Journal of Virological Methods 155 (2009) 175–181

Contents lists available at ScienceDirect

Journal of Virological Methods

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

Development of a real-time duplex TaqMan-PCR for the detection of Equine rhinitis A and B viruses in clinical specimens

Antonio Mori, Paola De Benedictis, Sabrina Marciano, Bianca Zecchin, Antonio Zuin, Barbara Zecchin, Ilaria Capua, Giovanni Cattoli*

Istituto Zooprofilattico Sperimentale delle Venezie, Research & Development Department, Viale dell'Universita' 10, 35020 Legnaro, Padova, Italy

Article history:
Received 27 February 2008
Received in revised form
30 September 2008
Accepted 7 October 2008
Available online 2 December 2008

Keywords:
Equine rhinitis virus, ERAV
ERBV
Real-time PCR

A B S T R A C T

Equine rhinitis A and B viruses (ERAV and ERBV) are respiratory viruses of horses belonging to the family *Picornaviridae*. Although these viruses are considered to cause respiratory disease in horses and are potentially infectious for humans, little is known about their prevalence and pathogenesis. Virus isolation is often unsuccessful due to their inefficient growth and lack of cytopathic effect in cell cultures. Therefore, molecular assays should be considered as the method of choice to detect infection in symptomatic or apparently healthy horses. In the present study, a novel real-time duplex PCR was developed for the detection and differentiation of both ERAV and ERBV. The method was evaluated for its ability to detect viral RNA in cell culture supernatants and nasal swabs, and lung and urine spiked with known quantities of virus. The assay demonstrated high analytical specificity, sensitivity and good reproducibility, with coefficients of variation (CV%) ranging from 1% to 7.4% and from 1.2% to 12% for intra- and inter-assay variability respectively. The assay detected ERBV in 14 of 86 nasal swabs collected from horses with respiratory disease. The real-time duplex PCR is a useful new diagnostic method for the rapid detection and differentiation of ERAV and ERBV.

1. Introduction

Equine rhinitis A and B viruses (formerly equine rhinovirus 1 and 2) are two respiratory viruses of horses belonging to the family *Picornaviridae*. Equine rhinitis A virus (ERAV) is the only non-foot-and-mouth disease virus member of the genus Aphtovirus (Stanway et al., 2005). The prototype for this virus was isolated in the United Kingdom (Plummer, 1962) and to date there is no evidence of extensive antigenic diversity among ERAV strains.

Equine rhinitis B virus (ERBV) is the only representative of the genus Erbovirus. Serologically, two distinct prototypes of ERBV were identified initially, namely ERBV1 and ERBV2 (formerly equine rhinovirus 2 and 3, respectively) (Huang et al., 2001). Both ERAV and ERBV are acid labile viruses, with the exception of a group of acid stable viruses belonging to ERBV1 group. These viruses have been grouped recently as members of a genetically distinguishable ERBV cluster (ERBV3), on the basis of sequence analysis of the viral P1 region, encoding for viral capsid structural proteins (Black et al., 2005; Black and Studdert, 2006).

Little is known about the pathogenesis and the prevalence of ERAV and ERBV, even though both infections are considered common in horses. Only a few studies on virus shedding from infected horses and on seroprevalence in equine populations are available in the literature, and most studies are fragmentary (Black et al., 2007b; Burrell et al., 1996; Carman et al., 1997; McCollumn and Timoney, 1992; Wernery et al., 1998; Willoughby et al., 1992). Seroprevalence for ERAV and ERBV is estimated to range from 50% to 80% in apparently healthy horses worldwide and the presence of neutralising antibodies to one or other of the viruses seems to be correlated with the age of the animals (Black et al., 2007b; Hartley et al., 2001; Kriegshauser et al., 2005; Studdert and Gleeson, 1978).

ERAV infection is confined commonly to the respiratory tract, but viraemia and persistent virus shedding from the pharyngeal region and in urine and faeces have been observed (Plummer, 1962, 1963). ERAV and ERBV infections could occur subclinically being detectable by seroconversion (Burrell et al., 1996; Willoughby et al., 1992), or as acute febrile diseases with mild to severe clinical signs. The ERAV host-range includes different mammalian species and humans, although horizontal infection in species other than equines seems unlikely (Plummer, 1962, 1963). Humans can be infected by both ERAV and ERBV, as antibodies against ERAV and ERBV1 have been demonstrated in sera of humans in regular contact with horses (Kriegshauser et al., 2005; Plummer, 1963). An experimental study showed the capability of ERAV to cause respiratory and systemic disease in humans, with clinical signs such as lymphadenitis, pharyngitis, fever and viraemia (Plummer, 1963).

* Corresponding author. Tel.: +39 049 8084384; fax: +39 049 8084360.
E-mail address: gcattoli@izsvenezie.it (G. Cattoli).

0166-0934/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2008.10.009

No other studies have been reported on the zoonotic significance of these viruses.

It is well known that the causative agents of these infections grow poorly in cell cultures and that ERAV and ERBV strains exist that show no cytopathic effect (Black et al., 2007a,b; Li et al., 1997). A modified culture medium supplemented with $MgCl_2$, could enhance the growth capability of some ERBV strains, but the lack of sensitivity of this technique makes it inadequate for diagnosis (Black et al., 2007a).

