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Original article

Use of an internal standard in a closed one-tube RT-PCR for the detection of equine arteritis virus RNA with fluorescent probes*

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Abstract – Routine detection of equine arteritis virus (EAV) can be achieved by virus isolation (VI) in cell culture, or by the amplification of viral genome by molecular methods. To simplify molecular diagnosis, a number of different Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and RT-nested PCR (RT-nPCR) assays were compared, and a one-tube method was developed and optimised utilizing a fluorogenic probe (TaqMan®). An artificial RNA template (Mimic) and associated probe were also constructed to provide in-tube validation of the RT-PCR system. To assess the utility of the RT-PCR TaqMan® assay, 28 different isolates of EAV representing different genetic groups of American and European strains were tested. Furthermore, the ability of VI and RT-PCR TaqMan® assay to detect EAV in different biological matrices such as semen, nasal and faecal swabs and blood was compared. All 28 EAV strains were detected by the RT-PCR TaqMan® assay. The results of TaqMan® and VI testing were in agreement for 30 of the 33 semen samples and all of the 50 other clinical specimens examined: the RT-PCR TaqMan® assay detected 18 positive semen samples, three more than VI. In conclusion, the one-tube RT-PCR TaqMan® assay is a rapid, reliable method for the detection of EAV.

equine arteritis virus / RT-PCR / mimic / fluorescent probe / TaqMan®

1. INTRODUCTION

Equine arteritis virus (EAV), which is the prototype of the family *Arteriviridae*, causes equine viral arteritis (EVA). EVA is a disease of variable severity affecting horses and donkeys. The virus replicates in the respiratory tract leading to viraemia and

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secondary replication in blood vessel walls. Most infections are mild or subclinical, but respiratory signs with or without dependent subcutaneous oedema may occur. Differences in the outcome of infection may be attributed to age and breed of horse, route of infection, virus dose or virulence [17]. The virus can infect the fetus and abortion in up to 60% of mares infected in pregnancy has been recorded [9].

The appearance of serum antibodies from 7–10 days post infection coincides with clinical recovery, which is usually complete by 14–21 days. A proportion of male animals shed virus in semen if infected after puberty, and some (30%–50%) may become long-term virus shedders [17]. These carrier stallions are very important reservoirs for the initiation of new infections, passing the virus to mares via natural mating or artificial insemination.

Clinical EAV can be mistaken for other febrile respiratory diseases, whilst carrier stallions appear normal. Therefore, laboratory tests are needed to provide a definitive diagnosis. Serological tests are routinely used to detect specific anti-viral antibodies and virus isolation is used to detect the agent in nasal swabs, blood or semen. Testing for the presence of the virus in the semen of seropositive stallions is a key requirement of any EVA disease control program.

RT-PCR has been described as an alternative to virus isolation (VI) for the detection of EAV [2, 6, 13–16], especially for screening semen. In order to improve the reliability and ease of use of RT-PCR, the aim of this work was to compare existing RT-PCR primer sets in order to select a suitable combination for use in conjunction with a fluorescent probe in a one-tube assay. The use of a probe confirms the specificity of the PCR product and allows automated reading of the test. To identify false negative results caused by failure of the RT-PCR assay, an RNA internal standard or "mimic" was constructed, with the same primer–recognition nucleotide sequences as the wild-type virus, but with a larger heterologous DNA fragment inserted between the primer sites. The amplicon of the mimic can be distinguished from that of the virus not only by its size, using agarose gel electrophoresis, but also, as described here, by the use of an additional fluorescent probe specific for the insertion.

2. MATERIALS AND METHODS

2.1. EAV isolates

In order to assess the ability of the TaqMan® RT-PCR assay to detect a wide range of EAV isolates, a panel was assembled, representative of the diversity revealed by published phylogenetic analyses (Tab. I), [1, 3, 7, 11, 12, 14, 16]. These viruses, which were propagated in RK-13 cells using established procedures [17], varied in virulence, came from different geographical locations and had been isolated over a period of 30 years.

