Arch Virol (1995) 140: 1483–1491

Archives of Virology

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Printed in Austria

Detection of equine arteritis virus (EAV) by polymerase chain reaction (PCR) and differentiation of EAV strains by restriction enzyme analysis of PCR products

Brief Report

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Accepted March 31, 1995

Summary. A polymerase chain reaction (PCR) based assay capable of detecting and differentiating seven strains of equine arteritis virus (EAV) from around the world was developed. The primers for the PCR were chosen from the ORF 6 gene encoding the unglycosylated membrane protein (M). Viral RNA from cell culture fluids infected with each of the seven EAV strains and RNA from the live vaccine, Arvac, was detected by PCR using four sets of primers. The sensitivity of detection was increased from 100 to 1 000 times by performing nested PCR enabling the detection of RNA at a level of 0.5–5 PFU. Differentiation among the virus strains and the live vaccine was achieved by cutting the PCR-amplified products from three sets of primers with six restriction endonucleases. Using this procedure it was possible to distinguish among the seven EAV strains used.

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The equine arteritis virus (EAV) has been classified as a member of the *Togaviridae* family [22, 24]. However, recently EAV has been found to resemble the coronavirus and torovirus in its genome organization and gene expression strategy [7], yet differing from them in virion genome size and virion morphology. Therefore EAV may be a member of a recently proposed new family of viruses, the *Arteriviridae*, consisting of the lactate dehydrogenase-elevating virus, porcine reproductive and respiratory syndrome virus, and simian hemorrhagic fever virus [7, 21]. The clinical signs of equine viral arteritis are very variable and subclinical infections are the most common sequel [12]. To date a single serotype has been recognized, and no major antigenic variation has been demonstrated among EAV isolated from disparatechronological and geographic origins [18]. Murphy et al. showed high

levels of genomic heterogeneity among isolates of EAV through oligonucleotide fingerprint comparisons [19, 20]. We determined the nucleotide sequence of ORF 6, which encodes the unglycosylated membrane protein (M), from several strains from around the world, and showed many genetic variations among the strains in spite of limited antigenic variations [23]. The objective of this study was to detect each of the EAV strains using PCR, and to differentiate the strains through restriction enzyme analysis of their PCR products, based on the M protein nucleotide sequence data of the strains, together with those of the Bucyrus [7] and modified Bucyrus strain [25].

EAV strains used in this study were as follows: Bucyrus (Ohio) [8], modified Bucyrus [9, 14], Red Mile (Kentucky) [15], 84 KY-A1 (Kentucky) [19], Wroclaw-2 (Poland) [11], Bibuna (Switzerland) [2] and Vienna (Austria) [3]. In addition, a modified live virus vaccine (Arvac) was used. All strains of EAV were propagated in an RK13 rabbit kidney cell line. RNA was extracted from 250 μl of culture fluid with 500 μl of RNAzol B (Biotecx Laboratories, Inc.). RNA was precipitated with isopropanol, using 2 μl of ethachinmate (Nippon gene Co., Japan) as a carrier. The RNA pellet was dissolved in 30 μl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Six primers were selected from the coding region for the M protein. The structure of the EAV genome, the location of the primer pairs and the restriction sites used are shown in Fig. 1. Upper primer; M1, 5′-CTGAGGTATGGGAGC-

