Journal of Virological Methods 234 (2016) 7–15

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Development and evaluation of a reverse transcription-insulated isothermal polymerase chain reaction (RT-iiPCR) assay for detection of equine arteritis virus in equine semen and tissue samples using the POCKIT™ system

Mariano Carossino[a], Pei-Yu A. Lee[b], Bora Nam[a], Ashley Skillman[a], Kathleen M. Shuck[a], Peter J. Timoney[a], Yun-Long Tsai[b], Li-Juan Ma[b], Hsiao-Fen G. Chang[b], Hwa-Tang T. Wang[b], Udeni B.R. Balasuriya[a,*]

[a] Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, College of Agriculture, Food and Environment, University of Kentucky, Lexington, KY, USA
[b] GeneReach USA, Lexington, MA, USA

Article history:
Received 18 September 2015
Received in revised form 11 February 2016
Accepted 12 February 2016
Available online 29 March 2016

Keywords:
Equine arteritis virus
Equine viral arteritis
Equine semen
Abortion
Real-time RT-PCR
Insulated isothermal RT-PCR

A B S T R A C T

Equine arteritis virus (EAV) is the causative agent of equine viral arteritis (EVA), a respiratory and reproductive disease of horses. Most importantly, EAV induces abortion in pregnant mares and can establish persistent infection in up to 10–70% of the infected stallions, which will continue to shed the virus in their semen. The objective of this study was to develop and evaluate a reverse transcription insulated isothermal polymerase chain reaction (RT-iiPCR) for the detection of EAV in semen and tissue samples. The newly developed assay had a limit of detection of 10 RNA copies and a 10-fold higher sensitivity than a previously described real-time RT-PCR (RT-qPCR). Evaluation of 125 semen samples revealed a sensitivity and specificity of 98.46% and 100.00%, respectively for the RT-qPCR assay, and 100.00% and 98.33%, respectively for the RT-iiPCR assay. Both assays had the same accuracy (99.2%, k = 0.98) compared to virus isolation. Corresponding values derived from testing various tissue samples (n = 122) collected from aborted fetuses, foals, and EAV carrier stallions are as follows: relative sensitivity, specificity, and accuracy of 88.14%, 96.83%, and 92.62% (k = 0.85), respectively for the RT-qPCR assay, and 98.31%, 92.06%, and 95.08% (k = 0.90), respectively for the RT-iiPCR assay. These results indicate that RT-iiPCR is a sensitive, specific, and a robust test enabling detection of EAV in semen and tissue samples with very considerable accuracy. Even though the RT-qPCR assay showed a sensitivity and specificity equal to virus isolation for semen samples, its diagnostic performance was somewhat limited for tissue samples. Thus, this new RT-iiPCR could be considered as an alternative tool in the implementation of EAV control and prevention strategies.

1. Introduction

Equine arteritis virus (EAV) is the causative agent of equine viral arteritis (EVA), a respiratory and reproductive disease of horses and other equids (Bryans et al., 1957; Doll et al., 1957). EAV is a small, enveloped virus with a positive-sense, single-stranded RNA genome that encodes for at least ten open reading frames (ORFs) (Snijder and Meulenberg, 1998). ORFs 6 and 7 encode for the major structural protein M and the nucleocapsid (N) protein, respectively and have been reported to be the most conserved genes in the viral genome (Balasuriya et al., 2013, 2004). However, previous studies have identified a conserved area in ORF 1b that encodes for the non-structural proteins (nsp) 9–12 (Balasuriya et al., 2004; Socha et al., 2015; Zhang et al., 2008). EAV is transmitted horizontally through respiratory secretions of acutely infected horses and through semen to naïve mares bred naturally or artificially to persistently infected carrier stallions (Balasuriya and MacLachlan, 2007; Timoney and McCollum, 1993). Most EAV

Abbreviations: EVA, equine viral arteritis; EAV, equine arteritis virus; VNT, virus neutralization test; OIE, World Organisation for Animal Health; ORF, open reading frame; RT-iiPCR, reverse transcription-insulated isothermal PCR; RT-qPCR, real-time RT-PCR; PFU, plaque-forming unit; EHV, equine herpesvirus; EMEM, Eagle's minimum essential medium; VBS, strain of EAV (ATCC® VR-796™); KY84, a virulent strain of EAV; FAM, 6-carboxyfluorescein; MGB, minor groove binder group; NFQ, non-fluorescent quencher; S/N, signal-to-noise ratio.

* Corresponding author.
E-mail address: ubalasuriya@uky.edu (U.B.R. Balasuriya).

http://dx.doi.org/10.1016/j.jviromet.2016.02.015
0166-0934/

infections are subclinical in nature, but occasional outbreaks occur and are characterized by influenza-like signs in adult horses, abortion, interstitial pneumonia or pneumoenteritis in young foals, and establishment of persistent infection in sexually mature stallions (Glaser et al., 1996; Johnson et al., 1991; Pronost et al., 2010; Timoney and McCollum, 1993; Vaala et al., 1992). Abortion rates during natural outbreaks of EVA vary from less than 10% to 71% of infected mares, and abortion can typically take place at any time between 3 and 10 months of gestation (Balasuriya, 2014; Cole et al., 1986; MacLachlan et al., 2000; Timoney and McCollum, 1993). Thus, outbreaks of EVA have the potential to cause severe economic losses for breeding farms. In addition, anywhere from 10 to 70% of EAV infected stallions can become persistently infected and will continue to shed the virus in their semen (Timoney and McCollum, 1993). Carrier stallions are the natural reservoir of EAV and ensure the maintenance of the virus in equine populations between breeding seasons (Timoney and McCollum, 1993; Timoney et al., 1986). The continued growth in international trade of horses and semen has served as a significant means of dissemination of EAV strains around the world (Balasuriya et al., 1998; Hullinger et al., 2001; Timoney, 2000b; Timoney and McCollum, 1993; Timoney et al., 1987, 1986). Identification of the carrier stallion is therefore of critical epidemiological importance in the prevention and control of EAV infections (Balasuriya and MacLachlan, 2004, 2007; Timoney, 2000a,b; Timoney and McCollum, 1993). Virus isolation (VI) is currently the World Organisation for Animal Health (OIE)-approved gold standard for the detection of EAV in semen and is the prescribed test for international trade (OIE, 2013, 2015). Most countries require stallions that have neutralizing antibodies to EAV and no prior history of vaccination be tested for EAV in semen prior to national and international movement.

The conserved regions within ORFs 1b, 6, and 7 have been successfully targeted to detect EAV nucleic acid using various molecular diagnostic assays. Different assays based on reverse transcription polymerase chain reaction (RT-PCR), RT-nested PCR, or real-time RT-PCR (RT-qPCR) have been developed for EAV detection in tissue culture fluid (TCF) and clinical samples including semen and nasal secretions (Balasuriya et al., 2002; Fukunaga et al., 2000; Gilbert et al., 1997; Lu et al., 2008; Mankoc et al., 2007; Ramina et al., 1999; St-Laurent et al., 1994; Starick, 1998; Westcott et al., 2003). In general, PCR-based assays are usually more sensitive and time-saving when compared to classical diagnostic assays such as virus isolation (VI), or when specific pathogens are difficult to isolate or culture *in vitro* (Espy et al., 2006). However, whether the nucleic acid-based assays can provide equivalent reliability to VI remains an important issue for EAV detection (Balasuriya et al., 2013). An RT-qPCR assay was shown to provide sensitivity comparable to VI for detecting EAV RNA in TCF, but reduced sensitivity when detecting EAV RNA in semen (Lu et al., 2008). Subsequently, the sensitivity of the RT-qPCR assay described by Balasuriya et al. (2002) was further improved to give equal or higher sensitivity to VI by Miszczak et al. (2011). Recently, a novel, unbiased amplification method using the isothermal Phi29 polymerase combined with a resequencing microarray was developed to detect and genotype EAV (Hans et al., 2015). This assay has shown a higher analytical sensitivity compared to the RT-qPCR developed by Balasuriya et al. (2002).

Rapid identification of abortigenic agents (*e.g.* EAV, equine herpesvirus 1 and 4 [EHV-1 and EHV-4]) is of primary importance on breeding facilities to minimize or prevent the risk of widespread abortion outbreaks. In this context, molecular based diagnostic assays constitute a useful tool that could provide highly sensitive and specific results within a very short time compared to traditional VI. Even though some studies assessed the efficacy of RT-PCR and RT-nested PCR for detection of EAV nucleic acid in tissue samples obtained from aborted fetuses (Starick, 1998; Szeredi et al., 2005), the number of samples evaluated was limited precluding the ability to validate these assays with certainty. Moreover, the performance of an EAV-specific RT-qPCR assay using nucleic acids extracted from tissue specimens has not been evaluated. Therefore, this study was undertaken to validate a molecular diagnostic assay based on RT-PCR for the detection of EAV nucleic acid in semen and tissue samples.