Clinical samples of heparinised blood (n = 9), nasal (n = 27) and faecal swabs (n = 14) for validation of the TaqMan® RT-PCR assay were obtained from eight ponies challenged with different biological clones of the Bucyrus strain of EAV. These clones were generated in our laboratory on passage in EEL cell culture of the original pleural fluid isolate of EAV. A panel of EAV VI positive (n = 18) and VI negative semen samples (n = 15) were also tested by TaqMan® RT-PCR, selected from semen samples sent in for routine EAV diagnosis by virus isolation.

2.2. Isolation of RNA

RNA was extracted from 280 μL volumes of RK-13 cell culture supernatants of the different virus strains, seminal plasma from centrifuged whole semen or

Table I. EAV isolates tested by TaqMan® PCR.

No	Strain	Year isolated	Group[1]	PCR-ORF 7 gel	TaqMan® RT-PCR
1	Polish-Wrowclaw	1978	II.B	+	+
2	McCollums- 1988	1988	I.B	+	+
3	French strain 1989	1989	II.A	+	+
4	Arvac F.D. (Sweden)	1997	I.B	+	+
5	U.K. Isolate G.B.1-1997	1997	II.A	+	+
6	U.K. Isolate G.B 2-1997	1997	I.A	+	+
7	U.K. Isolate G.B 3-1997	1997	II.B	+	+
8	U.K. Isolate G.B. 6-1997	1997	n.d.	+	+
9	CVL Bucyrus P.8	1971	I.B	+	+
10	U.K. Isolate G.B. 5-97	1997	n.d.	+	+
11	French Isolate 2000	2000	II.A	+	+
12	Ames -USA	1995	I.A	+	+
13	Velogenic Bucyrus P.fluid	1970	I.B	+	+
14	ATCC Strain USA	1995	I.B	+	+
15	ARVAC F.D. Vacc.	1996	I.B	+	+
16	Kentucky 1988	1988	I.B	+	+
17	Vienna Strain	1968	II.A	+	+
18	Leiden Strain 2000	2000	II.A	+	+
19	EAV 86 110 -USA	1986	II.A	+	+
20	A165 -USA	1995	II.A	+	+
21	S 1128 -USA	1996	I.B	+	+
22	1489v / 96 - US	1996	II.A	+	+
23	874v / 97 - USA	1997	II.A	+	+
24	1330ve99 - USA	1999	II.B	+	+
25	D526 - USA	1996	II.B	+	+
26	EAV86P - USA	1986	II.A	+	+
27	EAV 135 -USA	1996	II.B	+	+
28	86R - USA	1986	II.B	+	+

[1] Phylogenetic groups derived from classification proposed by Stadejek, [14]; n.d. not determined.

heparinised whole blood using QIAamp viral RNA kits (Qiagen, Crawley, UK). In addition, nasal and faecal swabs collected from experimentally infected ponies were put into 10 mL and 2 mL of PBS respectively and were squeezed with sterile forceps and centrifuged at 400 *g*. A sample of the resulting suspension (280 µL) was taken for RNA extraction. The RNA was stored at –80 °C until required, but was freeze-thawed several times in the course of testing the different PCR methods.

2.3. Evaluation of different RT-PCR and RT-nPCR methods

An initial evaluation of five existing RT-PCR and RT-nPCR methods was made (Tab. II). The relative sensitivity of tests was assessed using 10-fold dilutions of the Bucyrus strain of EAV, starting at a concentration of $10^{5.5}$ $TCID_{50}$/mL. RT and individual PCR steps were carried out in separate reaction tubes. First strand synthesis was carried out in 20 µL volumes in

Table II. Comparison of different RT-PCR methods used for the detection of EAV.