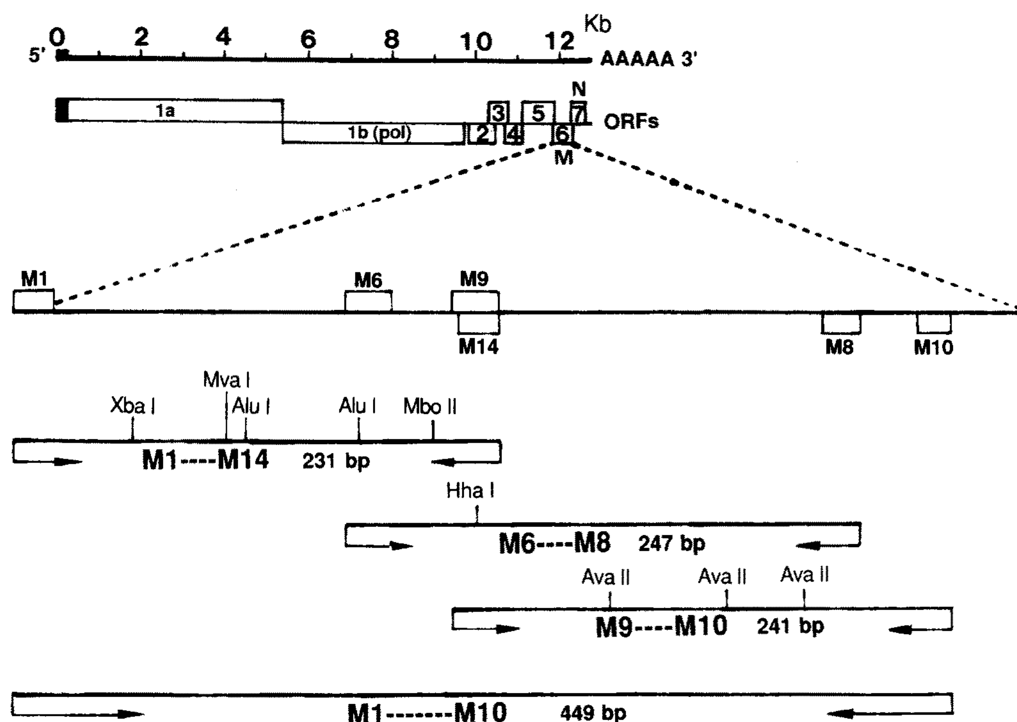

Fig. 1. Schematic representation of the EAV genome, and the locations of the PCR primers and restriction enzyme cleavage sites in the PCR products. The boxes with numbers indicate ORFs. The filled boxes indicate the 5′ leader sequence. The open boxes indicate primers

CATAG-3′ (identical to nucleotides 11895–11914 [7]). M6, 5′-TTAGCAGCTT-ATATTTGGTTTGT-3′ (identical to nucleotides 12052–12074). M9, 5′-TAT-GCTTTTGTGCTTTTGGCTGC-3′ (identical to nucleotides 12103–12125). Lower primer; M14, 5′-GCAGCCAAAAGCACAAAAGC-3′ (complementary to nucleotides 12125–12106). M8, 5′-CAACTGCGGTGTACCCGTT-3′ (complementary to nucleotides 12298–12280). M10, 5′-GGCCTGCGGACGTGATCG-3′ (complementary to nucleotides 12343–12326). Reverse transcription and PCR were performed in a final volume of 20 μl containing 3 μl of RNA using a GeneAmp RNA PCR kit (Perkin-Elmer-Cetus Co.) according to the protocol of the supplier. The tube was incubated at 42 °C for 15 min, at 94 °C for 5 min, and at 5 °C for 5 min. PCR was carried out in a final volume of 100 μl containing 20 μl of cDNA, 4 μl of $MgCl_2$ (25 mM), 2 μl of 10 × PCR buffer, 1 μl of upper stream primer (20 μM), 0.5 μl of AmpliTaq DNA polymerase (5U). The mixture was incubated at 94 °C for 2 min, followed by 35 cycles of denaturation (94 °C for 1 min) and annealing (60 °C for 1 min), and a final extension (60 °C for 7 min). Double nested PCR amplification was carried out using the external primer M1-M10 and various sets of inner primers. The second round amplification was carried out in a 50 μl reaction mixture containing 5 μl of 10 × PCR buffer, 0.2 U/μl of Taq polymerase, 2 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each inner primer and 1 μl of the first round PCR product. Denaturation and annealing consisted of 35 cycles 94 °C for 1 min, and 60 °C for 1 min, respectively, followed by a 7 min extension at 60 °C.