Molecular diagnostic techniques could, therefore, be suitable for the detection of viral shedding from symptomatic or apparently healthy horses and to study the pathogenesis of these viruses. At present, two distinct end point PCR-based diagnostic protocols are available for ERAV and ERBV (Black et al., 2007a; Dynon et al., 2001). However, their performances were not demonstrated clearly. Further, one of the protocols includes an RT-nested PCR method making it laborious, time consuming and susceptible to contamination. In the present study, a novel real-time duplex TaqMan-PCR was developed, which allows the detection and differentiation of ERAV/ERBV infections using two distinct sets of primers and probes.

2. Materials and methods

2.1. Viral, bacterial and protozoan strains

Two ERAV and two ERBV2 strains of European and North American origin were replicated in RK13 cells (ATCC CCL-37) and used as reference viruses (kindly provided by Dr. J. Daly and B. Geragthy, Animal Health Trust, Suffolk-UK, and by Prof. U. Balasuriya, University of Kentucky, USA). The viral titres were calculated as the mean tissue culture infectious dose/ml ($TCID_{50}$/ml) according to the Spearman–Karber method (Lorenz and Bogel, 1973). Several other micro-organisms, including RNA and DNA viruses, bacteria and protozoa were used to test the specificity of the assay (Table 1).

2.2. Primer and probe design

To minimize primer and probe mismatches, the nucleotide sequences available on GenBank for ERAV and ERBV 1, 2 and 3 were aligned using MEGA4 (Tamura et al., 2007) and CLC Free Workbench 3 software (CLC bio, Aarhus, Denmark) (see Figs. 1 and 2). Primers and probes were designed using the "Primer Express Software v2.0" according to the Applied Biosystems guidelines (Applied Biosystems, Foster City, CA).

2.3. RNA extraction, cDNA synthesis and real-time TaqMan Duplex PCR

RNA was extracted from 200 μl of sample using the High Pure™ RNA Isolation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. RNA was eluted in a final volume of 60 μl. The cDNA was synthesized by random hexamers using the "High capacity cDNA archive kit" (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions.

The real-time duplex PCR was carried out in 25 μl with 1× "TaqMan® Universal PCR Master Mix 2X" (Applied Biosystems, Foster City, CA), 240 nM of each primer, 250 nM of each probe and 5 μl of cDNA template. The reaction was performed in a 7300 real-time PCR System (Applied Biosystems, Foster City, CA) with the following protocol: 2 min at 50 °C and 10 min at 95 °C followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min.

2.4. Conventional RT-PCR and RT-nested PCR for the detection of ERAV and ERBV

To evaluate the performances of the real-time duplex PCR, its sensitivity and the results obtained with clinical specimens were compared to those obtained by the only two RT-PCR protocols described previously for the detection of ERAV and ERBV (Dynon et al., 2001; Black et al., 2007a). Primers for ERAV detection were located in the VP1-2A region (5′-3′ position 3098–3117 and 3881–3900, for forward and reverse primers, respectively. GenBank reference sequence: NC003982). For ERBV, primers were located in the 3D region (5′-3′ position 7986–8006 and 8746–8767, for outer forward and reverse primers, respectively; and 5′-3′ position 8117–8138 and 8722–8739, for inner forward and reverse primers, respectively. GenBank reference sequence: NC003983).

Table 1
Equine pathogens tested to assess the specificity of the real-time duplex PCR.

Pathogens tested	Results obtained by real-time duplex PCR
RNA viruses	
Equine rhinitis A virus (provided by Dr. Bob Geraghty, Animal Health Trust, UK, 1994)	Positive for ERAV; negative for ERBV
Equine rhinitis A virus (supplied by Prof. U. Balasuriya, Univ. of Kentucky, USA, 2001)	Positive for ERAV; negative for ERBV
Equine rhinitis B virus 2 (provided by Dr. Bob Geraghty, Animal Health Trust, UK, 1995)	Negative for ERAV; positive for ERBV
Equine rhinitis B virus 2 (supplied by Prof. U. Balasuriya, Univ. of Kentucky, USA, 2001)	Negative for ERAV; positive for ERBV
Equine arteritis virus (Bucyrus virus, provided by VLA-Weybridge, UK)	Negative
Equine influenza virus A, H3N8 (provided by the National Reference Laboratory for Equine Diseases, IZSLT-Rome, Italy)	Negative
Equine infectious anemia virus (provided by the National Reference Laboratory for EIA, IZSLT-Pisa, Italy)	Negative
Lyssavirus genotype 1, rabies virus (CVS-11 strain, provided by AFSSA-NANCY, France)	Negative
West Nile virus, Eg. 101 strain (provided by Prof. N. Nowotny, Univ. of Vienna, Austria)	Negative
DNA viruses	
Equine herpesvirus-1 (IZSVe repository, Italy)	Negative
Equine herpesvirus-4 (IZSVe repository, Italy)	Negative
Equine adenovirus 1 (provided by Dr. Bob Geraghty, Animal Health Trust, UK)	Negative
Bacteria	
Leptospira icterohaemorragiae (provided by the National Reference Laboratory for leptospirosis; IZSLER, Brescia, Italy)	Negative
Pseudomonas mallei (inactivated antigen, provided by CIDC-Lelystad, The Netherlands)	Negative
Protozoa	
Trypanosoma equiperdum (inactivated antigen, provided by the National Reference Laboratory for Exotic Diseases, IZSAM-Teramo, Italy)	Negative

The method demonstrated high specificity.