Ref Method	Genome target	1st round Primer location*	Nested primer location*	# cycles (1st and 2nd round)	Assay sensitivity (log_{10} dilution)	
					Single round	Nested PCR
Unpublished	ORF5	11087–11106	11146–11168	32	–4	–7
		11936–11917	11913–11893			
[4]	ORF7	12319–12338	12369–12388	40	–4	–7
		12628–12609	12552–12533			
Unpublished	ORF7	12310–12331	12342–12362	30	–4	–7
		12704–12675	12603–12582			
[15]	Leader	35–54	75–102	30	–2	–7
		203–184	180–163			
Unpublished (EAV 10 and EAV12)	ORF7	12284–12303	n.a.	40	–7	n.a.
		12682–12663	n.a.			

* Primer locations determined from GenBank accession number X53459; n.a. = not applicable.

RT buffer (Promega, Madison, USA) containing 2 µL of RNA, 25 pmol of random hexamers (Pharmacia, St Albans, UK), 0.5 mM of each dNTP, 20 U of RNAsin (Promega, Madison, USA), and 100 U of Molony Murine Leukemia Virus Reverse Transcriptase (M-MLV RT: Promega, Madison, USA). Samples were incubated at 42 °C for 30 min prior to inactivating RT at 95 °C for 5 min. Amplification of these cDNA preparations using the respective primers (25 pmol) was undertaken in fresh tubes and 50 µL volumes, using 2.5 µL of the prepared cDNA in a ABgene master mix (Epsom, UK) containing 1.25 units of *Taq* DNA polymerase, 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.01% (v/v) Tween 20, and 0.2 mM each of dATP, dCTP, dGTP and dTTP. One drop of mineral oil (Promega, Madison, USA) was added to the 0.5 mL reaction tubes. For the nested PCRs, 2.5 µL of the first round product was added to 45 µL of the ABgene master mix together with 25 pmol of the appropriate forward and reverse primers and made up to 50 µL with sterile distilled water. After the addition of mineral oil, the PCR assays were performed on a Biometra TRIO-Thermoblock (Whatman, Maidstone, UK). PCR cycling conditions for the published methods [4, 15] were as described. For the ORF-5 RT-nPCR (unpublished) the following cycling conditions were used: initial denaturation, 95 °C for 5 min; followed by 32 cycles of template denaturation at 95 °C for 1 min; primer annealing at 55 °C for 1 min and extension at 72 °C for 1 min. The reaction was terminated by a single extension step of 72 °C for 7 min after which the samples were held at 4 °C. For the unpublished ORF7 PCR, the following cycling conditions were used: initial denaturation at 95 °C for 5 min; and then 40 cycles of the following reaction parameters: template denaturation at 95 °C for 20 s; primer annealing at 58 °C for 20 s and extension at 72 °C for 30 s. The reaction was terminated by a single extension step of 72 °C for 7 min and was finally held at 4 °C.

2.4. Construction of an artificial template (Mimic)

A 399 bp fragment of a strain of EAV (isolated from an Eastern European stallion), containing the proposed diagnostic

Table III. Nucleotide mismatches at primer and probe recognition sites of 50 EAV ORF-7 Sequences (GenBank).