Using the primer pairs of M1-M10, M1-M14, M6-M8, or M9-M10, RNA from each of the strains and the live vaccine was equally and efficiently amplified producing a band of 449 bp, 231 bp, 247 bp, and 241 bp, respectively (Fig. 2). However, there was a single base mismatches in the M9 and M14 regions of the modified Bucyrus strain, in M6 of the Vienna strain, in M8 of the Bibuna and Vienna strains, and in M10 of the Bibuna strain. These mismatches were single internal mismatches, which probably had no significant effect on the PCR product yield. The mismatches in primer M9, M14, M6 and M10 were C:A (primer: template), A:C, C:T, and G:T, respectively. These mismatches were efficiently amplified even when located at the 3′ end of the primer [13]. The sensitivity of PCR for detecting EAV was determined by using serial ten-fold dilutions of infected cell fluid with a modified Bucyrus strain (2×10^7 PFU/ml). The first round PCR using the M1-M10 primer pair generated a faint though visible 449 bp product at a dilution level of 10^{-3}, corresponding to about 500 PFU (Fig. 3A). When the PCR product obtained from the first round PCR was reamplified with one of the three sets of inner primers M1-M14, M6-M8, or M9-M10, all of the respective 231 bp, 247 bp and 241 bp products were detected at a 10^{-6} dilution level of cell fluid (Fig. 3B). The results showed that reamplification with the nested or hemi-nested primers increased sensitivity by at least 100–1 000 times, enabling us to detect at a level of 0.5–5 PFU. However, amplified DNA was not detected when the first round PCR product was amplified using an equine herpesvirus (EHV)-1 gC primer, even though a 712 bp product of EHV was produced using the primer in the presence of an EHV genome (Fig. 3B). The genome of EHV and equine influenza virus yielded no amplification band using the EAV primer pairs.

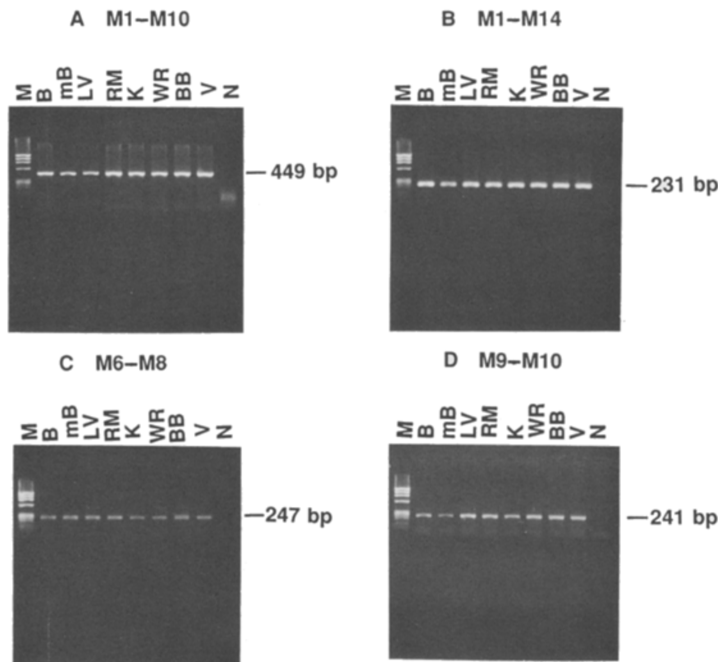

Fig. 2. Analysis of PCR amplification products derived from viral RNA extracted from culture infected with the EAV strains and from the live vaccine, using the four primer pairs: **A** M1-M10, **B** M1-M14, **C** M6-M8, **D** M9-M10. Five μl of product were electrophoresed through a 2% agarose gel in a TAE buffer (40 mM Tris-acetate, 1 mM EDTA) containing 0.5 μg/ml of ethidium bromide. EAV strains; *B* Bucyrus, *mB* modified Bucyrus, *LV* live vaccine, *RM* Red Mile, *K* 84 Y-A1, *WR* Wroclaw-2, *BB* Bibuna, *V* Vienna, *M* molecular weight marker ϕX174/Hae III digest, *N* no template