	Sense (5',3')	Probe (5',3')	Antisense (3',5')
	(6782-6798)	(6801-6824)	(6827-6848)
	CC**R**CCGATGCCCCATGT	T**R**GTTGGTAGGCA**R**CT**Y**TTGGGCC	AAACACCGTTT**Y**AAAGTACT**Y**C
gi|21328570|ref|NC_003982.1|[a]	CC**A**CCGATGCCCCATGTGGT**G**GTTGGTAGGCA**G**CT**T**TTGGGCCGGTTTGTGGCAAA**G**TTTCATGA**G**G
gi|82468888|gb|DQ268580.1|	CC**G**CCGATGCCCCATGTGGT**A**GTTGGTAGGCA**G**CT**T**TTGGGCCGGTTTGTGGCAAA**A**TTTCATGA**A**G
gi|82468892|gb|DQ272128.1|	CC**G**CCGATGCCCCATGTGGT**A**GTTGGTAGGCA**G**CT**T**TTGGGCCGGTTTGTGGCAAA**A**TTTCATGA**A**G
gi|82468894|gb|DQ272577.1|	CC**G**CCGATGCCCCATGTGGT**A**GTTGGTAGGCA**G**CT**T**TTGGGCCGGTTTGTGGCAAA**A**TTTCATGA**A**G
gi|82468890|gb|DQ272127.1|	CC**G**CCGATGCCCCATGTGGT**A**GTTGGTAGGCA**A**CT**C**TTGGGCCGGTTTGTGGCAAA**A**TTTCATGA**A**G
gi|2231133|gb|L43052.1|	CC**G**CCGATGCCCCATGTGGT**G**GTTGGTAGGCA**G**CT**C**TTGGGCCGGTTTGTGGCAAA**A**TTTCATGA**A**G
gi|1262766|emb|X96870.1|	CC**A**CCGATGCCCCATGTGGT**G**GTTGGTAGGCA**G**CT**T**TTGGGCCGGTTTGTGGCAAA**G**TTTCATGA**G**G
gi|82468896|gb|DQ272578.1|	CC**A**CCGATGCCCCATGTGGT**G**GTTGGTAGGCA**G**CT**T**TTGGGCCGGTTTGTGGCAAA**G**TTTCATGA**G**G

[a] Reference sequence

Fig. 1. Multiple sequence alignment of the ERAV 3D region amplified by the real-time duplex PCR. Non-conserved nucleotides are bold and underlined. The ERAV primer and probe sequences used in the real-time duplex PCR are shown underlined on the top line.

2.5. Analytical specificity

The specificity of the method was assessed by testing the primer–probe sets on common equine RNA and DNA viruses and other micro-organisms listed in Table 1.

2.6. Analytical sensitivity

To test the sensitivity of the real-time duplex PCR and to establish whether the different type of sample matrices could influence the analytical sensitivity of the assay, tissue culture supernatants, lung homogenates, urine and nasal swab suspensions were spiked with 10-fold serial dilutions of titrated viruses (ERAV and ERBV from Animal Health Trust, UK; Table 2) and the total RNA was then extracted.

Briefly, lung, urine and nasal swabs were collected at the slaughterhouse from asymptomatic horses negative for ERAV/ERBV by methods described previously (Black et al., 2007a; Dynon et al., 2001). Lung was weighed (0.1 g), homogenised with sterile quartz sand in 1 ml of phosphate buffer solution (PBS, pH 7.4) to make 1:10 (w/v). Nasal swabs were suspended in 1 ml of PBS. After virus spiking, the total RNA was extracted from the suspension of each