A rapid and user-friendly EAV nucleic acid detection test could facilitate rapid diagnosis of EAV in semen and cases of abortion, and thus reduce the risk of dissemination of the virus at time of breeding or embryo transfer in addition to controlling abortion outbreaks. The POCKIT™ system (GeneReach USA, Lexington, MA) was developed as a portable device for animal point-of-need testing based on an insulated-isothermal PCR (iiPCR) (Chang et al., 2012; Tsai et al., 2012a,b). The principle of the iiPCR is that target cDNA is amplified by cycling the reaction components through different temperature zones to achieve the denaturation, annealing, and extension steps of PCR (Chang et al., 2012; Tsai et al., 2012a). This is accomplished in a capillary vessel heated through the bottom end of the tube, and the reaction is completed in a short time (~1 h). Integration of fluorescent probe hydrolysis-based iiPCR with automatic detection of light signals by an optical module eliminated all post-amplification processing steps of PCR amplicons required for conventional, gel-based PCR assays (Tsai et al., 2014). Furthermore, it provides an automated interpretation of the results using a default algorithm that generates “positive” and “negative” readouts (Tsai et al., 2014, 2012b). Recently, it has been shown that the performance of iiPCR assays is comparable to nested PCR and real-time PCR in detecting white spot syndrome virus, equine influenza virus (H3N8 subtype), canine distemper virus, canine parvovirus, feline immunodeficiency virus, classical swine fever virus, and bluetongue virus (Ambagala et al., 2015; Balasuriya et al., 2014; Lung et al., 2015; Tsai et al., 2014; Wilkes et al., 2015a,b, 2014). In this study, we developed a point-of-care one-step RT-iiPCR assay targeting ORF 7 of the EAV genome. A previously described RT-qPCR (Miszczak et al., 2011) was evaluated in parallel with the RT-iiPCR for analytical sensitivity and specificity. Accuracy of both assays in detecting EAV in semen and tissue samples was also compared to the VI test. In addition, a column-based extraction method (PetNAD™ Nucleic Acid Co-prep kit [PetNAD™ Co-prep], GeneReach USA, Lexington, MA) was also compared to a miniaturized automatic extraction device (taco™ mini Automatic Nucleic Acid Extraction System [taco™ mini], GeneReach USA, Lexington, MA) based on magnetic bead-based technology for nucleic acid extraction from semen samples.

2. Materials and methods

2.1. Viruses, equine semen samples, tissue samples, and cells

A total of 28 TCF samples were included in the study. It was comprised of one sample containing the modified live virus (MLV) vaccine strain of EAV (ARVAC®), 26 samples containing North American and European strains of EAV, and a sample of mock infected TCF (Balasuriya et al., 1998, 1999a, 2004, 1999b, 1995; Hedges et al., 1999). All EAV-positive TCF samples used in the present study were previously confirmed in a one-way serum neutralization assay using polyclonal anti-EAV equine serum or an immunofluorescent assay using monoclonal antibodies to the major envelope glycoprotein GP5 of EAV (Glaser et al., 1995; OIE, 2013). To determine the specificity of these two PCR assays, TCF or bacterial culture medium containing the following equine viral pathogens were included in the study: EHV-1 (ATCC® VR-700™; ATCC, Manassas, MA), EHV-2 (ATCC® VR-701™), EHV-3 (ATCC® VR-352™),

EHV-4 (ATCC® VR-2230™), and EHV-5 (KD-05, (Bell et al., 2006)); equine rhinitis virus A (NVSL-0600EDV8501) and B (NVSL-0610EDV85010); equine adenoviruses 1 (NVSL-001EDV8401) and 2; equine influenza virus type A1 (A/equine/Prague/1/56 [H7N7], ATCC® VR-297™) and A2 (A/equine/Miami/63 [H3N8, NVSL-060IDV0501], A/equine/Kentucky/81 [H3N8, NVSL-040IDV0001], A/equine/Alaska/29759/91 [H3N8, NVSL-020IDV9101]); Salem virus, a novel paramyxovirus of horses (Glaser et al., 2002); *Leptospira* spp. (*L. interrogans* serovar Pomona type kennewicki and serovar Grippotyphosa [Maxwell H. Gluck Equine Research Center, Lexington, KY]); *Taylorella equigenitalis* (ATCC® 35865™); *Taylorella asinigenitalis* (ATCC® 700933™); *Streptococcus zooepidemicus;* and *Streptococcus equi* (Maxwell H. Gluck Equine Research Center, Lexington, KY).

A panel of archived semen samples (n = 125) from EAV carrier stallions that had been stored at −80 °C was tested in this study. These included 118 sequential semen samples from seven carrier stallions that were previously infected with the KY84 strain of EAV and 7 semen samples from stallions that were submitted to the EVA OIE Reference Laboratory at the Maxwell H. Gluck Equine Research Center, Lexington, KY. The experimental inoculation and establishment of the EAV carrier state along with collection of sequential semen samples have been previously described by Campos et al. (2014).

In addition, a total of 122 archived tissue samples (stored at −80 °C) collected at necropsy from aborted fetuses, foals, and the reproductive tract of EAV long-term carrier stallions were included in this study. Samples collected from aborted fetuses and foals (n = 94) were lung, liver, spleen, thymus, lymph nodes (mainly bronchial and mesenteric lymph nodes), kidney, heart, and fetal membranes (chorioallantoic and amniotic membranes). Samples collected from EAV long-term carrier stallions (n = 28) included testis, epididymis, vas deferens, accessory sex glands (ampulla, vesicular glands, prostate, and bulbourethral glands), kidney, and urinary bladder. The EAV strains associated with the occurrence of abortions or establishment of the carrier state in stallions from which tissues were used in this study included KY84, AZ87, NE89, PA96, MLV, and viruses responsible for the 2006–2007 US multistate outbreak (Balasuriya et al., 2004; Hedges et al., 1999; Zhang et al., 2010).

The high passage RK-13 cell line, designated as RK-13 (KY) and used from passage levels 399–409), was maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% ferritin-supplemented bovine calf serum, 1% penicillin and streptomycin, and 0.1% amphotericin B (1000 μg/ml). The overlay medium used for inoculated cultures was 0.75% medium viscosity carboxymethylcellulose (CMC; Sigma-Aldrich, St. Louis, MO) in supplemented EMEM.

2.2. Virus isolation

Isolation of EAV from equine semen samples was attempted in a high passage RK-13 cell line, RK-13 (KY), according to the standard laboratory protocol used by the OIE Reference Laboratory (OIE, 2013). Viral titers were calculated and expressed as plaque-forming units per ml of seminal plasma (PFU/ml). Similarly, VI from tissue samples was also attempted in RK-13 (KY) cells. Briefly, 10% tissue homogenates were prepared in 1X EMEM (1 g of tissue homogenized in a final volume of 10 ml 1X EMEM [Mediatech Inc., Herndon, VA]), and centrifuged at 2500*g* for 15 min at 4 °C. Serial ten-fold dilutions (10^{-1}–10^{-5}) were prepared in 1X EMEM, and 1 ml of each dilution was inoculated into each of two 25 cm^2 flasks containing confluent monolayers of RK-13 (KY) cells. Additionally, 1 ml of the undiluted 10% tissue homogenate was also inoculated in duplicate flasks. Flasks were incubated at 37 °C for 1 h before being overlaid with supplemented EMEM containing 0.75% CMC. Flasks were checked for the appearance of cytopathic effect (CPE) on days 3 and 4 post inoculation. If there was no detectable CPE, a second blind passage was performed on day 4 post inoculation into new RK-13 (KY) monolayers using 1 ml of TCF as inoculum and incubated for an additional 4 days. Tissue culture supernatants from both passages were harvested and stored at −80 °C to confirm the identity of the isolates in a one-way serum neutralization assay using polyclonal anti-EAV equine serum and the previously validated RT-qPCR assay described below. Monolayers were fixed and stained with a 1% crystal violet solution containing 1% neutral buffered formalin, washed, and plaques were counted to determine viral titers. Viral titers were calculated and expressed as plaque-forming units per gram of tissue (PFU/g).