Each of the PCR products amplified using the M1-M14 primer was digested with each of the four restriction enzymes; Xba I, Mva I, Mbo II, and Alu I. Digestion of PCR products from the Bucyrus, modified Bucyrus, live vaccine, and Wroclaw-2 strains with Xba I gave rise to fragments of about 174 bp and 57 bp (Fig. 4a). Products from Red Mile and 84 KY-A1 digested with Mva I produced fragments of about 131 bp and 100 bp (Fig. 4b). Digestion of PCR products with Mbo II gave rise to fragments of about 200 bp and 31 bp in all strains except for the Wroclaw-2 and Vienna strains (Fig. 4c). The PCR products from all strains were cleaved by digestion with Alu I into two fragments of 164 bp and 67 bp. In Bibuna strain a 164 bp fragment was further cleaved into 110 bp and 54 bp fragments, allowing for three fragments of 110 bp, 67 bp and 54 bp to be produced (Fig. 4d). Of the PCR products using M6-M8 primer only PCR products derived from modified Bucyrus and the live vaccine were digested by Hha I, to give fragments of about 185 bp and 62 bp (Fig. 4e). PCR products from 84 KY-A1 and Red Mile strains were shown to share the same fragment patterns by the above 5 restriction enzymes. These strains were discriminated by digestion of PCR products using M9-M10 with Ava II, i.e. the digestion generated fragments of about 75 bp in all strains, plus fragments of about 56 bp and 110 bp in the Bucyrus, modified Bucyrus, live vaccine and Wroclaw-2 strains, about 94 bp and 72 bp in the Red Mile strain and about 166

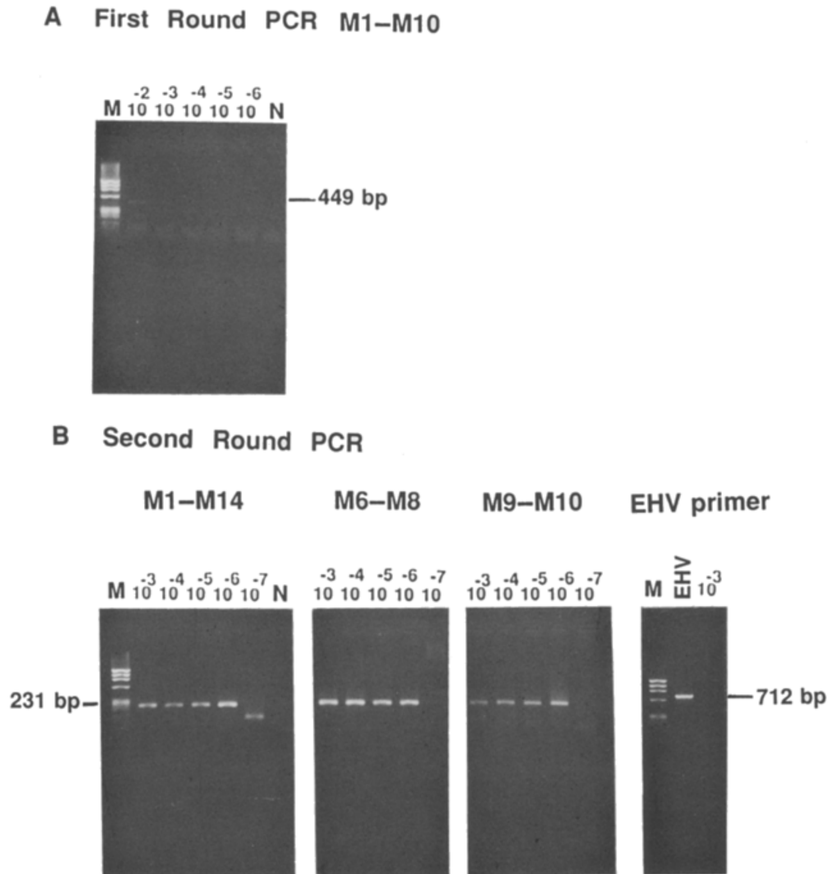

Fig. 3. Nested PCR and its sensitivity in detecting EAV. **A** Analysis of the first round PCR products after amplification of ten-fold serially diluted culture fluid of cells infected with the modified Bucyrus EAV strain (2×10^7 PFU/ml) using the M1-M10 primer. **B** Analysis of the second round PCR products. The first round PCR products of each dilution were reamplified using each of the M1–M14, M6–M8, and M9–M10 inner primer sets. *N* no template, *M* molecular weight marker, *EHV* equine herpesvirus-1 DNA