	Sense (5',3')	Probe (5',3')	Antisense (3',5')
	(8385- 8407)	(8409- 8441)	(8464- 8443)
	AACTTTGGCAGACGTTGTGTTTC	CAAACGTC**R**ATTTAAACCTGATGAAGAATTCCC	AGGACAA**RT**YTGGTCACTACC
gi|21335364|ref|NC_003983.1[a]	AACTTTGGCAGACGTTGTGTTTCTCAAACGTC**G**ATTTAAACCTGATGAAGAATTCCCATTCCTGTT**C**A**A**ACCAGTGATGG
gi|15192761|gb|AF361253.1|AF361253	AACTTTGGCAGACGTTGTGTTTCTCAAACGTC**A**ATTTAAACCTGATGAAGAATTCCCATTCCTGTT**T**A**A**ACCAGTGATGG
gi|15192761|gb|AF361253.1|AF361253-1	AACTTTGGCAGACGTTGTGTTTCTCAAACGTC**A**ATTTAAACCTGATGAAGAATTCCCATTCCTGTT**T**A**A**ACCAGTGATGG
gi|16156223|ref|NC_003077.1|	AACTTTGGCAGACGTTGTGTTTCTCAAACGTC**A**ATTTAAACCTGATGAAGAATTCCCATTCCTGTT**T**A**A**ACCAGTGATGG
gi|1262769|emb|X96871.1|	AACTTTGGCAGACGTTGTGTTTCTCAAACGTC**G**ATTTAAACCTGATGAAGAATTCCCATTCCTGTT**C**A**A**ACCAGTGATGG
gi|51242898|gb|AY606999.1|	AACTTTGGCAGACGTTGTGTTTCTCAAACGTC**G**ATTTAAACCTGATGAAGAATTCCCATTCCTGTT**T**A**G**ACCAGTGATGG
gi|51242902|gb|AY607001.1|	AACTTTGGCAGACGTTGTGTTTCTCAAACGTC**G**ATTTAAACCTGATGAAGAATTCCCATTCCTGTT**T**A**A**ACCAGTGATGG
gi|51242916|gb|AY607008.1|	AACTTTGGCAGACGTTGTGTTTCTCAAACGTC**G**ATTTAAACCTGATGAAGAATTCCCATTCCTGTT**T**A**G**ACCAGTGATGG
gi|51242910|gb|AY607005.1|	AACTTTGGCAGACGTTGTGTTTCTCAAACGTC**G**ATTTAAACCTGATGAAGAATTCCCATTCCTGTT**T**A**G**ACCAGTGATGG
gi|51242904|gb|AY607002.1|	AACTTTGGCAGACGTTGTGTTTCTCAAACGTC**G**ATTTAAACCTGATGAAGAATTCCCATTCCTGTT**T**A**G**ACCAGTGATGG
gi|51242900|gb|AY607000.1|	AACTTTGGCAGACGTTGTGTTTCTCAAACGTC**G**ATTTAAACCTGATGAAGAATTCCCATTCCTGTT**T**A**G**ACCAGTGATGG
gi|51242914|gb|AY607007.1|	AACTTTGGCAGACGTTGTGTTTCTCAAACGTC**G**ATTTAAACCTGATGAAGAATTCCCATTCCTGTT**T**A**G**ACCAGTGATGG
gi|51242906|gb|AY607003.1|	AACTTTGGCAGACGTTGTGTTTCTCAAACGTC**G**ATTTAAACCTGATGAAGAATTCCCATTCCTGTT**T**A**A**ACCAGTGATGG
gi|51242908|gb|AY607004.1|	AACTTTGGCAGACGTTGTGTTTCTCAAACGTC**G**ATTTAAACCTGATGAAGAATTCCCATTCCTGTT**T**A**A**ACCAGTGATGG
gi|51242912|gb|AY607006.1|	AACTTTGGCAGACGTTGTGTTTCTCAAACGTC**G**ATTTAAACCTGATGAAGAATTCCCATTCCTGTT**T**A**G**ACCAGTGATGG

[a] Reference sequence

Fig. 2. Multiple sequence alignment of the ERBV 3D region amplified by the real-time duplex PCR. Non-conserved nucleotides are bold and underlined. The ERBV primer and probe sequences used in the real-time duplex PCR are shown underlined on the top line.

Table 2
Comparison of the limits of detection (LoD) of the molecular methods applied in the present study.

Equine rhinitis virus	Matrix	Methods		
		Real-time duplex PCR	Conventional PCR[a]	RT-nested PCR[b]
		Limit of detection		
ERAV	Tissue culture supernatant	10^1 $TCID_{50}$/ml	10^5 $TCID_{50}$/ml	
	Urine	10^1 $TCID_{50}$/ml	10^5 $TCID_{50}$/ml	
	Lung	10^1 $TCID_{50}$/ml	10^5 $TCID_{50}$/ml	
	Nasal swabs	10^1 $TCID_{50}$/ml	10^5 $TCID_{50}$/ml	
ERBV	Tissue culture supernatant	1 $TCID_{50}$/ml		10^1 $TCID_{50}$/ml
	Urine	1 $TCID_{50}$/ml		10^1 $TCID_{50}$/ml
	Lung	1 $TCID_{50}$/ml		10^1 $TCID_{50}$/ml
	Nasal swabs	1 $TCID_{50}$/ml		10^1 $TCID_{50}$/ml

[a] Dynon et al., 2001.
[b] Black et al., 2007a.

viral dilution and tested in triplicate by the real-time duplex PCR and the PCR reference methods described previously (Black et al., 2007a; Dynon et al., 2001).

2.7. Intra- and inter-assay variability

To calculate the intra- and inter-assay variability, three different concentrations (high, medium and low; see Table 3) for both ERAV and ERBV were analysed in triplicate within the same run (intra-assay) and on three different days (inter-assay). The coefficient of variation (CV) was determined according to a protocol published previously (EPA, 2004).