2.3. Bacterial nucleic acid isolation

T. equigenitalis, T. asinigenitalis, Leptospira spp, *S. zooepidemicus,* and *S. equi* nucleic acid was extracted from cultures using Direct-zol™ RNA Kits (Zymo Research Corp., Irvin, CA) according to the manufacturer's protocol in order to determine the specificity of the RT-qPCR and RT-iiPCR assays.

2.4. Generation of in vitro transcribed RNA

The analytical sensitivity of the RT-iiPCR was determined using a dilution series (100, 50, 10, and 0 molecules/reaction) of *in vitro* transcribed (IVT) RNA containing a region in ORF7 of EAV genome (nt12313-12704 of GenBank accession number DQ846750). The IVT ORF7 RNA was stored at −80 °C until used. The concentration of the IVT ORF7 RNA (number of molecules per microliter) was calculated according to the following formula:

$$\text{No. of IVT ORF7 RNA molecules}/\mu\text{l} = \frac{\text{Avogadro number}\left(6.022 \times 10^{23}\right) \times \text{IVT ORF7 RNA concentration}\ \left(\text{g}/\mu\text{l}\right)}{\text{IVTO RF7 RNA molecular weight (g)}}$$

The dilution of RNA transcripts was carried out in 40 μg/μl of tRNA.

2.5. Nucleic acid extraction

The performance of three different nucleic acid extraction kits (two magnetic bead-based methods and one column-based method) was evaluated in this study. Initially, EAV nucleic acid was extracted from 125 equine semen and 27 TCF samples using *MagMAX*™-96 Viral RNA Isolation Kit (Ambion®, Life Technologies, Grand Island, NY). Subsequently, 14 semen samples were used to compare *MagMAX*™-96 Viral RNA Isolation Kit with a magnetic bead-based (taco™ mini) and a column-based (PetNAD™ Co-prep) extraction method. Each method of extraction was carried out according to the manufacturer's instructions. Semen and TCF samples were microcentrifuged at 13,800*g* for 2 min prior to nucleic acid extraction. The supernatants were subjected to the following extraction procedures: (A) For viral RNA extraction with *MagMAX*™-96 Viral RNA Isolation Kit, 50 μl of the supernatant was mixed with 150 μl of Total Lysis Buffer/Binding Buffer and subjected to the extraction procedure described in the manufacturer's user manual. Nucleic acids were eluted in 50 μl of Elution buffer and stored at −80 °C for future use. (B) For the taco™ mini, 200 μl of the supernatant was added into the first well of a taco™ Preloaded DNA/RNA Extraction plate (GeneReach USA) and subjected to the extraction steps as described in the manufacturer's user manual. Elution was performed with 200 μl of Elution buffer,

Table 1
Sequences of primers and probes used in the RT-qPCR and RT-iiPCR assays targeting the conserved ORF 7 of EAV genome.

Name	Sequence (5′ to 3′)	Target	Position[a]	Function	Reference
EAV7.53F	GGCGACAGCCTACAAGCTACA	ORF7	nt12365-12385	RT-qPCR primer	(Balasuriya et al., 2002)
EAV7.256R	CGGCATCTGCAGTGAGTGA	ORF7	nt12550-12568	RT-qPCR primer	(Balasuriya et al., 2002)
EAV7.92P	FAM-TTGCGGACCCGCATCTGACCAA-TAMRA	ORF7	nt12404-12425	RT-qPCR probe	(Balasuriya et al., 2002)
EAV_iiF	AGTTGGGTTCCTACCAAACAAAT	ORF7	nt12589-12611	RT-iiPCR primer	Current study
EAV_iiR	CACACAGGAGAATATCCACGTCTT	ORF7	nt12644-12667	RT-iiPCR primer	Current study
EAV_iiP	FAM-CAGCGCAAAGTTGCGCCT-MGB	ORF7	nt12613-12630	RT-iiPCR probe	Current study

[a] Nucleotide position is based on GenBank accession number DQ846750.

and stored at −80 °C for future use. (C) In the case of PetNAD™ Coprep, 100 μl of the supernatant was mixed with 300 μl of buffer PB1 for 1 min. After the addition of 300 μl of PB2, the 700 μl mixture was transferred into the spin column. Wash steps were performed as described in the manufacturer's user manual. Nucleic acids were eluted in 100 μl of elution buffer PB5. All nucleic acid preparations were stored at −80 °C prior to testing. In addition, viral nucleic acids were extracted from 10% tissue homogenates using the taco™ mini as indicated above, which allowed a higher input volume of sample (200 μl). Elution was performed with 200 μl of Elution buffer, and nucleic acids were stored at −80 °C for future use.

2.6. EAV ORF7 real-time RT-PCR (RT-qPCR)

The TaqMan® fluorogenic probe-based one-tube RT-qPCR assay targeting a 204 base pair region of EAV ORF7 (Table 1) was utilized as previously described (Balasuriya et al., 2002). The RT-qPCR reagents, primers, probe concentrations, and thermocycling conditions used in the detection of EAV nucleic acid by RT-qPCR using the QuantiFast kit (Qiagen, Valencia, CA) was identical to the method described by Miszczak et al. (2011). Briefly, the 25 μl reaction contained 12.5 μl of 2X QuantiFast Probe RT-PCR Master Mix, 0.25 μl QuantiFast RT Mix, 0.5 μl 50X ROX Dye Solution, 200 nM (final concentration) of TaqMan® fluorogenic probe, 800 nM (final concentration) of each primer, 5.45 μl of nuclease free water, and 5 μl of template RNA. Reverse transcription and amplification were carried out in an ABI 7500 Fast Real-time PCR System (Applied Biosystems®, Life Technologies, Grand Island, NY). The program included 10 min at 50 °C (reverse transcription step), 5 min at 95 °C, followed by 45 cycles at 95 °C for 10 s and 60 °C for 30 s.

2.7. EAV ORF7 RT-iiPCR with POCKIT™

The EAV RT-iiPCR was designed based on a previously described probe hydrolysis-based iiPCR method (Tsai et al., 2012b). The program of the POCKIT™ device includes a 42 °C/10 min step for reverse transcription and a 95 °C/30 min step for iiPCR. Reaction signals are processed by an optical detection module similar to the one described in the original iiPCR device (Chang et al., 2012; Tsai et al., 2012a) and are shown automatically on the display screen. Signal-to-noise (S/N) ratios were calculated by dividing light signals collected after iiPCR by those from before iiPCR (Tsai et al., 2012b). Results are converted automatically to "+" (positive), "−" (negative), or "?" (inconclusive), according to the default S/N thresholds of POCKIT™.

Nucleotide sequences (n = 263) of EAV ORF7 from the GenBank database were aligned to identify regions with a conserved sequence specific for EAV. The EAV virulent Bucyrus sequence was used as the reference strain (GenBank accession number DQ846750) for comparative nucleotide analysis (Balasuriya et al., 2007). RT-iiPCR primers and probes (Table 1) were designed with Primer Express software (Applied Biosystems®), following principles recommended for the iiPCR (http://www.iipcr.com/eweb/uploadfile/20130522114104277.pdf). Annealing temperatures of the probe were about 10 °C higher than those of the primers. Analysis with MFold program (http://mfold.rna.albany.edu/?q=mfold) was performed to avoid amplicons with major secondary structures. The final reaction mixture contained 1X Uni-ii HS Buffer (GeneReach USA), 0.5 μM of forward and reverse primers, 0.1 μM of the probe, 4 U of MMLV RTase (BioMi, Taichung, Taiwan), 2 U of ribonuclease inhibitor (Takara, Shiga, Japan), and 120 U of Taq DNA polymerase (BioMi). All components except for Uni-ii HS Buffer were freeze-dried (Qu et al., 2010) and stored at room temperature. After rehydrating the lyophilized pellet with 50 μl of Uni-ii HS Buffer, 5 μl of the sample nucleic acid was added to the reaction. Subsequently, 50 μl of the final mixture was transferred to an R-tube™ (GeneReach USA). The R-tube™ was sealed with a cap, spun for 10 s in a cubee™ centrifuge (GeneReach USA), and placed into a POCKIT™ device.

Table 2
Evaluation of the analytical sensitivity of EAV RT-iiPCR assay using EAV ORF 7 *in vitro* transcribed RNA (nt 12313-12704 of GenBank accession number DQ846750).

RNA copies/reaction	No. positive/No. tested	Rate (%)
100	20/20	100
50	20/20	100
10	19/20	95
0	0/18	0

2.8. Statistical analysis

Limits of detection with 95% confidence (LOD 95%) were determined by statistical probit analysis (a non-linear regression model) using commercial software SPSS 14.0 (SPSS Inc., Chicago, IL). Amplification efficiencies (%) were calculated using the following formula: $E = \left[10^{-1/\text{slope}} - 1\right] \times 100$. Sensitivity and specificity were determined by 2×2 contingency tables with 95% confidence intervals. The degree of agreement between two assays was assessed by calculating Cohen's Kappa (k) values.