bp in the 84 KY-A1, Bibuna and Vienna strains (Fig. 4f). The results of the digestion of PCR products using M1-M14, M6-M8, and M9-M10, with the appropriate restriction enzymes, are summarized in Table 1. The Bucyrus, modified Bucyrus and live vaccine, the Red Mile and 84 KY-A1, and the Wroclaw-2, Bibuna and Vienna strains were differentiated by the digestion of PCR products using the M1–M14 primer with the four restriction enzymes XbaI, Mva I, Mbo II and AluI. The modified Bucyrus strain and live vaccine were discriminated from the others by the digestion of their PCR products using the M6–M8 primer with Hha I. Ava II digestion of PCR products using M9–M10 enabled us to distinguish between the Red Mile and 84 KY-A1 strains.

In PCR based detection systems for EAV, Chirnside et al. selected primer from the leader sequence, polymerase (ORF 1-b) and nucleocapsid gene (ORF 7) [4], and Belák et al. chose from the nucleocapsid gene [1]. We selected oligonucleotide

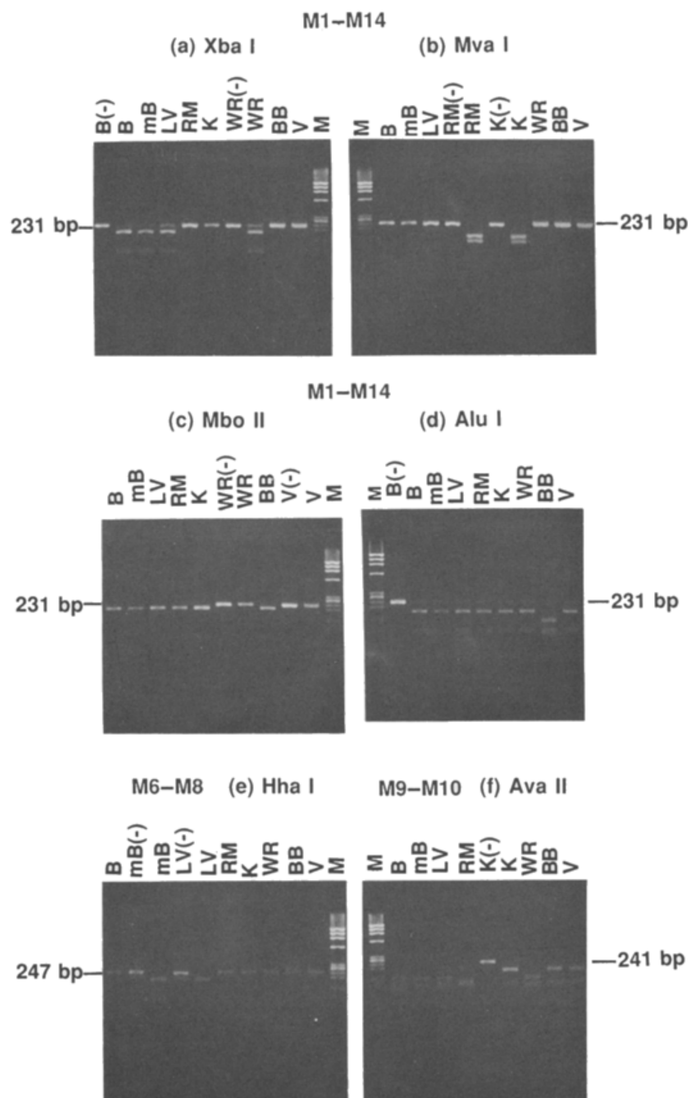

Fig. 4. Restriction enzyme analysis of PCR amplification products. PCR products using the primer pair M1-M14 (Fig. 2B) were digested with Xba 1 (**a**), Mva I (**b**) Mbo II (**c**), and Alu I (**d**). Product with the primer M6-8, (Fig. 2C) was digested with Hha I (**e**), and the primer M9-M10 (Fig. 2D) with Ava II (**f**). Ten μl of the final PCR products were digested in a 20 μl mixture containing 3 to 3.6 U of the restriction enzyme at 37 °C for an hour. Five μl of restriction enzyme digests were analyzed by electrophoresis on 3% agarose gel. Strains: *B* Bucyrus, *mB* modified Bucyrus, *LV* live vaccine, *RM* Red Mile, *K* 84 KY-A1, *WR* Wroclaw-2, *BB* Bibuna, *V* Vienna, *M* molecular weight marker ϕX174/Hae III digest, – no enzyme

primers from the ORF 6 gene because it has been shown to be an area with a higher sequence homology than ORF 7 among the members of the *Arterivirus* group [6, 16, 17]. We designed primer pairs so that there would be no mismatches or at most, a single base pair mismatch, and ensured that the area of amplification product contained the strain specific restriction site for the endonuclease.

Table 1. Differentiation of EAV strains by restriction enzyme analysis of PCR products

Primers	M1-M14					M6-M8	M9-M10		
Enzyme site[a]	Xba I	Mva I	Mbo II	Alu I		HhaI	Ava II		
				1	2		1	2	3
Bucyrus	+	–	+	–	+	–	+	+	–
m Bucyrus	+	–	+	–	+	+	+	+	–
Live vaccine	+	–	+	–	+	+	+	+	–
Red Mile	–	+	+	–	+	–	+	–	+
84 KY-A1	–	+	+	–	+	–	+	–	–