GenBank accession number	Sense primer (EAV10) GTACACCGCAGTTGGTAACA	TaqMan® probe site TGGTTCACTCACTGCAGATGCCGG	Anti-sense primer (EAV12) ACTTCAACATGACGCCACAC
22 EAV sequences	--------------------	------------------------	-------------------- (12 sequences)
X78498	--------------------	----------T---------	--------------------
X78492	--------------------	----A---------------	--------------------
U81029	------------------T-	------------------------	n.a.
U81025	------------------T-	------------------------	n.a.
U81015	------------------T-	------------------------	n.a.
AF065824	------A-------------	------------------------	n.a.
AF118770	------A-------------	------------------------	n.a.
AF118771	------A-------------	------------------------	n.a.
AF118772	------A-------------	------------------------	n.a.
AF118775	------A-------------	C-----------------------	n.a.
AF118776	------A-------------	------------------------	n.a.
AF118778	------A-------------	------------------------	n.a.
AF118779	------A-------------	C-----------------------	n.a.
AF118780	------A-------------	------------------------	n.a.
AF118781	------A-------------	------------------------	n.a.
AF107266	---------------G--T-	------------------------	n.a.
AF107267	---------------G--T-	------------------------	n.a.
AF107268	---------------G--T-	------------------------	n.a.
AF107269	---------------G--T-	------------------------	n.a.
AF107270	---------------G--T-	------------------------	n.a.
AF107272	---T-----------G--T-	------------------------	n.a.
AF107273	------------C--G--T-	------------------------	n.a.
AF107274	---T-----------G--T-	------------------------	n.a.
AF107275	---------------G--T-	---C--------------------	n.a.
AF107276	---------------G--T-	------------------------	n.a.
AF107277	---------------G--T-	------------------------	n.a.
AF107278	---------------G--T-	------------------------	n.a.
AF107279	---------------G--T-	------------------------	n.a.

n.a. sequence data not available.

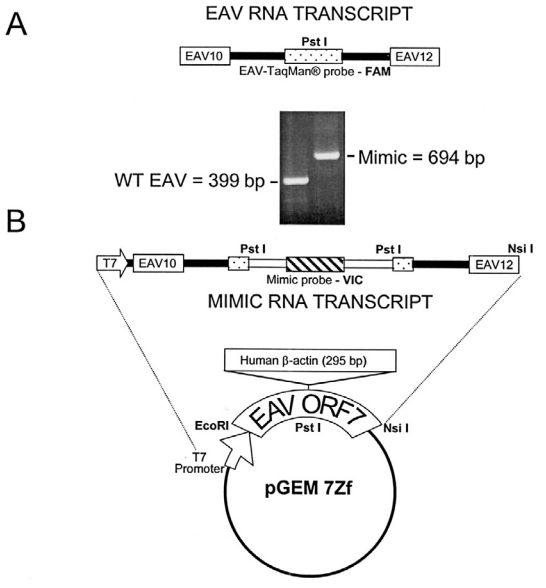

Figure 1. A schematic diagram of the artificial EAV TaqMan® mimic RNA construct. RNA was prepared by in-vitro transcription using T7 RNA polymerase. The EAV TaqMan® probe site (⁘) was disrupted in the mimic by the insertion of a 295 bp fragment of human β-actin and was detected by a second probe (▨). The location of PCR primers (EAV10 and EAV12) are shown.

ORF-7 PCR amplicon, was amplified using primers EAV10 and EAV12 (Tab. III) modified at the 5' end to provide cleavage sites for *EcoR* I and *Nsi* I respectively, and subsequently cloned into pGEM-7f (+) (Promega, Madison, USA). Plasmid DNA was purified using a QIAprep spin minikit (Qiagen, Crawley, UK) and the presence of inserts of the appropriate size was confirmed by restriction enzyme digestion and by sequencing (Big Dye, Applied Biosystems, Foster City, USA). The strategy for the design of the artificial template (Fig. 1) exploited the presence of a single *Pst* I site located within the region targeted by the EAV TaqMan® probe (12546–12569). After linearisation with *Pst* I, the plasmid was incubated with calf alkaline phosphatase (Promega, Madison, USA) to prevent recircularisation. A 295 bp fragment of human beta-actin gene generated by PCR using primers with 5'-*Pst* I linkers: (5'-TTT CTG CAG TCA CCC ACA CTG TGC CCA TCT ACG A-3' and (5'-TTT CTG CAG CAG CGG AAC CGC TCA TTG CCA ATG G-3'), was ligated into the plasmid, and transfected into *E. coli* DH5α (Invitrogen, Paisley, UK). Purified plasmids (QIAprep spin minikit, Qiagen, Crawley, UK) were linearised with *Nsi* I and purified (Qiagen gel kit) for use as templates for in vitro transcription. RNA transcripts were prepared using the Megascript T7 RNA polymerase kit (Ambion, Austin USA) according to the manufacturer's instructions, treated with DNAse I and stored at –70 °C for use as an artificial template (mimic) in the single tube RT-PCR. Successful amplification of this mimic in the samples was detected using a TaqMan® probe containing a different fluorochrome to that used for detection of the viral amplicon (5'-VIC™ GCC ACG TCC AGA CAC AGG ATG GCA -3'- [6-carboxy-tetramethyl-rhodamine (TAMRA)].