3. Results

3.1. Analytical sensitivity of EAV RT-qPCR and RT-iiPCR

The primer and probe sets were designed to target a highly conserved region within EAV ORF7 for the RT-iiPCR (Table 1). Conditions including primer and probe sequences, primer, probe, dNTP, Taq DNA polymerase, and MMLV RT concentrations were optimized to establish the assays (see Section 2 for details). The analytical sensitivity of the RT-iiPCR was determined by using replicates of a dilution series containing 100, 50, 10 and 0 EAV IVT RNA transcripts. Probit analysis determined that the LOD 95% of the EAV RT-iiPCR was about 10 copies of EAV IVT RNA per reaction (Table 2).

3.2. Comparison of detection limits of RT-qPCR and RT-iiPCR

Detection limits of RT-qPCR and RT-iiPCR were compared side by side with RNA extracted from two EAV strains (highly virulent prototype VBS and strain KY84) using *MagMAX*™-96 Viral RNA

Table 3
Evaluation of the analytical sensitivity of the EAV RT-qPCR and RT-iiPCR assays using EAV RNA extracted from serial dilutions (10^0–10^{-10}) of the VBS and KY84 strains of EAV.

Dilutions	EAV VBS				EAV KY84			
	RT-qPCR (Ct value)		RT-iiPCR		RT-qPCR (Ct value)		RT-iiPCR	
10^0	15.4	15.2	ND	ND	14.0	13.8	ND	ND
10^{-1}	19.2	18.9	ND	ND	17.8	17.1	ND	ND
10^{-2}	22.9	22.7	ND	ND	21.1	21.1	ND	ND
10^{-3}	26.3	26.2	Pos	Pos	24.3	24.7	Pos	Pos
10^{-4}	29.5	29.4	Pos	Pos	28.0	27.6	Pos	Pos
10^{-5}	**32.7**	**32.7**	Pos	Pos	31.0	31.4	Pos	Pos
10^{-6}	Neg	36.0	**Pos**	**Pos**	**35.0**	**34.2**	Pos	Pos
10^{-7}	Neg	Neg	Neg	Neg	Neg	37.1	**Pos**	**Pos**
10^{-8}	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg
10^{-9}	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
10^{-10}	Neg	Neg	Neg	Neg	Neg	37.2	Neg	Neg

Limits of detection (LOD) are indicated in bold. Neg, negative; Pos, positive; ND, not determined; VBS, virulent Bucyrus strain of EAV (ATCC® VR-796); KY84, a moderately pathogenic strain of EAV.

isolation kit (Miszczak et al., 2011). RNA extractions were serially diluted (ten-fold, 10^0 to 10^{-10}) and tested in duplicate. The RT-qPCR assay detected EAV RNA in 10^{-5} and 10^{-6} dilutions prepared from VBS and KY84, respectively. Regression analysis indicated that good linearity was achieved with both strains (VBS: slope = −3.47, R^2 = 0.999; KY84: slope = −3.44, R^2 = 0.999) in the RT-qPCR. Amplification efficiencies of the RT-qPCR with VBS and KY84 were 93.96% and 95.32%, respectively. Similarly, the RT-iiPCR method detected EAV RNA in 10^{-6} and 10^{-7} dilutions prepared from VBS and KY84 virus strains, respectively (Table 3). These results indicate that the sensitivity of the RT-iiPCR was approximately 10-fold higher than that of the previously described RT-qPCR assay.

3.3. Specificity of the EAV RT-qPCR and RT-iiPCR

To evaluate the specificity of the RT-qPCR and the RT-iiPCR, viral RNA extracted from archived EAV strains from North America and Europe (n = 27), and the MLV vaccine strain were tested. The exclusivity panel was comprised of nucleic acids extracted from 20 other equine pathogens including EHV-1, -2, -3, -4, and -5, equine rhinitis A and B viruses, equine adenoviruses 1 and 2, equine influenza viruses (H7N7 and H3N8 subtypes), Salem virus, *Leptospira* spp. (*L. interrogans* serovar Pomona type kennewicki and serovar Grippotyphosa), *T. equigenitalis*, *T. asinigenitalis*, *S. zooepidemicus,* and *S. equi*. Both assays detected viral RNA extracted from all EAV strains, but not the nucleic acids of other equine pathogens in the exclusivity panel (Tables 4 and 5). These data clearly indicate that both RT-qPCR and RT-iiPCR assays were highly specific for EAV.

3.4. Evaluation of two viral RNA extraction methods for PCR-based detection of EAV in equine semen

Methods for viral nucleic acid extraction can have a significant effect on the sensitivity of molecular diagnostic assays. Therefore, the *MagMAX*™-96 Viral RNA isolation kit that has been previously verified for EAV RNA extraction from equine semen samples (Miszczak et al., 2011) was used as the reference method to evaluate a portable magnetic bead-based method (taco™ mini) and a column-based method (PetNAD™ Co-prep) for extraction of EAV nucleic acids in semen samples. The nucleic acids were extracted using all three methods in parallel and tested by the EAV RT-qPCR and the RT-iiPCR assays. Fourteen equine semen samples were included in the study. Among these were 11 VI-positive samples with viral titers ranging from 10^1 to $\geq 10^5$ PFU/ml, and three VI-negative samples from carrier stallions that stopped shedding

Table 4
Inclusivity test panel including the diverse North American and European strains of EAV used to evaluate the specificity of the EAV RT-qPCR and RT-iiPCR assays.

Sample ID	Clade	RT-qPCR (Ct value)	RT-iiPCR
VBS	North American	16.15	Pos
KY84	North American	20.15	Pos
CA95G	North American	19.31	Pos
EAV03011	North American	14.47	Pos
EAV MLV vaccine	North American	23.06	Pos
KY93TB	North American	22.70	Pos
IL93TB	North American	19.37	Pos
NE89	North American	20.45	Pos
MI93STB	North American	25.59	Pos
MT89	North American	27.47	Pos
KY77	North American	21.26	Pos
PA76	North American	23.20	Pos
IL93ARAB	North American	22.02	Pos
NEB88	North American	23.12	Pos
ALBCAN86	North American	22.07	Pos
CA95I	North American	21.19	Pos
M405	European	22.40	Pos
M477	European	23.80	Pos
M547	European	22.48	Pos
S-3685	European	23.79	Pos
S-3583	European	22.19	Pos
Austria68	European	20.44	Pos
KY63	European	26.31	Pos
Italy92	European	25.86	Pos
AZ87	European	25.87	Pos
CW01	European	27.99	Pos
CW96	European	26.43	Pos

Pos, positive.

Table 5
Exclusivity test panel including diverse equine viral and bacterial pathogens used to evaluate the specificity of the EAV RT-qPCR and RT-iiPCR assays.

Sample ID	RT-qPCR	RT-iiPCR
EHV-1 [ATCC® VR-700™]	Neg	Neg
EHV-2 [ATCC® VR-701™]	Neg	Neg
EHV-3 [ATCC® VR-352™]	Neg	Neg
EHV-4 [ATCC® VR-2230™]	Neg	Neg
EHV-5 KD-05	Neg	Neg
Equine rhinitis A virus (NVSL-0600EDV8501)	Neg	Neg
Equine rhinitis B virus (NVSL-0610EDV85010)	Neg	Neg
Equine adenovirus 1 (NVSL-001EDV8401)	Neg	Neg
Equine adenovirus 2	Neg	Neg
Equine influenza A1 (A/equine/Prague/1/56 [H7N7]; ATCC® VR-297™)	Neg	Neg
Equine influenza A2 (A/equine/Miami/63 [H3N8], NVSL-060IDV0501; A/equine/Kentucky/81 [H3N8], NVSL-040IDV0001; A/equine/Alaska/29759/91 [H3N8], NVSL-020IDV9101)	Neg	Neg
Salem virus	Neg	Neg
Leptospira spp. (*Leptospira interrogans* serovar Pomona type kennewicki and serovar Grippotyphosa)	Neg	Neg
Taylorella equigenitalis (ATCC® 35865™)	Neg	Neg
Taylorella asinigenitalis (ATCC® 700933™)	Neg	Neg
Streptococcus zooepidemicus	Neg	Neg
Streptococcus equi	Neg	Neg

Neg, negative.