2.8. Detection of ERAV/ERBV in clinical specimens

To evaluate the reliability of the method during clinical investigations, 86 nasal swabs collected from horses suffering from mild to severe respiratory symptoms were tested for ERAV/ERBV by real-time duplex PCR and virus isolation was attempted. For molecular tests, RNA was extracted from the clinical specimens as described for nasal swabs in Section 2.6. For virus isolation attempts, swabs were resuspended in 1 ml of phosphate-buffered saline (0.05 M to pH 7.0–7.4) with antibiotics (penicillin: 10000 IU/ml, streptomycin: 10 mg/ml, nystatin: 5000 IU/ml, gentamycin sulphate: 250 μg/ml) and clarified by centrifugation. Nearly confluent RK13 (ATCC CCL-37) and VERO (ATCC CCL-81) cell monolayers in 12.5 cm^2 flasks were used. Cell culture growth medium (Eagle Minimum Essential Medium, SIGMA, with 10% of foetal calf serum and 1% of L-glutamine) was removed, and 300 μl of sample was inoculated onto the cells, and then incubated 1 h at 37(±2)°C and in presence of 5% CO_2. 5 ml of maintenance medium (Eagle Minimum Essential Medium, SIGMA) were added to each flask. Inoculated cells were incubated at 37(±2)°C in presence of 5% CO_2 for 6 days, and were observed daily for the presence of cytopathic effect using an inverted microscope. Four blind passages were attempted both in VERO and RK13 cells. Cell culture lysates obtained at the end of the second, third and fourth passage were tested by real-time duplex PCR, in order to detect any growth of viruses in the absence of cytopathic effect.

Samples positive for ERAV or ERBV were confirmed by the PCR methods described previously.

3. Results

3.1. Primer and probe design

The 3D genomic region, encoding for the viral RNA polymerase, was selected for primers and probes design. It has been previously reported to be sufficiently conserved among strains belonging to the same virus type but distinguishable genetically in ERAV and ERBV (Huang et al., 2001). This was confirmed through the alignments of all the sequences available in the public database GenBank (n = 8 and 15 for ERAV and ERBV, respectively Figs. 1 and 2). Two distinct sets of primers and probe were designed for ERAV and ERBV. Primer and probe details and the reference sequences used for numbering of the oligonucleotides location are shown in Figs. 1 and 2.

Table 3
Intra-assay repeatability and inter-assay reproducibility for ERAV and ERBV. Coefficient variation values are expressed as percentages (CV%).

Matrix	Virus concentration ($TCID_{50}$/ml)	ERAV (CV%)		Virus concentration ($TCID_{50}$/ml)	ERBV (CV%)	
		Intra-assay	Inter-assay		Intra-assay	Inter-assay
Tissue culture supernatant	10^7	<5.8	<12	10^5	<1.8	<1.2
	10^4	<1	<4.6	10^2	<1.4	<2
	10^1	<3.5	<3	10^0	<2	<4
Urine	10^7	<4.4	<8.8	10^5	<1.3	n.d.[a]
	10^4	<4.2	<6	10^2	<3	<5.2
	10^1	<3.7	<4.7	10^0	<1.5	<1.5
Lung	10^7	<2.7	<5	10^5	<2	<6
	10^4	<4.5	<4.3	10^2	<2.1	<1.7
	10^1	<4.2	<2.3	10^0	<5.2	<6.4
Nasal swabs	10^7	<1.7	<4.4	10^5	<1.2	<1.8
	10^4	<2.1	<2.4	10^2	<1	<1.3
	10^1	<7.4	<4.1	10^0	<2.9	<2.7

[a] n.d.: no determined.

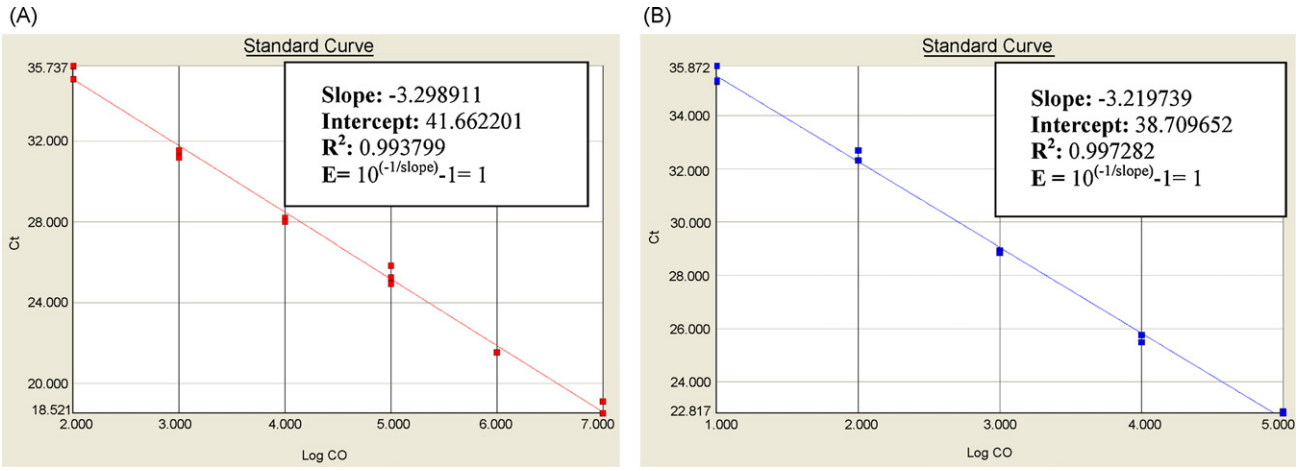

Fig. 3. Standard curves and dynamic ranges (R^2) of real-time duplex PCR. Viral RNA extracted from 10-fold dilutions of each virus strain was tested. Each plot represents the mean of three replicate amplifications of each dilution. The *x*-axis indicates the virus concentration expressed in $TCID_{50}$/ml. The *y*-axis indicates the Ct values. The examples show standard curves of the amplification of ERAV (A) and ERBV (B) in 'spiked' nasal swabs.