Wroclaw	+	–	–	–	+	–	+	+	–
Bibuna	–	–	+	+	+	–	+	–	–
Vienna	–	–	–	–	+	–	+	–	–

+ Cleaved at restriction site, – not cleaved
[a]Alu I and Ava II have two and three cleavage sites in the PCR amplified region, respectively. Numbering the sites is from the 5′ terminal

Recently, Chirnside et al. presented M and N gene sequences of 10 EAV isolates including the isolates used by us [5]. There were some differences in the M gene nucleotide sequence of Kentuncky 84 and Wroclaw-2 isolates from our data. If the restriction fragment length polymorphism (RFLP) patterns determined by the 6 restriction enzymes are assessed according to their nucleotide sequences, Pleural Fluid, MAFF Ireland, 185/83, NEA V2, Kentucky 84, and Wroclaw-2 isolates should show the same RFLP patterns as the Bucyrus isolate. These isolates exhibited a 96–98% nucleotide identity with Bucyrus isolate [5]. However, our RFLP patterns were exactly as predicted from our nucleotide sequence data.

In this paper we showed the RFLP patterns of single amplified PCR products from seven disparate strains using three sets of primers (Fig. 4). The same restriction enzyme patterns were also obtained by cutting the second round PCR products with restriction enzymes (data not shown).

Nested PCR and the restriction enzyme analysis of product can allow for the detection and identification of virus in small amounts of clinical samples. The live vaccine was provided after repeated passages of the modified Bucyrus strain in tissue culture. RNA from the live vaccine showed the same behavior in restriction enzyme digestion of PCR products as did the RNA from the modified Bucyrus virus. The restriction site of Hha I in the M6-M8 amplified area seems to be unique to the live vaccine, Arvac, and to the modified Bucyrus strains, according to our study and to reports by Chirnside et al. [5]. Fukunaga et al. demonstrated some serological differences between the modified Bucyrus strain and the Bucyrus, 84 KY-A1, and Wroclaw-2 strains using a serum cross neutralization test [10]. The differentiation between horses vaccinated with live vaccine and horses infected with wild strain should be reliable through RFLP analysis by using restriction sites from other ORFs, such as ORF 2, 4, and 5, in combination with serological differentiation using the neutralization. The application of PCR for EAV detection in clinical samples is presently under investigation.

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Received January 25, 1995