EAV in their semen. Based on the RT-qPCR results obtained from the *MagMAX*™-96 extractions from VI-positive samples, a total of 7/11 (64%) PetNAD™ Co-prep extractions and 9/11 (82%) taco™ mini extractions generated comparable Ct values (ΔCt <1–2) while the remaining samples (4 and 2 PetNAD™ Co-prep and taco™ mini extractions, respectively) showed higher Ct values (ΔCt >2–4) (Table 6). Overall, the performance of the two extraction methods tested on VI-positive samples was comparable to that of the

Table 6
Performance evaluation of a column-based (PetNAD™) and an automatic magnetic bead-based (taco™ mini) nucleic acid extraction methods for PCR-based detection of EAV in equine semen samples as compared to the *MagMAX*™ automatic magnetic bead-based extraction method.

Sample ID	Virus isolation (PFU/ml)	PetNAD™		taco™ mini		*MagMAX*™
		RT-qPCR (Ct value)	RT-iiPCR	RT-qPCR (Ct value)	RT-iiPCR	RT-qPCR (Ct value)
L136	Pos (2.7×10^3)	25.62	Pos	24.43	Pos	24.87
L140(A)	Pos (2.3×10^3)	28.17	Pos	27.33	Pos	26.58
L141(A)	Pos (7.5×10^3)	24.07	Pos	23.11	Pos	22.33
L141(B)	Pos (1.0×10^1)	28.38	Pos	28.81	Pos	27.10
L139	Pos (3.5×10^1)	35.59	Pos	34.01	Pos	32.65
L140(B)	Pos (2.5×10^1)	26.98	Pos	27.08	Pos	26.04
S-5662	Pos (1.4×10^2)	34.21	Pos	34.21	Pos	33.17
S-5654	Pos (2.6×10^4)	31.17	Pos	31.91	Pos	27.84
S-5646	Pos (2.8×10^4)	28.10	Pos	28.03	Pos	25.78
S-5637 A/B	Pos ($\geq 1 \times 10^5$)	26.72	Pos	25.59	Pos	23.61
S-5636	Pos (3.5×10^3)	31.34	Pos	30.51	Pos	29.36
S-5661	Neg	Neg	Neg	Neg	Neg	Neg
S-5653	Neg	38.04	Pos	Neg	Neg	Neg
S-5647	Neg	Neg	Neg	38.06	Neg	Neg

Neg, negative; Pos, positive.

reference method, and side-by-side testing of PetNAD™ Co-prep and taco™ mini extractions with RT-qPCR and RT-iiPCR showed that both PCR assays generated equivalent qualitative results independently of the extraction method used (Table 6). Among the VI-negative samples from carrier stallions that stopped shedding of virus in their semen, PetNAD™ Co-prep extraction of S-5653 tested positive by qPCR and RT-iiPCR while taco™ mini extraction of S-5647 tested positive by RT-qPCR. In both cases, Ct values were >36. This shows that the detection of viral nucleic acid is possible after ceasing the shedding of infective virus in semen and thus, these PCR-based assays present a higher sensitivity than VI for the detection of EAV nucleic acid in semen samples.

3.5. Detection of EAV in semen samples by EAV RT-qPCR and RT-iiPCR

The RT-qPCR and RT-iiPCR assays were compared with the OIE prescribed VI test to evaluate their sensitivity and accuracy in detecting EAV in semen samples. For this purpose, RNA extracted from 125 archived sequential equine semen samples using *MagMAX*™-96 Viral RNA isolation kit was used. Of these, 65 samples were positive by VI in RK-13 (KY) cells with viral titers ranging from 2.0×10^1 to $\geq 1.0 \times 10^5$ PFU/ml of seminal plasma. The EAV RT-qPCR assay successfully detected EAV nucleic acid in 64/65 VI positive semen samples, while the total number of VI negative semen samples (60/60) were confirmed negative by the RT-qPCR (Table 7a and Supplementary Table 1). In comparison, the EAV RT-iiPCR assay successfully detected EAV nucleic acid in all the semen samples that tested positive by VI (65/65; Table 7b). However, this assay detected a positive amplification signal from a semen sample that was negative by VI (false positive [#98, Supplementary Table 1]). In summary, 64/65 and 65/65 semen samples were positive by the RT-qPCR and RT-iiPCR respectively as compared to VI. The sensitivity, specificity, and accuracy of the EAV RT-qPCR and RT-iiPCR were determined from 2×2 contingency tables as compared to VI (gold standard). Sensitivity and specificity were 98.46% (95% confidence interval [CI]: 93.56–100%) and 100% (95% CI: 95.68%–100%) for the EAV RT-qPCR (Table 7a) while the RT-iiPCR (Table 7b) showed 100.00% (95% CI: 96–100%) and 98.33% (95% CI: 93.05–100%), respectively. Both assays were found of equal accuracy (99.2% [95% CI: 96.59–100%], k = 0.98) when compared to VI. Therefore, the RT-qPCR and RT-iiPCR were in high agreement with VI in detecting EAV nucleic acid in semen samples. Finally, the RT-iiPCR was 98.4% in agreement with the RT-qPCR (Table 7c) with a kappa value of 0.97, thus confirming a substantial level of agreement between the RT-qPCR and RT-iiPCR in detecting EAV in semen samples.

Table 7
Contingency tables for the comparison of (a) the RT-qPCR and (b) the RT-iiPCR assays for the detection of EAV in equine semen samples with virus isolation (gold standard). (c) Contingency table for analysis of the level of agreement between the RT-qPCR and the RT-iiPCR assays for the detection of EAV in equine semen samples.

(a)

		Virus isolation		
		Positive	Negative	Total
RT-qPCR	Positive	64	0	64
	Negative	1	60	61
	Total	65	60	125

(b)

		Virus isolation		
		Positive	Negative	Total
RT-iiPCR	Positive	65	1	66
	Negative	0	59	59
	Total	65	60	125

(c)

		RT-qPCR		
		Positive	Negative	Total
RT-iiPCR	Positive	64	2	66
	Negative	0	59	59
	Total	64	61	125

3.6. Detection of EAV in tissue samples by EAV RT-qPCR and RT-iiPCR

A total of 122 tissue samples collected from aborted fetuses, foals, and EAV long-term carrier stallions were tested to evaluate clinical performance of the EAV RT-qPCR and RT-iiPCR assays side by side. The samples collected from aborted fetuses and foals (n = 94) were comprised of lung, liver, spleen, thymus, lymph nodes (mainly bronchial and mesenteric lymph nodes), kidney, heart, and fetal membranes (chorioallantoic and amniotic membranes). In contrast, the samples collected from EAV long-term carrier stallions (n = 28) included testis, epididymis, vas deferens, accessory sex glands (ampulla, vesicular glands, prostate, and bulbourethral glands), kidney, and urinary bladder. A total of 59 samples were EAV positive by VI, while 63 samples were negative (Supplemen-

Table 8
Contingency tables for the comparison of (a) the RT-qPCR and (b) the RT-iiPCR assays for the detection of EAV in equine tissue samples with virus isolation (gold standard). (c) Contingency table for analysis of the level of agreement between the RT-qPCR and the RT-iiPCR assays for the detection of EAV in equine tissue samples.

(a)

		Virus isolation		
		Positive	Negative	Total
RT-qPCR	Positive	52	2	54
	Negative	7	61	68
	Total	59	63	122

(b)

		Virus isolation		
		Positive	Negative	Total
RT-iiPCR	Positive	58	5	63
	Negative	1	58	59
	Total	59	63	122

(c)