The appropriate dilution of mimic RNA used in the RT-PCR TaqMan® assay was determined by serial dilution so that RT-PCR TaqMan® assay sensitivity was not affected.

2.5. TaqMan® assay method

A TaqMan® probe (5'- [6-carboxy-fluorescein (FAM)]-TGG TTC ACT CAC TGC AGA TGC CGG-3'-TAMRA) was designed based on an alignment of 50 EAV sequences available in GenBank (Tab. III) for the proposed amplicon in ORF7. This probe spans the single *Pst* I site exploited during the construction of the mimic (Fig. 1). Preliminary experiments indicated that TaqMan® signal would be compromised using 2.5 mM $MgCl_2$. In order to increase the magnesium ion concentration for optimal TaqMan® assay signal, the Abgene mastermix was replaced with 10 × reaction buffer (Promega, Madison, USA).

A single-tube RT-PCR assay was performed containing 50 mM KCl, 10 mM Tris-HCl pH 9.0, 5 mM $MgCl_2$, 0.4 mM of each dNTPs, and 5 U *Taq* DNA polymerase (All reaction components were obtained from Promega, Madison, USA unless stated otherwise). To the mastermix was added 20 U RNAsin, 100 U M-MLV-RT, 25 pmol of EAV10 (5'-GTA CAC CGC AGT TGG TAA CA-3') and EAV12 (5'-ACT TCA ACA TGA CGC CAC AC-3') primers, 5 pmol EAV probe (FAM reporter), 5 pmol mimic probe (VIC™ reporter). HPLC water was added to make the final volume up to 50 µL. The mastermix containing the buffers, probes and mimic RNA were aliquoted into a 96 well plate (MicroAmp Perkin-Elmer, Foster City, USA) and then the sample RNA was added last in a separate work area. In addition to wells containing RNA from test samples, control wells containing only mimic template were also setup between each sample well, substituting HPLC water for the sample RNA. Further control wells without either sample or mimic RNA templates were also set up. After sealing with optical caps (MicroAmp, Perkin Elmer, Foster City, USA), the plate was placed in a 9700 GeneAMP PCR thermal cycler (Applied Biosystems, Warrington, UK) and subjected to the following cycling conditions, 42 °C, 30 min, inactivation at 95 °C for 5 min, then 40 cycles of the following reaction parameters: template denaturation at 95 °C for 20 s, primer annealing at 60 °C for 60 s and extension at 72 °C for 30 s. The reaction was terminated by a single extension step of 72 °C for 7 min and finally held at 4 °C. The TaqMan® reactions were read on an ABI PRISM 7200 sequence detector (Applied Biosystems, Warrington, UK).

2.6. Statistical analysis

Statistical analysis of the TaqMan® data was performed in order to determine which samples were EAV positive, EAV negative and reaction failures. 2D scatter-plots of the FAM and VIC™ fluorescent signals were generated using Statistica (ver 6, Statsoft, Tulsa, USA). This software package was used in order to define 99.9% confidence ellipses around the water control and no template control wells. EAV negative samples were grouped with the water control wells, while EAV positive samples were identified as points that were outside of the water control 99% confidence ellipse. Sample failures were grouped with readings from no template controls.

