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On-site detection of equid alphaherpesvirus 3 in perineal and genital swabs of mares and stallions

Vissani M.A.[a], Tordoya M.S.[a], Tsai Y.-L.[b], Lee P.-Y.A.[b], Shen Y.-H.[b], Lee F.-C.[b], Wang H.-T.T.[b], Parreño V.[a], Barrandeguy M.[a,c,*]

[a] Instituto Nacional de Tecnología Agropecuaria (INTA), Instituto de Virología, Argentina
[b] GeneReach USA, Lexington, MA, USA
[c] Cátedra de Enfermedades Infecciosas, Escuela de Veterinaria, Universidad del Salvador, Champagnat 1599, Ruta Panamericana km54.5 (B1630AHU), Pilar, Buenos Aires, Argentina

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ABSTRACT

Equine coital exanthema (ECE) is an infectious, venereally transmitted muco-cutaneous disease affecting mares and stallions, caused by equid alphaherpesvirus 3 (EHV3). Diagnostic tools for rapid identification of EHV3 are of primary importance to diminish the risk of EHV3 dissemination at the time of breeding. In the last years, it has been shown that the performance of the insulated-isothermal polymerase chain reaction (iiPCR) is comparable to virus isolation, nested PCR and real-time PCR (qPCR) in detecting pathogens of various animal species. Analytical sensitivity and specificity of the iiPCR were compared with a qPCR, using a plasmid containing the target region of the EHV3 glycoprotein G gene and an Argentinian EHV3 isolate (E/9283/07 C3A). In order to evaluate the diagnostic performance of the iiPCR, nucleic acids of 85 perineal and genital swabs (PGS) of mares and stallions were extracted by taco™ mini and tested by both techniques. EHV3 was detected in 46 and 45 of the 85 PGS by the iiPCR and qPCR, respectively. There was almost perfect agreement between the two diagnostic methods (98.82%; 95% CI: 95.03–100%; $\kappa = 0.98$). The iiPCR had a limit of detection of 95.00% at 6 genome equivalents per reaction and a detection endpoint for viral DNA comparable to that of the qPCR, and did not react with six non-targeted equine pathogens. The iiPCR represents a sensitive and specific method for the rapid on-site diagnosis of EHV3 infection. Its routinely implementation in breeding facilities, and artificial insemination and embryo transfer centers, will contribute to prevent the dissemination of this venereal, highly contagious disease in horses.

1. Introduction

Equid alphaherpesvirus 3 (EHV3) is a member of the order *Herpesvirales*, family *Herpesviridae* and belongs to the genus *Varicellovirus* (Davison, 2010). EHV3, which is the causative agent of equine coital exanthema (ECE), has a worldwide distribution and is antigenically, genetically, and pathogenically distinct from the other equid herpesviruses. ECE is a venereal and highly contagious disease, characterized by the formation of papules, vesicles, pustules and ulcers on the external genitalia of mares and stallions. Stallions with extensive ECE lesions may walk stiffly, display general discomfort and loss of the libido, and refuse to mate mares (Allen and Umphenour, 2004; Barrandeguy and Thiry, 2012). The virus is primarily transmitted by sexual intercourse or direct contact with contaminated objects such as inspection gloves, ultrasound scanner or other used for the hygiene of the genital area (Allen and Umphenour, 2004; Barrandeguy and Thiry, 2012). As for other herpesvirus infections, latency and reactivation are critical events in their epidemiology. With respect to EHV3, in latently infected horses, episodes of reactivation and re-excretion, either with or without the manifestation of typical lesions, and together with variable levels of serum antibodies, have been demonstrated, being these horses the source of virus for other animals (Allen and Umphenour, 2004; Barrandeguy, 2010; Barrandeguy et al. 2008; Barrandeguy et al. (2010)).

The negative impact of ECE on the equine industry, mainly in the thoroughbred breed, is the forced and temporary disruption of the mating activities of affected stallions and mares, which is translated into significant end-of-season decreases in the number of entries into the mare book of the affected stallions, and also delaying foaling dates or reducing pregnancy rates in mares that miss breeding opportunities due to the disease (Allen and Umphenour, 2004; Barrandeguy and Thiry, 2012). Similarly, in artificial insemination and embryo transfer

* Corresponding author at: Instituto Nacional de Tecnología Agropecuaria (INTA), Instituto de Virología, Argentina.
E-mail address: Barrandeguy.maria@inta.gob.ar (M. Barrandeguy).

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Table 1
Sequences of primers and probes used in the qPCR and iiPCR assays targeting the conserved *gG* gene of EHV3 genome.