		RT-qPCR		
		Positive	Negative	Total
RT-iiPCR	Positive	54	9	63
	Negative	0	59	59
	Total	54	68	122

tary Table 2). EAV positive samples had viral titers ranging from <10 to 8.5×10^8 PFU/g of tissue. Nucleic acids extracted by taco™ mini were subjected to RT-qPCR and RT-iiPCR analyses (Table 8). Using the RT-qPCR, 54 and 68 samples tested positive and negative, respectively. Seven of the VI positive samples were negative by RT-qPCR, all of which exhibited low ($\leq 1.5 \times 10^3$ PFU/g) or very low (100 to <10 PFU/g) infectivity titers. Four of these samples were accessory sex glands (ampulla, vesicular gland, and bulbourethral gland) collected from EAV long-term carrier stallions with viral titers ranging from 300 PFU/g to <10 PFU/g of tissue (Supplementary Table 2). In contrast, 2 of the VI negative samples were positive by the RT-qPCR (average Ct value = 31.46 and 37.56) and also by RT-iiPCR. When tested by the EAV RT-iiPCR method, 63 and 59 samples tested positive and negative, respectively. A single VI positive sample (viral titer of 1.5×10^3 PFU/g) tested negative by RT-iiPCR and 5 of the VI negative samples were RT-iiPCR positive. The sensitivity, specificity, and accuracy of both EAV RT-qPCR and RT-iiPCR assays were determined from 2×2 contingency tables as compared to VI (gold standard). Furthermore, Cohen's kappa values were determined to assess the magnitude of agreement between assays. The EAV RT-qPCR showed a sensitivity, specificity, and accuracy of 88.14% (95% CI: 79.23–97.04%), 96.83% (95% CI: 91.02%–100%), and 92.62% (95% CI: 87.63–97.62%), respectively (k = 0.85, Table 8a). On the other hand, the sensitivity, specificity, and accuracy of the EAV RT-iiPCR assay was 98.31% (95% CI: 92.93–100%), 92.06% (95% CI: 84.54–99.59%), and 95.08% (95% CI: 90.77–99.39%), respectively (k = 0.9, Table 8b). The findings using either the RT-qPCR or the RT-iiPCR were in high agreement with VI for the detection of EAV in equine tissue samples. Performance comparing the RT-iiPCR and RT-qPCR showed that all 54 RT-qPCR positive samples were also RT-iiPCR positive, but 7 of the RT-qPCR negative samples tested positive by RT-iiPCR (Table 8c). Six of the RT-qPCR negative and RT-iiPCR positive samples were also VI positive. Contingency analysis found 92.62% agreement (k = 0.85) between the results obtained with both assays, indicating that the performances of the RT-iiPCR and the RT-qPCR were in high agreement with each other. However, the RT-iiPCR had a higher sensitivity (98.31%) and a slightly higher agreement (k = 0.9) with VI as compared to the RT-qPCR assay.

4. Discussion

In this study, we developed an RT-iiPCR for the rapid detection of EAV nucleic acid in equine semen and tissues. Similar to the RT-qPCR, this assay is also based on TaqMan® probe hydrolysis to generate a fluorescent signal that requires no post-reaction handling of amplicons, thus reducing the risks of carry-over contamination of samples during laboratory testing. The analytical sensitivity of the RT-iiPCR was at least 10-fold more sensitive than the previously described RT-qPCR.

The RT-iiPCR is performed in a portable device (POCKIT™) using lyophilized reagents, and results are obtained after automatic data processing with a default algorithm, without the need for manual data analysis and interpretation as required for RT-qPCR assays. These allow its point-of-care use in veterinary clinics, on horse farms, and at racetracks. Thus, the use of RT-iiPCR point-of-care assay could allow the rapid detection of EAV nucleic acid in a variety of clinical and tissue samples from abortions. Thus, the use of this type of molecular diagnostic assay on breeding farms could facilitate the rapid identification of EAV related abortions. Similarly, its use at racetracks would allow the rapid and accurate diagnosis of outbreaks of respiratory disease and immediate identification of the causative agent (i.e. differential diagnosis of EVA from other respiratory diseases caused by equine influenza, EHV-1, EHV-4, among others).

Successful target amplification by PCR generally requires purification of nucleic acids from samples because *Taq* DNA polymerase is sensitive to various inhibitors present in different clinical samples (Schrader et al., 2012). The choice of the nucleic acid extraction method could result in significant variations in diagnostic sensitivity of an assay for EAV detection. Manual nucleic acid extraction methods are less expensive but more labor-intensive than automatic nucleic acid extraction methods. The reference *MagMAX*™ method demonstrated to achieve satisfactory purification of EAV RNA from equine semen samples (Miszczak et al., 2011). The manual column-based method (PetNAD™ Co-prep) and the field-deployable automatic extraction device (taco™ mini) can extract nucleic acid from 200 μl of sample volume, which could enhance the sensitivity of downstream assays. Therefore, the extraction efficiency of both methods was compared with that of the *MagMAX*™ Express method using the manufacturers' protocols. The RT-qPCR testing of nucleic acid extracts confirmed that both systems gave comparable results to the reference *MagMAX*™ Express method for the majority of the VI-positive samples evaluated (Table 6). Nucleic acids extracted from this set of VI-positive samples with PetNAD™ Co-prep and taco™ mini gave positive results in both assays. Interestingly, viral nucleic acid was detected from two semen samples (S-5653 and S-5647) collected from carrier stallions that stopped shedding virus in their semen either by RT-qPCR or both RT-qPCR and RT-iiPCR (Table 6) even though no infective virus was isolated. This demonstrates that PCR-based assays offer a higher sensitivity when compared to VI, which requires the presence of infective virus.

The sensitivity, specificity, and accuracy of the newly developed EAV RT-iiPCR assay were evaluated on equine semen and tissue samples with reference to the OIE-prescribed VI test and, in addition, compared to the previously validated EAV RT-qPCR assay. Both RT-qPCR and RT-iiPCR assays proved to be reliable tests for detecting EAV nucleic acids in equine semen, and each was in high agreement with the OIE recommended VI assay (99.2% accuracy, k = 0.98). However, there were two discrepancies in samples with very low infectivity titer or negative by VI (S-5662 and S-5609).

Even though several RT-PCR, RT-nested PCR, and RT-qPCR assays have been developed and validated for detection of EAV in semen and nasopharyngeal swabs in the past, the performance of these assays has never been systematically evaluated on an extensive

number of tissue samples. This study has shown that the RT-iiPCR assay was more efficacious and highly accurate (95.08%, k = 0.9) for detecting EAV in tissues from aborted fetuses, foals, and EAV carrier stallions when compared to the VI test than the RT-qPCR assay (accuracy = 92.62%, k = 0.85). Even though 7/59 and 1/59 VI positive tissue samples tested negative by RT-qPCR and RT-iiPCR, respectively, these samples exhibited low ($\leq 1.5 \times 10^3$ PFU/g) or very low (100 to <10 PFU/g) viral titers that could have potentially hindered efficient detection of EAV RNA by both molecular diagnostic assays. Additionally, tissue samples frequently contain PCR inhibitors that reduce the reaction efficiency and constitute a well-recognized challenge encountered with tissue material (Bustin and Nolan, 2004; Das et al., 2009; Fleige and Pfaffl, 2006; Schrader et al., 2012; Tichopad et al., 2004). The combination of low/very low viral titers along with the presence of PCR inhibitors may very well compromise the sensitivity of molecular diagnostic assays.

In conclusion, the use of different extraction procedures along with different PCR reagents and/or different molecular methods for signal detection might improve the diagnostic sensitivity of qPCR or RT-qPCR assays. This study showed that the use of the RT-iiPCR/POCKIT™ system in combination with either manual or automatic point-of-need extraction methods has the potential to aid in the timely identification of EAV carrier stallions, EAV associated cases of abortion, and outbreaks of respiratory disease. This can help in reducing the risk of transmission and dissemination of the virus through the semen and assist in the control of outbreaks of abortion. The higher sensitivity demonstrated by this assay supports its use as a rapid screening test. Thus, the EAV RT-iiPCR/POCKIT™ system could be considered as an alternative tool in the implementation of EAV control and prevention strategies.

Ethical approval

The experimental inoculation and establishment of the EAV carrier state along with collection of sequential semen samples have been carried out in accordance with an Institutional Animal Care and Use Committee approved protocol at the University of Kentucky, Lexington, KY (protocol number 2011-0888).

Funding

This work was partially supported by Agriculture and Food Research Initiative competitive grant no. 2013-68004-20360 from the USDA National Institute of Food and Agriculture and gifts and contracts to Dr. Udeni Balasuriya at the Maxwell. H. Gluck Equine Research Center, Department of Veterinary Science, College of Agriculture, Food and Environment, University of Kentucky.

Conflict of interests

MC, BN, AS, KMS, PJT, and UBRB declare no competing interests. PAL, YT, LM, HGC, and HTW are affiliated to GeneReach USA. However, this does not alter our adherence to the Journal of Virological Methods policies on sharing data and materials.

Acknowledgements

The authors would like to thank the late Dr. William H. McCollum for archiving and cataloging the tissue samples from aborted fetuses and EAV long-term carrier stallions, and Dr. Sergey Artiushin for providing the nucleic acids from various equine bacterial pathogens used in this study.

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.2016.02.015.

References

Ambagala, A., Pahari, S., Fisher, M., Lee, P.A., Pasick, J., Ostlund, E.N., Johnson, D.J., Lung, O., 2015. A rapid field-deployable reverse transcription-insulated isothermal polymerase chain reaction assay for sensitive and specific detection of bluetongue virus. Transbound. Emerg. Dis., http://dx.doi.org/10.1111/tbed.12388.