2.7. Virus detection by virus isolation (VI)

Virus titres within semen and nasal swabs were determined by virus isolation (VI), essentially as described previously [8]. Briefly, in the case of semen samples, an initial 1:10 dilution of sample, starting with 0.5 mL was made in Minimal Essential Medium (MEM) and then doubling dilutions made up to 1:3560. Finally, 0.5 mL of each sample dilution was inoculated into 25 cm^2 flasks of

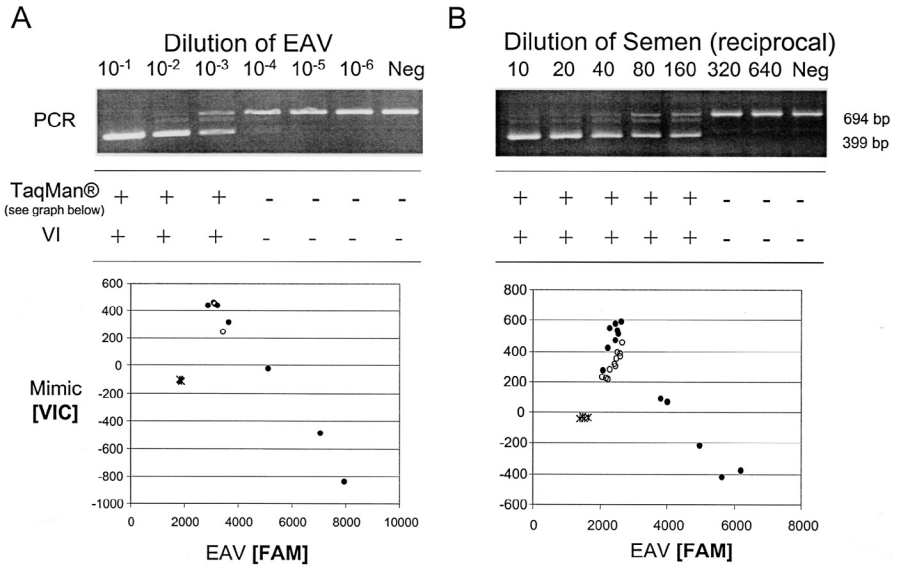

Figure 2. Comparative sensitivity of the TaqMan® RT-PCR compared with gel-based detection of PCR product and virus isolation. Serial dilutions were prepared using EAV from tissue culture supernatant (A) and EAV positive semen titrated into EAV negative semen (B). 2D scatter-plots show the EAV-FAM and MIMIC VIC™ relative fluorescent signals (derived from multicomponent analysis) for these samples. Test samples (•), water controls (NEG:○) and no template controls (NTC wells: *) are shown.

confluent monolayers of RK-13 cells. Flasks were incubated for 1 h at 37 °C in 5% CO_2 and overlaid with 10 mL MEM supplemented with 2% bovine serum. The cells were cultured for a minimum of 7 days at 37 °C, before being passaged twice more by trypsinisation (split ratio 1:3). Half the cells from the third passage were also set out in 96-well microtitre plates. The cells were cultured for 3 days at 37 °C prior to fixation and antigen detection with either an EAV polyclonal antibody (LS 7005) or EAV-specific monoclonal antibody (WB20 [22]) in conjunction with an indirect immunoperoxidase staining method [8]. In the case of nasal swabs, 150 µL of nasal swab eluate was titrated (0.5 $\log_{10}$) in quadruplicate in 96-well plates. Then, 100 µL of EMEM containing 3×10^5 RK-13 cells and 10% FBS was added to each well and plates incubated for 3 days at 37 °C in 5% CO_2. Wells showing CPE were recorded as positive and virus titres were calculated according to the Karber formula [10] and expressed as $TCID_{50}$/mL of swab extract. Heparinised blood and faecal swabs were only diluted 1:10 for cell culture, but otherwise treated in the same way as for the semen and nasal swabs. Amplification of the RNA isolated from the clinical samples was carried out using the single tube RT-PCR TaqMan® method as described above.