The ERBV set was designed taking into account the genetic variations in order to detect ERBV genotypes 1, 2 and 3. Designed primers amplified 69 and 82 bp products for ERAV and ERBV, respectively. TaqMan probes were labelled with fluorescent reporter dyes at the 5′end: FAM for ERAV and VIC for ERBV. TAMRA dye was used as quencher at the 3′end (Livak et al., 1995).

3.2. Analytical specificity

The primer and probe sets were able to detect and distinguish ERAV and ERBV tested by real-time duplex PCR. The assays were specific and no positive fluorescence signal was detected when the other micro-organisms listed in Table 1 were tested.

3.3. Analytical sensitivity and intra- and inter-assay variability

The limit of detection (LoD) of the methods was calculated as the last dilution of virus that was detected in the assay. Results are summarised in Table 2. The LoDs of the real-time duplex PCR were 10 $TCID_{50}$/ml for ERAV and 1 $TCID_{50}$/ml for ERBV in all matrices tested. The LoD of the reference ERAV PCR (Dynon et al., 2001) was calculated as 10^5 $TCID_{50}$/ml. The LoD of the reference RT-nested PCR for ERBV (Black et al., 2007a) was 10 $TCID_{50}$/ml.

To determine the linearity of the reaction and the influence of the different matrices on the PCR efficiency, the Ct values of the individual dilution steps for each matrix were plotted against the logarithm of the $TCID_{50}$/ml resulting in a typical standard curve.

The reaction efficiencies for ERAV ranged between 92% (tissue culture supernatants) and 100% (other tested matrices). For ERBV, the reaction efficiencies ranged between 83% (urine) and 100% (all other tested matrices). The linear range was between 10^7 and 10^2 for ERAV, and 10^5 and 10^1 for ERBV. The correlation coefficient (R^2) for ERAV was between 0.96 (urine) and 0.99 (nasal swabs), and for ERBV the correlation coefficient varied between 0.91 (urine) and 0.99 (nasal swabs and lung) (see Fig. 3 for an example).

The results of the intra- and inter-assay variability are summarized in Table 3. The assay demonstrated good reproducibility with the highest CV values in the inter-assay tests.

3.4. Detection of ERAV/ERBV in clinical specimens

None of the 86 samples tested by real-time duplex PCR were positive for ERAV. Fourteen samples (16.3%) were positive for ERBV by real-time duplex PCR. The reference protocol applied (Black et al., 2007a) confirmed the presence of ERBV in 11 out of 14 (78.6%) samples. In the three samples tested negative by RT-nested PCR, high Ct values (>36) were detected by the real-time PCR. Based on the standard reference curve developed for ERBV (Fig. 3B), these Ct values corresponded to viral loads ranging from 1 to 10 $TCID_{50}$/ml. These values were below the LoD established in the present study for the RT-nested PCR. Sequence analysis of the ERBV VP1 region indicated the correct detection of the virus, and identified the positive samples as type 1 ERBVs (Black et al., 2005). The representative sequence was deposited in the GenBank database (accession number EU246848). All samples were negative by virus isolation.

4. Discussion

Although ERAV and ERBV infections are considered a common event in horses, limited data exist on the pathogenesis and prevalence of these infections (Black et al., 2007b; Burrell et al., 1996; Carman et al., 1997; Hartley et al., 2001; Kriegshauser et al., 2005; Li et al., 1997; McCollumn and Timoney, 1992; Studdert and Gleeson, 1978; Wernery et al., 1998; Willoughby et al., 1992). This lack of knowledge could be attributable in part to the absence of suitable diagnostic techniques for these infections. Virus isolation cannot be considered a valid diagnostic tool due to the poor replication of these viruses in the tissue cultures examined so far. In addition, successful virus isolation often requires multiple blind passages and subsequent confirmation through the application of additional techniques such as electron microscopy and immunofluorescence for non-cytopathic strains. All these reasons make this technique time consuming and not practical for clinicians.

Few rapid molecular tests have been developed for the detection of these viruses in clinical specimens, essentially one RT-PCR for ERAV and one RT nested-PCR for ERBV (Black et al., 2007a; Dynon et al., 2001). The original papers did not describe their specificity and only the sensitivity of the ERBV RT-nested PCR assay was assessed at 0.7 $TCID_{50}$/ml. Up to now, these protocols represented the only rapid molecular methods for ERAV/ERBV detection in clinical specimens. In the present investigation the sensitivity of these assays was tested and they were used as reference methods for comparison of results.

In the real-time duplex PCR for ERAV/ERBV developed in this study, primers and probes were designed targeting the highly conserved 3D region (Huang et al., 2001). The method demonstrated

high type-related specificity with no cross-reactions to other micro-organisms that could be detected commonly in samples of equine origin. The method also demonstrated excellent sensitivity, the LoDs being 10 $TCID_{50}$/ml for ERAV and 1 $TCID_{50}$/ml for ERBV for each tested matrix. Compared to the methods described previously (Black et al., 2007a; Dynon et al., 2001), the real-time PCR protocol developed in this study showed higher sensitivity, particularly in the ERAV detection. For ERBV, the real-time PCR protocol was 10 times more sensitive than the RT nested-PCR protocol. In addition, RT nested-PCR is time consuming and prone to cross-contamination compared with the real-time PCR protocol.