Balasuriya, U.B., MacLachlan, N.J., 2004. The immune response to equine arteritis virus: potential lessons for other arteriviruses. Vet. Immunol. Immunopathol. 102, 107–129.

Balasuriya, U.B., MacLachlan, N.J. (Eds.), 2007. Saunders/Elsevier, St. Louis, MO.

Balasuriya, U.B., Timoney, P.J., McCollum, W.H., MacLachlan, N.J., 1995. Phylogenetic analysis of open reading frame 5 of field isolates of equine arteritis virus and identification of conserved and nonconserved regions in the GL envelope glycoprotein. Virology 214, 690–697.

Balasuriya, U.B., Evermann, J.F., Hedges, J.F., McKeirnan, A.J., Mitten, J.Q., Beyer, J.C., McCollum, W.H., Timoney, P.J., MacLachlan, N.J., 1998. Serologic and molecular characterization of an abortigenic strain of equine arteritis virus isolated from infective frozen semen and an aborted equine fetus. J. Am. Vet. Med. Assoc. 213, 1586––1589, 1570.

Balasuriya, U.B., Hedges, J.F., Nadler, S.A., McCollum, W.H., Timoney, P.J., MacLachlan, N.J., 1999a. Genetic stability of equine arteritis virus during horizontal and vertical transmission in an outbreak of equine viral arteritis. J. Gen. Virol. 80, 1949–1958.

Balasuriya, U.B., Snijder, E.J., van Dinten, L.C., Heidner, H.W., Wilson, W.D., Hedges, J.F., Hullinger, P.J., MacLachlan, N.J., 1999b. Equine arteritis virus derived from an infectious cDNA clone is attenuated and genetically stable in infected stallions. Virology 260, 201–208.

Balasuriya, U.B., Leutenegger, C.M., Topol, J.B., McCollum, W.H., Timoney, P.J., MacLachlan, N.J., 2002. Detection of equine arteritis virus by real-time TaqMan reverse transcription-pCR assay. J. Virol. Methods 101, 21–28.

Balasuriya, U.B., Hedges, J.F., Smalley, V.L., Navarrette, A., McCollum, W.H., Timoney, P.J., Snijder, E.J., MacLachlan, N.J., 2004. Genetic characterization of equine arteritis virus during persistent infection of stallions. J. Gen. Virol. 85, 379–390.

Balasuriya, U.B., Snijder, E.J., Heidner, H.W., Zhang, J., Zevenhoven-Dobbe, J.C., Boone, J.D., McCollum, W.H., Timoney, P.J., MacLachlan, N.J., 2007. Development and characterization of an infectious cDNA clone of the virulent Bucyrus strain of Equine arteritis virus. J. Gen. Virol. 88, 918–924.

Balasuriya, U.B., Go, Y.Y., MacLachlan, N.J., 2013. Equine arteritis virus. Vet. Microbiol. 167, 93–122.

Balasuriya, U.B., Lee, P.Y., Tiwari, A., Skillman, A., Nam, B., Chambers, T.M., Tsai, Y.L., Ma, L.J., Yang, P.C., Chang, H.F., Wang, H.T., 2014. Rapid detection of equine influenza virus H3N8 subtype by insulated isothermal RT-pCR (iiRT-pCR) assay using the POCKIT nucleic acid analyzer. J. Virol. Methods 207, 66–72.

Balasuriya, U.B., 2014. Equine viral arteritis. Vet. Clin. North Am. Equine Pract. 30, 543–560.

Bell, S.A., Balasuriya, U.B., Nordhausen, R.W., MacLachlan, N.J., 2006. Isolation of equine herpesvirus-5 from blood mononuclear cells of a gelding. J. Vet. Diagn. Invest. 18, 472–475.

Bryans, J.T., Doll, E.R., Knappenberger, R.E., 1957. An outbreak of abortion caused by the equine arteritis virus. Cornell Vet. 47, 69–75.

Bustin, S.A., Nolan, T., 2004. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. J. Biomol. Tech. 15, 155–166.

Chang, H.F.G., Tsai, Y.L., Tsai, C.F., Lin, C.K., Lee, P.Y., Teng, P.H., Su, C., Jeng, C.C., 2012. A thermally baffled device for highly stabilized convective PCR. Biotechnol. J. 7, 662–666.

Cole, J.R., Hall, R.F., Gosser, H.S., Hendricks, J.B., Pursell, A.R., Senne, D.A., Pearson, J.E., Gipson, C.A., 1986. Transmissibility and abortogenic effect of equine viral arteritis in mares. J. Am. Vet. Med. Assoc. 189, 769–771.

Campos, J.R., Breheny, P., Araujo, R.R., Troedsson, M.H., Squires, E.L., Timoney, P.J., Balasuriya, U.B., 2014. Semen quality of stallions challenged with the Kentucky 84 strain of equine arteritis virus. Theriogenology 82, 1068–1079.

Das, A., Spackman, E., Pantin-Jackwood, M.J., Suarez, D.L., 2009. Removal of real-time reverse transcription polymerase chain reaction (RT-pCR) inhibitors associated with cloacal swab samples and tissues for improved diagnosis of avian influenza virus by RT-pCR. J. Vet. Diagn. Invest. 21, 771–778.

Doll, E.R., Bryans, J.T., McCollum, W.H., Crowe, M.E., 1957. Isolation of a filterable agent causing arteritis of horses and abortion by mares; its differentiation from the equine abortion (influenza) virus. Cornell Vet. 47, 3–41.

Espy, M.J., Uhl, J.R., Sloan, L.M., Buckwalter, S.P., Jones, M.F., Vetter, E.A., Yao, J.D., Wengenack, N.L., Rosenblatt, J.E., Cockerill, F.R., 2006. Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clin. Microbiol. Rev. 19, 165–256.

Fleige, S., Pfaffl, M.W., 2006. RNA integrity and the effect on the real-time qRT-pCR performance. Mol. Aspects Med. 27, 126–139.

Fukunaga, Y., Wada, R., Sugita, S., Fujita, Y., Nambo, Y., Imagawa, H., Kanemaru, T., Kamada, M., Komatsu, N., Akashi, H., 2000. In vitro detection of equine arteritis

