays had equivalent analytical sensitivity in detecting viral DNA (Table 3) and comparable clinical sensitivity in detecting viral nucleic acids in nasal swab samples (Tables 4 and 5).

Manual column-based (PetNAD^TM Nucleic Acid Co-prep kit, GeneReach USA) and automatic silica-coated magnetic bead-based nucleic acid extraction (taco^TM mini Automatic Nucleic Acid Extraction System, GeneReach USA) methods are available to allow PON applications of the iiPCR tests. However, some modifications to these methods may be necessary to make this assay more user friendly at the field level. Subsequently, in the United States this assay has to be accepted by the NAHLAN (National Animal Health Laboratory Network) to be used in racetracks, horse shows and farms as a PON to detect EHV-1 infection.

Table 4

EHV-1 detection in horse clinical samples: The comparison of qPCR and iiPCR results from testing clinical samples.

		qPCR[a]		Total
		Positive	Negative	
iiPCR	Positive	53	5	58
	Negative	0	46	46
	Total	53	51	104

iiPCR, insulated isothermal PCR; qPCR, real-time PCR.

[a] Smith et al., 2012.

Previously described LAMP assays could be used to conduct PON diagnostics during outbreaks of EHV-1 or EHM (Nemoto et al., 2011; Nemoto et al., 2010). The LAMP methodology has the advantage of having a simple nucleic acid extraction step, followed by isothermal amplification of the nucleic acid template at a constant temperature that does not require a thermal cycler where the amplified product is detected via photometry for turbidity caused by an increasing quantity of magnesium pyrophosphate precipitate in solution as a byproduct of amplification. However, LAMP tends to produce non-specific amplicons and detection of LAMP products via turbidity and generic fluorescent dyes could lead to false positive result interpretation. Furthermore, clinical performance of the available EHV-1 LAMP assays (Nemoto et al., 2011; Nemoto et al., 2010) was shown to have similar sensitivity conventional PCR that has a lower sensitivity than that of real-time PCR in general. Besides, analytical sensitivity and specificity of the EHV-1 LAMP assays were not verified. Thus, the EHV-1 specific iiPCR assay describe here is likely to have several advantages over the LAMP assays (i.e. better sensitivity and specificity).

Upon reactivation of latent infections, viral shedding could lead to horizontal EHV-1 transmission. Detection of both EHV-1 genotypes at veterinary clinics, racetracks, and breeding facilities helps alert the owners and trainers to take immediate precautions to minimize the risks of an EHV-1 outbreak. Interestingly, the comparison of the results from the allelic discrimination real-time PCR assays targeting ORF30 (A/G_{2254}) with clinical histories provided by the attending veterinarians had revealed a number of significant discrepancies between the genotype of ORF30 (A_{2254} or G_{2254}) and reported clinical findings (Pronost et al., 2010; Smith et al., 2012). Thus, it is important to identify EHV-1 nucleic acids in clinical samples regardless of their genotype (i.e. the EHV-1 iiPCR does not discriminate between the two genotypes but able detect both genotypes in clinical speciments). Offering a performance (speci-

Table 5
Analysis of samples with discrepant results between iiPCR and qPCR.

Sample		iiPCR				qPCR[a]			
No.	identification	readout			No.+/total	result	C^T		No.+/total	result
36	MB 1332 NS	+	−	−	1/3	Pos	ND	ND	0/2	Neg
41	MB 1338 NS	+	−	−	1/3	Pos	ND	ND	0/2	Neg
68	MB1202 NS	+	−	−	1/3	Pos	ND	ND	0/2	Neg
90	MB 1280 NS	+	−	−	1/3	Pos	ND	ND	0/2	Neg
97	MB 1240 NS	+	−	−	1/3	Pos	ND	ND	0/2	Neg

iiPCR, insulated isothermal PCR; qPCR, real-time PCR; C^T, threshold cycle, ND, not determined; +, positive; −, negative; Pos, positive; Neg, negative.

[a] Smith et al. (2012).

ficity and sensitivity) comparable to that of the reference qPCR assays within one hour, the newly developed iiPCR system, including the lyophilized EHV-1 iiPCR reagents along with the portable POCKIT^{TM} device, has the potential to be used at these PON for routine diagnosis of EHV-1 infection in horses. The iiPCR assay could be completed with a simple protocol, including rehydration of the lyophilized reagents, addition of sample nucleic acids, transfer of reaction mixture into reaction tubes, and placing of the tubes into the POCKIT^{TM}. Risks of carry-over contamination are minimal because no post amplification manipulations are required. Furthermore, lyophilized reagents could be shipped at ambient temperatures, minimizing shipping trouble and greatly reducing shipping costs for PON, especially in rural regions. This assay is specially designed to be used on a compatible field-deployable iiPCR instrument which is intended for use by trained personnel in veterinary clinics, racetracks, breeding facilities, and diagnostic laboratories. Thus, the method described herein provides a simple, rapid, and robust PON diagnostic assay for the detection of EHV-1 in clinical specimens under field conditions.

Competing interests

Acknowledgments

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.2016.12.010.

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