To assess the accuracy of the TaqMan® assay in differentiating EAV from other arteriviruses, a series of 10-fold dilutions of viral RNA were carried out on the isolate H2 (Humberside) of porcine reproductive and respiratory syndrome virus (PRRSV) [5]. In addition, DNA (Qiagen, Crawley, UK) from equine herpesvirus-1 (RACH strain) and a field isolate of equine herpesvirus-2 were tested by the TaqMan® assay.

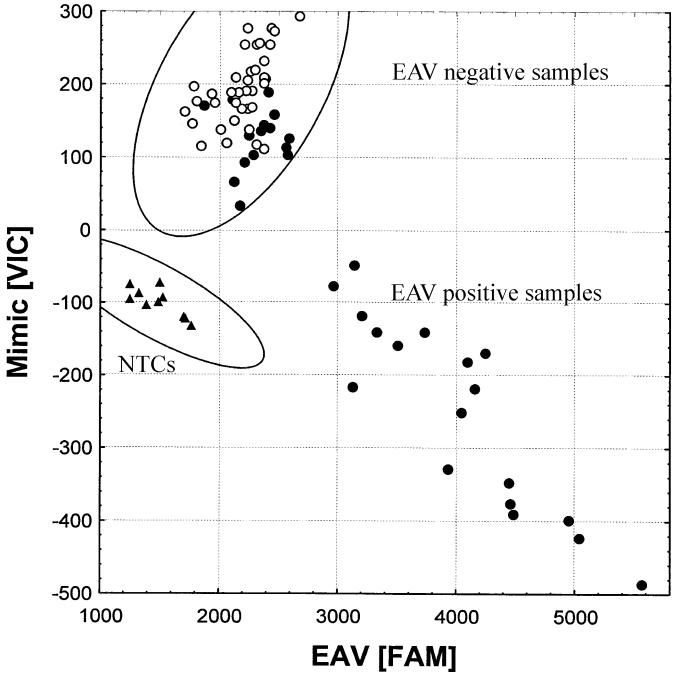

Figure 3. Detection of EAV in semen by TaqMan® RT-PCR. 2D scatter-graph (Statistica) shows relative fluorescent EAV-FAM and MIMIC-VIC™ signals (derived from multicomponent analysis) for semen samples (n = 33, •), water controls (NEG: n = 36, ○) and no template controls (NTC: n = 10, ▲). A 99.9% confidence ellipse was drawn around NEGs to discriminate EAV positive from EAV negative samples. RT-PCR failures would be contained within the NTC ellipse.

3. RESULTS

3.1. Evaluation of different RT-PCR and RT-nPCR methods

The same buffer system was employed with all 5 different RT-PCR and RT-nPCR methods, in order to determine the set of primers that give optimum sensitivity with a laboratory strain of EAV. Interestingly, an un-nested RT-PCR (using primers EAV 10 and 12) had similar analytical sensitivity as the 4 nested RT-PCRs. This method targeting ORF-7, the most homogeneous and stable region of EAV was therefore selected for adaptation to a fluorogenic assay. Interestingly, the unpublished ORF-5 (G_L) RT-nPCR which was included in this comparative exercise also detected all of the 28 diverse strains of EAV analysed in this study (data not shown). The ORF-5 responsible for the coding of the membrane region of EAV is subject to genetic variation. Therefore, since ORF-7 is recognised to be the most conserved gene for EAV, it was chosen in preference to ORF-5 as the diagnostic target.

3.2. TaqMan® assay optimisation

Amplification of the mimic template was inhibited when EAV RNA was present (shown in Fig. 2). This was not unexpected, since the smaller EAV amplicon will be amplified preferentially. At a dilution of 10^{-5} of mimic template, there was no detectable competition of EAV template. This concentration of mimic RNA

template was therefore added to subsequent TaqMan® assay reactions as an internal control.