Name	Sequence (5´–3´)	Position[a]	Target	Function	Reference
EHV-3 Fw	GGGTATCGGCTTTCTCATCTTG	133342–133363	*gG* gene	qPCR	Barrandeguy et al. (2008)
EHV-3 Rv	CCGCAGGACGCAAACG	133411–133396	*gG* gene	qPCR	
EHV-3 Probe	6-FAM-TGTGTCTCCTCATCGGCCTCATTGTCT-TAMRA	133368–133394	*gG* gene	qPCR	
EHV-3 ii-F	GGGCCCGACTTGTATAGCG	132632–132650	*gG* gene	iiPCR	this paper
EHV-3 ii-R	TCCCGTGGGTCATTTTGCA	132710–132692			
EHV-3 ii-Probe	ATCTCCCTGCGCGTAAGCC	132656–132674			

[a] Nucleotide position is based on GenBank accession number KM051845.

centers, affected mares are reluctant to be inspected, inseminated or transferred with the consequent loss of opportunity to be pregnant. Moreover, additional time and necessary precautions which are required to manage the mares also have a substantial negative impact (Barrandeguy and Thiry, 2012).

The diagnosis of ECE relies mainly on clinical inspection, and it can be confirmed, so far, by virus isolation or EHV3 DNA detection, by conventional or real-time PCR (qPCR). However, both types of diagnosis (clinical inspection and laboratory tests) are not proper enough to detect EHV3 before mating. On one hand, sub-clinical infections cannot be detected by clinical inspection before mating; on the other hand, EHV3 diagnosis can only be conducted in a specialized virology laboratory, and is not useful before mating. Diagnostic tools for on-site identification of EHV3 are of primary importance to prevent the risks of EHV3 dissemination at the time of breeding or of embryo transfer procedures, from mares which are shedding the virus sub-clinically. In recent years, a PCR detection platform based on the fluorescent probe hydrolysis-based insulated isothermal PCR (iiPCR) technology has become commercially available. With the field-deployable PCR device (POCKIT™ Nucleic Acid Analyzer; POCKIT™; GeneReach USA, Lexington, MA, USA) the entire reaction, known as iiPCR, can automatically be developed obtaining the results within one hour (Tsai et al., 2014, 2012). In the last years, it has been shown that the performance of iiPCR assays is comparable to virus isolation in tissue culture, nested PCR, and/or qPCR in detecting equine viruses (specifically Equid herpesvirus 1 [EHV1], equine influenza virus [H3N8 subtype], equine arteritis virus [EAV]), other etiological agents of economic and companion animals, and human pathogens (Ambagala et al., 2015; Balasuriya et al., 2017, 2014; Carossino et al., 2016; Go et al., 2017, 2016; Lung et al., 2016; Soltan et al., 2016; Wilkes et al., 2015b, 2017, 2015a; Zhang et al., 2016). Essentially, the POCKIT™ system is ready in a format for point-of-need applications. The reagents are lyophilized to facilitate long-term storage and easy shipping; a field-deployable, automated nucleic acid extraction system (taco™ mini Nucleic Acid Automatic Extraction System, taco™ mini; GeneReach USA) is also provided for easy and quick on-site nucleic acids extraction.

The aim of this study was to develop and validate the iiPCR test for EHV3 detection, assessing its analytical sensitivity and specificity, as well as its performance with clinical samples, comparing it with a real-time PCR.

2. Materials and methods

2.1. Clinical samples

The well characterized EHV3 strain (E/9283/07 C3A) (Barrandeguy, 2010) was used as reference (positive control). The virus was ten-folded diluted (10^{-1} up to 10^{-7}) in D-MEM, being the initial titer $10^{5.36}$ plaque forming units/ml.

A panel of 85 perineal-genital swabs (PGS) of mares and stallions with and without clinical ECE lesions (n: 40) and of experimentally EHV3 infected mares (n: 45) (Barrandeguy et al., 2012) were tested in this study. All these clinical samples had been tested as EHV3 positive and negative by conventional and qPCR (Barrandeguy et al., 2008; Dynon et al., 2001).

In order to determine the specificity of the iiPCR assay, other equine pathogens (one reference strain of EHV1 [Cornell University, Ithaca, Nueva York, USA]; one Equid herpesvirus 4 [EHV4, Cornell University, Ithaca, Nueva York, USA]; one Equid herpesvirus 2 [EHV2, Cornell University, Ithaca, Nueva York, USA]; six EHV1 and three EHV4 field isolates [Argentina]; the modified live virus [MLV] vaccine strain of EAV ARVAC®; one EAV contaminated semen; a field isolate of *Salmonella enterica* serovar Abortusequi, and two isolates of *Streptococcus equi* Beta hemolytic Group C Lancefield) were included in the study.