Concerning the RT nested-PCR, the different levels of sensitivity obtained in this study could be due to the different characteristics of virus strains used as reference material and/or to the different performances of the RNA extraction kits applied.

The real-time duplex PCR protocol developed in this study appears repeatable, robust and applicable to different types of samples likely to be collected during clinical investigations (e.g., nasal swabs, respiratory tissues and urine). Indeed, similar PCR efficiencies were recorded regardless of the different matrices used, the only exception being urine, where the effect of PCR inhibitors was the likely cause of the reduced efficiency (83%) for ERBV detection. Evidence for the reproducibility of the method was demonstrated with CV values contained within 7.4% and 12% for intra- and inter-assay tests respectively. As expected, the highest CV values were recorded in the extreme dilutions and in the inter-assay tests. The results obtained indicate that this protocol might also be applied to quantitative studies on viral pathogenesis and viral shedding.

Clinical specimens tested for ERAV/ERBV by the real-time duplex PCR were also tested by other PCRs (Black et al., 2007a; Dynon et al., 2001). Of 14 samples positive for ERBV by real-time duplex PCR, 11 were also positive by RT nested-PCR. In the three discrepant samples, high Ct values (>36) were detected by real-time indicative of viral loads lower than 10 $TCID_{50}$/ml, thus below the LoD of the RT-nested PCR. The lower sensitivity of the RT nested-PCR compared to real-time duplex PCR observed in this study, may well explain the discrepancy in these results. The specificity of the ERBV detection in the clinical samples was confirmed by partial sequencing of the VP1 viral genomic region and identification of a type 1 ERBV (Black et al., 2005). This finding excluded laboratory contamination of the samples since both positive control viruses were type 2 ERBVs. The ability of the protocol to detect different genotypes of ERBV was, therefore, also confirmed.

Unfortunately, virus isolation was unsuccessful with the 14 samples positive by the molecular methods confirming the difficulties reported in replicating these viruses *in vitro* (Dynon et al., 2007; Black et al., 2007a; Li et al., 1997).

Due to the relatively low number of samples tested and the absence of clinical samples positive for ERAV in the present study, more extensive field investigation studies are necessary to validate further this protocol. For clinical investigations, the use of an internal amplification control (IAC) is recommended to identify false negative samples resulting from the presence of PCR inhibitors in the sample or degradation of the nucleic acid (Hoorfar et al., 2004). In this regard, endogenous (i.e. a housekeeping-gene) or exogenous nucleic acid (i.e. a micro-organism which is usually not found in the sample type to be tested) can be used as internal control. These types of internal controls can be applied to many different tests, running independently of the specific virus assay and are available in many diagnostic laboratories. Alternatively, a competitive IAC (i.e. the target and the IAC are amplified with one common set of primers and under the same conditions in the same PCR tube) can be developed bearing in mind that all of these approaches have advantages and limitations (Hoorfar et al., 2004; EPA, 2004). For example, in case a competitive IAC approach is applied, the different amplification assays will be competing for the same reagents in the same tube, thus target detection sensitivity may be affected adversely (Hoorfar et al., 2004; EPA, 2004). However, the results of the evaluation study described above will be useful to assess the impact of this type of IAC on the test performances and to optimize the assay. In conclusion, these results indicate that the real-time duplex PCR developed can be considered a valid tool for the detection of ERAV/ERBV infections in horses.

Acknowledgments

This work was supported financially by the Italian Ministry of Health. The authors wish to thank Udeni B.R. Balasuriya, University of Kentucky, USA, Janet Daly and Bob Geragthy, Animal Health Trust, Suffolk-UK, for kindly supplying ERAV and ERBV strains used in this work. Marco Salvadori and Edwin Lagas are gratefully acknowledged for sample collection and submission.

References

Black, W.D., Hartley, C.A., Ficorilli, N.P., Studdert, M.J., 2005. Sequence variation divides Equine rhinitis B virus into three distinct phylogenetic groups that correlate with serotype and acid stability. J. Gen. Virol. 86, 2323–2332.

Black, W.D., Studdert, M.J., 2006. Formerly unclassified, acid-stable equine picornaviruses are a third equine rhinitis B virus serotype in the genus Erbovirus. J. Gen. Virol. 87, 3023–3027.

Black, W.D., Hartley, C.A., Ficorilli, N.P., Studdert, M.J., 2007a. Reverse transcriptase-polymerase chain reaction for the detection of equine rhinitis B viruses and cell culture of the virus. Arch. Virol. 152, 137–149.

Black, W.D., Wilcox, R.S., Stevenson, R.A., Hartley, C.A., Ficorilli, N.P., Gilkerson, J.R., Studdert, M.J., 2007b. Prevalence of serum neutralising antibody to equine rhinitis A virus (ERAV), equine rhinitis B virus 1 (ERBV1) and ERBV2. Vet. Microbiol. 119, 65–71.