After one step RT-PCR, all 28 RNA samples representing North American and European strains of EAV gave the expected 399-bp fragment in the agarose gels (Tab. I). Preliminary experiments with these primers, in the absence of the TaqMan® probe but using 2.5 mM $MgCl_2$ gave sharp well defined bands upon agarose gel examination, but produced poor results in the TaqMan® assay. It was found that by increasing the concentration of $MgCl_2$ (5 mM), the intensity of reporter fluorescence signal was optimised. This protocol was followed for all subsequent TaqMan® assays.

The detection sensitivity of the TaqMan® RT-PCR was compared with gel-based visualisation of PCR product and VI. Samples used were serial $\log_{10}$ dilutions of tissue culture derived EAV (Bucyrus strain) and EAV positive semen titrated in negative semen. Using these samples, there was good agreement between the TaqMan® PCR and the visualisation of PCR product in a gel. Furthermore, EAV detection by VI also had similar detection limits. The EAV TaqMan® assay gave negative results with template extracted from equine herpesvirus-1 and 2, and PRRSV.

3.3. Demonstration of EAV in clinical samples

Selected semen samples that had been received at the laboratory for VI in the last 10 years were also tested by the single tube TaqMan® assay. Of the 33 semen samples tested, 18 were positive by the RT-PCR TaqMan® assay (Fig. 3), three more than by VI. All of the nasal swabs, faecal swabs and blood samples (50 samples in all) taken from infected ponies were both positive by VI (mostly on first passage) and by single-tube RT-PCR TaqMan® assay (data not shown).

4. DISCUSSION

This report describes the development of a TaqMan® assay to combine one step RT-PCR amplification of the EAV ORF7 with an automated method of product detection using a specific fluorogenic probe. The method does not require time-consuming electrophoresis and/or hybridization to visualise and confirm specificity. Furthermore, since the PCR plates never had to be opened after adding the reaction components, the risk of contamination was reduced. RNA samples prepared on different occasions from replicate cell cultures and clinical samples gave very similar TaqMan® results, suggesting good reproducibility of the assay. Negative control samples were routinely interspersed between the samples tested, demonstrating that cross-contamination had been avoided. The method outlined in this report also utilizes an artificial RNA template (mimic) in order to validate negative results. Successful amplification of the mimic was detected by a second reporter dye (VIC™). Although there is considerable overlap between the emission spectra of the two reporter dyes used (FAM emax = 527 nm and VIC™ emax = 558 nm), the use of 2D scatter-plots was able to discriminate the signals due to the respective dyes.

All 28 EAV isolates tested were successfully detected by the TaqMan® assay. The TaqMan® probe recognition site matched all 50 EAV GenBank accessions except for 5 sequences which had single nucleotide substitutions (Tab. III). The sense primer (EAV 10) mismatched at one nucleotide position with 10 European strains of EAV and at two and three nucleotide positions with 13 and 3 US strains, respectively. The EAV 12 anti-sense

primer was located outside ORF-7, but matched at all the EAV nucleotide positions where sequence has been determined.

The aim of this study was to develop a TaqMan® PCR assay to detect EAV in semen and clinical samples. Proposed advantages of the TaqMan® PCR assay are that its amplification of virions often makes it more sensitive than VI, its single tube format decreases carry-over contamination and its ability to be standardized and quantitated overcome difficulties present in current PCR techniques. In addition, TaqMan® is very rapid (one day for results versus up to three weeks for VI) and capable of high throughput, which increases efficiency and decreases cost when compared to the standard test of virus isolation. The most practical use of the assay will be for the rapid diagnosis, typing and confirmation of EAV in equine semen. A further development of this TaqMan® assay could exploit the use of recently developed robotic extraction protocols to automate the RNA extraction and aliquoting procedures into a 96-well format. This approach would permit a greater throughput to be achieved, and may further reduce the risk of cross-contamination. In addition, the method could readily be adapted to quantify EAV RNA in semen and clinical samples. Rapid, sensitive and accurate detection of positive semen and clinical samples can be used to control this economically important disease for the horse industry.

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