2.2. Nucleic acid extraction

Nucleic acids were extracted from clinical perineal-genital swab samples using the taco™ mini method. Briefly, 200 µl of the samples were 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 user manual. The nucleic acids were eluted in 200 µl Eluting Buffer, transferred to fresh tubes, and stored at −70 °C for later use. Nucleic acids from virus and bacteria isolates were extracted using the High Pure PCR Template Preparation Kit® (Cat No. 11 796 828 001; ROCHE, Indianapolis, Indiana, USA) following the instructions of the manufacturer.

2.3. EHV3 qPCR and iiPCR

The TaqMan® fluorogenic probe-based qPCR assay targeting EHV3 glycoprotein G (*gG*) gene was performed, using TaqMan Universal Master Mix (Applied Biosystems®, Foster City, California, USA). Primers and probe are described in Table 1, and the program (one step at 50 °C for 2 min, one step at 95 °C for 10 min and 40 steps at 95 °C 15 s and at 60 °C 1 min) was run in the Applied Biosystems® ABI-7500 (Barrandeguy et al., 2008).

The EHV3 iiPCR was designed based on a previously described probe hydrolysis-based iiPCR method (Chang et al., 2012). Primer pair and probe (Table 1) were designed to target a region of the *gG* gene of EHV3 according to the recommended principles for iiPCR (http://www.iipcr.com/eweb/uploadfile/20130522114104277.pdf). The POCKIT™ device has one default program for both PCR and reverse transcription (RT)-PCR, including a step at 50 °C for 10 min (for RT) and a step at 95 °C for 30 min (for PCR). The reaction is completed in 50 min. The POCKIT™ device collected optical signals through an image sensor. Signal-to-noise (S/N) ratios were calculated by dividing light signals collected after iiPCR by those from before iiPCR (Tsai et al., 2012). According to the default S/N thresholds, results were converted automatically to 'positive' or 'negative' and shown on the display screen (Chang et al., 2012).

2.4. Statistical analysis

Limit of detection 95% (LoD 95%) of the reaction was determined by probit analysis at 95% confidence interval by SPSS version 14 (SPSS, Chicago, IL, USA). The 2 × 2 contingency tables were analyzed by

Table 2
Comparison of the analytical sensitivity between the EHV3 iiPCR and qPCR assays using a reference strain of EHV3 (E/9283/07 C3A).

Sample Dilutions[a]	POCKIT PCR			Real-time PCR[a] (result/Ct value)		
10^{-2}	+	+	nd	+/28.5	+/28.4	+/28.5
10^{-3}	+	+	+	+/32.16	+/31.96	+/31.82
10^{-4}	+	+	+	+/36.39	+/36.18	+/36.47
10^{-5}	+	+	–	+/39.42	+/38.21	−/ND
10^{-6}	–	–	–	+/39.32	+/39.28	−/ND

[a] Nucleic acids extracted from serial dilutions (10^{-1} to 10^{-7}) of EHV3 (E/9283/07 C3A) made in DMEM; +, positive; −, negative; nd, not done; ND, not detected.

kappa statistic using SPSS version 14 (SPSS, Chicago, IL, USA) to determine the inter-rater agreement (Cortés-Reyes et al., 2010; Eliasson et al., 2017).

3. Results

3.1. Analytical sensitivity of EHV3 iiPCR

A serial dilution of the plasmid containing the target sequences was used to determine the sensitivity of the EHV3 iiPCR assay. The percentage of positive results was 100% (10/10), 100% (20/20), 100% (20/20), 80% (16/20) and 0% (0/24) for 100, 50, 20, 5 and 0 copies of plasmid DNA, respectively. Calculation by probit regression analysis determined that the LoD 95% of the reaction was 6 copies genome equivalents per reaction. In addition, the analytical sensitivity of the iiPCR for EHV3 detection was compared to that of the published EHV3 qPCR (Barrandeguy et al., 2008). Both PCR methods tested positive with all the replicates of the dilution 10^{-4} (3/3) (Table 2), indicating that the two had comparable detection limits for EHV3 genomic DNA.

3.2. Analytical specificity of EHV3 iiPCR

Analytical specificity of the EHV3 iiPCR assay on the POCKIT™ device was verified with a panel of six equine pathogens (seven EHV1, one EHV2, four EHV4, two EAV, one *S. enterica* serovar. Abortusequi and two *S. equi*). None of the six pathogens were detected indicating that the iiPCR has high analytical specificity.