Burrell, M.H., Wood, J.L.N., Whitwell, K.E., Chanter, N., Mackintosh, M.E., Mumford, J.A., 1996. Respiratory disease in thoroughbred horses in training: the relationships between disease and viruses, bacteria and environment. Vet. Rec. 139, 308–313.

Carman, S., Rosendal, S., Huber, L., Gyles, C., McKee, S., Willoughby, R.A., Dubovi, E., Thorsen, J., Lein, D., 1997. Infectious agents in acute respiratory disease in horses in Ontario. J. Vet. Diagn. Invest. 9, 17–23.

Dynon, K., Varrasso, A., Ficorilli, N., Holloway, S.A., Reubel, G.H., Li, F., Hartley, C.A., Studdert, M.J., Drummer, H.E., 2001. Identification of equine herpesvirus 3 (equine coital exanthema virus), equine gammaherpesviruses 2 and 5, equine adenoviruses 1 and 2, equine arteritis virus and equine rhinitis A virus by polymerase chain reaction. Aust. Vet. J. 79, 695–702.

Dynon, K., Black, W.D., Ficorilli, N., Hartley, C.A., Studdert, M.J., 2007. Detection of viruses in nasal swab samples from horses with acute, febrile, respiratory disease using virus isolation, polymerase chain reaction and serology. Aust. Vet. J. 85, 46–50.

EPA, United States Environmental Protection Agency, 2004. Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples. U.S. Environmental Protection Agency, Cincinnati, OH.

Hartley, C.A., Ficorilli, N., Dynon, K., Drummer, H.E., Huang, J.-A., Studdert, M.J., 2001. Equine rhinitis A virus: structural proteins and immune response. J. Gen. Virol. 82, 1725–1728.

Hoorfar, J., Malorny, B., Abdulmawjood, A., Cook, N., Wagner, M., Ach, P., 2004. Practical considerations in design of internal amplification controls for diagnostic PCR assay. J. Clin. Microbiol. 42, 1863–1868.

Huang, J.-A., Ficorilli, N., Hartley, C.A., Wilcox, R.S., Weiss, M., Studdert, M.J., 2001. Equine rhinitis B virus: a new serotype. J. Gen. Virol. 82, 2641–2645.

Kriegshauser, G., Deutz, A., Kuechler, E., Skern, T., Lussy, H., Nowotny, N., 2005. Prevalence of neutralizing antibodies to Equine rhinitis A and B virus in horses and man. Vet. Microbiol. 106, 293–296.

Li, F., Drummer, H.E., Ficorilli, N., Studdert, M.J., Crabb, B.S., 1997. Identification of noncytopathic equine rhinovirus 1 as a cause of acute febrile respiratory disease in horses. J. Clin. Microbiol. 35, 937–943.

Livak, K.J., Flood, S.J., Marmaro, J., Giusti, W., Deetz, K., 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl. 4, 357–362.

Lorenz, R.G., Bogel, K., 1973. Calculation of titres and their significance A. Spearman–Karber, B. Method of Reed and Muench, C. Infectivity titration based on plaque counts. In: Kaplan, M.M., Koprowski, H. (Eds.), Laboratory Techniques in Rabies, 3rd ed. World Health Organization, Geneva, Switzerland, pp. 321–335.

McCollumn, W.H., Timoney, P.J., 1992. Studies on seroprevalence and frequency of rhinovirus I and rhinovirus II infection in normal horse. In: Plowright, W., Rossdale, P.D., Wade, J.F. (Eds.), Equine Infectious Diseases VI. Proceedings of the 6th International Conference. R&W Publications, Newmarket, UK, Ltd., pp. 83–87.

Plummer, G., 1962. An equine respiratory virus with enterovirus properties. Nature 195, 519–520.
Plummer, G., 1963. An equine respiratory enterovirus. Some biological and physical properties. Arch. Gesamte Virusforsch. 12, 694–700.
Stanway, G., Brown, F., Christian, P., Hovi, T., Hyypiä, T., King, A.M.Q., Knowles, N.J., Lemon, S.M., Minor, P.D., Pallansch, M.A., Palmenberg, A.C., Skern, T., 2005. Family *Picornaviridae*. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy. 8th Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, London, UK, pp. 757–778.
Studdert, M.J., Gleeson, L.J., 1978. Isolation and characterisation of an equine rhinovirus. Zentralbl. Veterinarmed. B 25, 225–237.
Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol., doi:10.1093/molbev/msm092.
Wernery, U., Wernerey, R., Zachariah, R., Haydn-Evans, J., 1998. Serological survey of some equine infectious diseases in the United Arab Emirates. In: Wernery, U., Wade, J.F., Mumford, J.A., Kaaden, O. (Eds.), Equine Infectious Diseases VIII. Proceedings of the 8th International Conference. R&W Publications, Newmarket, UK, Ltd., pp. 367–370.
Willoughby, R., Ecker, G., McKee, S., Riddolls, L., Vernaillen, C., Dubovi, E., Lein, D., Mahony, J.B., Chernesky, M., Nagy, E., Staempfli, H., 1992. The effects of equine rhinovirus, influenza virus and herpesvirus infection on tracheal clearance rate in horses. Can. J. Vet. Res. 56, 115–121.