virus from seminal plasma for identification of carrier stallions. J. Vet. Med. Sci. 62, 643–646.
Gilbert, S.A., Timoney, P.J., McCollum, W.H., Deregt, D., 1997. Detection of equine arteritis virus in the semen of carrier stallions by using a sensitive nested PCR assay. J. Clin. Microbiol. 35, 2181–2183.
Glaser, A.L., de Vries, A.A., Dubovi, E.J., 1995. Comparison of equine arteritis virus isolates using neutralizing monoclonal antibodies and identification of sequence changes in GL associated with neutralization resistance. J. Gen. Virol. 76, 2223–2233.
Glaser, A.L., de Vries, A.A., Rottier, P.J., Horzinek, M.C., Colenbrander, B., 1996. Equine arteritis virus: a review of clinical features and management aspects. Vet. Q. 18, 95–99.
Glaser, A.L., Renshaw, R.W., Trock, S.C., Brady, R.C., Dubovi, E.J., 2002. Isolation of Salem virus, a novel equine paramyxovirus, and assessment of its etiologic role in a disease outbreak. Vet. Microbiol. 87, 205–212.
Hans, A., Gaudaire, D., Manuguerra, J.C., Leon, A., Gessain, A., Laugier, C., Berthet, N., Zientara, S., 2015. Combination of an unbiased amplification method and a resequencing microarray for detecting and genotyping equine arteritis virus. J. Clin. Microbiol. 53, 287–291.
Hedges, J.F., Balasuriya, U.B., Timoney, P.J., McCollum, W.H., MacLachlan, N.J., 1999. Genetic divergence with emergence of novel phenotypic variants of equine arteritis virus during persistent infection of stallions. J. Virol. 73, 3672–3681.
Hullinger, P.J., Gardner, I.A., Hietala, S.K., Ferraro, G.L., MacLachlan, N.J., 2001. Seroprevalence of antibodies against equine arteritis virus in horses residing in the United States and imported horses. J. Am. Vet. Med. Assoc. 219, 946–949.
Johnson, B., Baldwin, C., Timoney, P.J., Ely, R., 1991. Arteritis in equine fetuses aborted due to equine viral arteritis. Vet. Pathol. 28, 248–250.
Lu, Z., Branscum, A.J., Shuck, K.M., Zhang, J., Dubovi, E.J., Timoney, P.J., Balasuriya, U.B., 2008. Comparison of two real-time reverse transcription polymerase chain reaction assays for the detection of equine arteritis virus nucleic acid in equine semen and tissue culture fluid. J. Vet. Diagn. Invest. 20, 147–155.
Lung, O., Pasick, J., Fisher, M., Buchanan, C., Erickson, A., Ambagala, A., 2015. Insulated isothermal reverse transcriptase PCR (iiRT-pCR) for rapid and sensitive detection of classical swine fever virus. Transbound. Emerg. Dis., http://dx.doi.org/10.1111/tbed.12318.
MacLachlan, N.J., Conley, A.J., Kennedy, P.C., 2000. Bluetongue and equine viral arteritis viruses as models of virus-induced fetal injury and abortion. Anim. Reprod. Sci. 60–61, 643–651.
Mankoc, S., Hostnik, P., Grom, J., Toplak, I., Klobucar, I., Kosec, M., Barlic-Maganja, D., 2007. Comparison of different molecular methods for assessment of equine arteritis virus (EAV) infection: a novel one-step MGB real-time RT-PCR assay, PCR-ELISA and classical RT-PCR for detection of highly diverse sequences of Slovenian EAV variants. J. Virol. Methods 146, 341–354.
Miszczak, F., Shuck, K.M., Lu, Z., Go, Y.Y., Zhang, J., Sells, S., Vabret, A., Pronost, S., Fortier, G., Timoney, P.J., Balasuriya, U.B., 2011. Evaluation of two magnetic-bead-based viral nucleic acid purification kits and three real-time reverse transcription-PCR reagent systems in two TaqMan assays for equine arteritis virus detection in semen. J. Clin. Microbiol. 49, 3694–3696.
OIE, 2013. Chapter 2.5.10: equine viral arteritis. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. World Organisation for Animal Health, Paris, France.
OIE, 2015. Chapter 12.9: infection with equine arteritis virus. In: Terrestrial Animal Health Code. World Organisation for Animal Health, Paris, France.
Pronost, S., Pitel, P.H., Miszczak, F., Legrand, L., Marcillaud-Pitel, C., Hamon, M., Tapprest, J., Balasuriya, U.B., Freymuth, F., Fortier, G., 2010. Description of the first recorded major occurrence of equine viral arteritis in France. Equine Vet. J. 42, 713–720.
Qu, S., Shi, Q., Zhou, L., Guo, Z., Zhou, D., Zhai, J., Yang, R., 2010. Ambient stable quantitative PCR reagents for the detection of *Yersinia pestis*. PLoS Negl. Trop. Dis. 4, e629.
Ramina, A., Dalla Valle, L., De Mas, S., Tisato, E., Zuin, A., Renier, M., Cuteri, V., Valente, C., Cancellotti, F.M., 1999. Detection of equine arteritis virus in semen by reverse transcriptase polymerase chain reaction-ELISA. Comp. Immunol. Microbiol. Infect. Dis. 22, 187–197.
Schrader, C., Schielke, A., Ellerbroek, L., Johne, R., 2012. PCR inhibitors—occurrence, properties and removal. J. Appl. Microbiol. 113, 1014–1026.
Snijder, E.J., Meulenberg, J.J., 1998. The molecular biology of arteriviruses. J. Gen. Virol. 79, 961–979.
Socha, W., Rola, J., Zmudzinski, J.F., 2015. Variability of non-structural proteins of equine arteritis virus during persistent infection of the stallion. Pol. J. Vet. Sci. 18, 255–259.
St-Laurent, G., Morin, G., Archambault, D., 1994. Detection of equine arteritis virus following amplification of structural and nonstructural viral genes by reverse transcription-PCR. J. Clin. Microbiol. 32, 658–665.
Starick, E., 1998. Rapid and sensitive detection of equine arteritis virus in semen and tissue samples by reverse transcription-polymerase chain reaction, dot blot hybridisation and nested polymerase chain reaction. Acta Virol. 42, 333–339.
Szeredi, L., Hornyak, A., Palfi, V., Molnar, T., Glavits, R., Denes, B., 2005. Study on the epidemiology of equine arteritis virus infection with different diagnostic techniques by investigating 96 cases of equine abortion in Hungary. Vet. Microbiol. 108, 235–242.
Tichopad, A., Didier, A., Pfaffl, M.W., 2004. Inhibition of real-time RT-PCR quantification due to tissue-specific contaminants. Mol. Cell. Probes 18, 45–50.
Timoney, P.J., McCollum, W.H., 1993. Equine viral arteritis. Vet. Clin. North Am. Equine Pract. 9, 295–309.
Timoney, P.J., McCollum, W.H., Roberts, A.W., Murphy, T.W., 1986. Demonstration of the carrier state in naturally acquired equine arteritis virus infection in the stallion. Res. Vet. Sci. 41, 279–280.
Timoney, P.J., McCollum, W.H., Murphy, T.W., Roberts, A.W., Willard, J.G., Carswell, G.D., 1987. The carrier state in equine arteritis virus infection in the stallion with specific emphasis on the venereal mode of virus transmission. J. Reprod. Fertil. 35 (Suppl), 95–102.
Timoney, P.J., 2000a. Factors influencing the international spread of equine diseases. Vet. Clin. North Am. Equine Pract. 16, 537–551.
Timoney, P.J., 2000b. The increasing significance of international trade in equids and its influence on the spread of infectious diseases. Ann. N. Y. Acad. Sci. 916, 55–60.
Tsai, Y.L., Lin, Y.C., Chou, P.H., Teng, P.H., Lee, P.Y., 2012a. Detection of white spot syndrome virus by polymerase chain reaction performed under insulated isothermal conditions. J. Virol. Methods 181, 134–137.
Tsai, Y.L., Wang, H.T., Chang, H.F., Tsai, C.F., Lin, C.K., Teng, P.H., Su, C., Jeng, C.C., Lee, P.Y., 2012b. Development of TaqMan probe-based insulated isothermal PCR (iiPCR) for sensitive and specific on-site pathogen detection. PLoS One 7, e45278.
Tsai, Y.L., Wang, H.C., Lo, C.F., Tang-Nelson, K., Lightner, D., Ou, B.R., Hour, A.L., Tsai, C.F., Yen, C.C., Chang, H.F., Teng, P.H., Lee, P.Y., 2014. Validation of a commercial insulated isothermal PCR-based POCKIT test for rapid and easy detection of white spot syndrome virus infection in *Litopenaeus vannamei*. PLoS One 9, e90545.
Vaala, W.E., Hamir, A.N., Dubovi, E.J., Timoney, P.J., Ruiz, B., 1992. Fatal, congenitally acquired infection with equine arteritis virus in a neonatal thoroughbred. Equine Vet. J. 24, 155–158.
Westcott, D.G., King, D.P., Drew, T.W., Nowotny, N., Kindermann, J., Hannant, D., Belak, S., Paton, D.J., 2003. Use of an internal standard in a closed one-tube RT-PCR for the detection of equine arteritis virus RNA with fluorescent probes. Vet. Res. 34, 165–176.
Wilkes, R.P., Tsai, Y.L., Lee, P.Y., Lee, F.C., Chang, H.F., Wang, H.T., 2014. Rapid and sensitive detection of canine distemper virus by one-tube reverse transcription-insulated isothermal polymerase chain reaction. BMC Vet. Res. 10, 213.
Wilkes, R.P., Kania, S., Tsai, Y.L., Lee, P.Y., Chang, H.H., Ma, L.J., Chang, H.F., Wang, H.T., 2015a. Rapid and sensitive detection of Feline immunodeficiency virus using an insulated isothermal polymerase chain reaction-based assay with a point-of-need PCR detection platform. J. Virol. Methods 27, 510–515.
Wilkes, R.P., Lee, P.Y., Tsai, Y.L., Tsai, C.F., Chang, H.H., Chang, H.F., Wang, H.T., 2015b. An insulated isothermal PCR method on a field-deployable device for rapid and sensitive detection of canine parvovirus type 2 at points of need. J. Virol. Methods 220, 35–38.
Zhang, J., Go, Y.Y., MacLachlan, N.J., Meade, B.J., Timoney, P.J., Balasuriya, U.B., 2008. Amino acid substitutions in the structural or nonstructural proteins of a vaccine strain of equine arteritis virus are associated with its attenuation. Virology 378, 355–362.
Zhang, J., Timoney, P.J., Shuck, K.M., Seoul, G., Go, Y.Y., Lu, Z., Powell, D.G., Meade, B.J., Balasuriya, U.B., 2010. Molecular epidemiology and genetic characterization of equine arteritis virus isolates associated with the 2006–2007 multi-state disease occurrence in the USA. J. Gen. Virol. 91, 2286–2301.