3.3. Diagnostic sensitivity and specificity of EHV3 iiPCR

The diagnostic performance of the EHV3 iiPCR/POCKIT™ assay to detect EHV3 in equine clinical samples was compared to that of the reference qPCR. After testing 85 PGS from mares and stallions with or without ECE lesions, EHV3 was detected in 46 and 45 of the PGS samples by the iiPCR and qPCR, respectively (Table 1). The two methods had 98.82% agreement (95% confidence interval [CI]: 95.03%–100%, $\kappa = 0.98$). Both specificity and sensitivity were determined as 98.34% (95% CI: 91.20%–100%) and 100% (95% CI: 93.30%–100%), respectively (Table 3).

Table 3
Contingency table for analysis of the agreement between the qPCR and the iiPCR assays for the detection of EHV3 in perineal and genital swabs of mares and stallions.

		EHV3 real-time PCR		
		Positive	Negative	Total
EHV3 POCKIT PCR	Positive	45	1	**46**
	Negative	0	39	**39**
	Total	**45**	**40**	**85**

Agreement: 98.82% (95%CI: 95.03–100%).
Kappa Value (agreement beyond chance) = 0.98.
Specificity: 98.34% (95%CI: 91.20%–100%); Sensitivity: 100% (95%CI: 93.30% - 100%).
Bold values signifies the total value of each group.

4. Discussion

In the present study, we developed and validated an iiPCR for the rapid detection of EHV3 nucleic acid in PGS of mares and stallions. Similar to the qPCR, this assay is based on TaqMan® probe hydrolysis to generate a fluorescent signal and requires no post- reaction handling of amplicons, thus reducing the risks of carry-over contamination of samples.

In addition to the advantages of the iiPCR reaction, the nucleic acid extraction system is very simple. Most of the available nucleic acid extraction methods are labour-intensive and require trained technicians to be performed. Simplified column-based extraction systems are available to isolate nucleic acids from clinical samples in the field (Ambagala et al., 2015), but still require a considerable manual handling with the potential risks of target contaminations and/or nucleic acid degradation. The fully automated field-deployable taco™ mini system can be used to facilitate easy and consistent nucleic acid extraction in the field, greatly reducing hands-on time from samples to results.

The analytical sensitivity of the iiPCR (98.34%) was similar to that of the previously described qPCR (Barrandeguy et al., 2008); however, iiPCR has more than one additional advantage: a very simple carry-out protocol; performance in a portable device (POCKIT™) using lyophilized reagents; and results obtained, as positive or negative, after automatic data processing in only 50 min.

Furthermore, the iiPCR assay was highly specific for EHV3, as it did not cross-react with other common equine viral (EHV1, 4, 2 and EAV) and bacterial (*S. enterica* serovar Abortusequi and *S. equi* Beta hemolytic Group C Lancefield) pathogens.

The diagnostic sensitivity and specificity of the newly developed EHV3 iiPCR assay on PGS samples of mares and stallions were comparable to those of the previously described EHV3 qPCR assay, with an agreement of 98.82%. The only one discrepant result corresponded to a PGS sample from a mare experimentally infected in its last day of EHV3 shedding period (iiPCR+/nested PCR+/conventional PCR−/real time−/virus isolation -; data not shown).

Overall, the combination of a quick nucleic acid extraction (25 min) with taco™ mini and the DNA amplification with iiPCR/POCKIT™ (POCKIT™ Combo, GeneReach USA) which are available in a durable suitcase to serve as a mobile laboratory, allow the detection of EHV3 in PGS of mares or stallions, and can generate qualitative test results within 90 min. The high sensitivity and specificity of the EHV3 iiPCR on the POCKIT™ Combo system demonstrated by this study, support its use as a rapid screening test before mating activities in breeding farms. Thus, the use of this methodology in breeding farms would facilitate the rapid identification of EHV3 in animals which are sub-clinically shedding the virus at the time of mating. Similarly, its use in artificial insemination and embryo transfer centers would allow the rapid diagnosis of outbreaks of ECE. In both cases, the use of the EHV3 iiPCR on the mobile PCR/POCKIT™ system can be considered an important tool in the implementation of ECE control and prevention strategies, and therefore diminish the risks of virus transmission in breeding facilities, and in artificial insemination and embryo transfer centers.

Conflict of interests

YT, YS, FL, HTW, and PAL are affiliated to GeneReach USA. However, this does not alter our adherence to the Journal of Virological Methods policies on sharing data and materials.

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