RAPID AND SENSITIVE DETECTION OF EQUINE ARTERITIS VIRUS IN SEMEN AND TISSUE SAMPLES BY REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION, DOT BLOT HYBRIDISATION AND NESTED POLYMERASE CHAIN REACTION

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Summary. – A reverse transcription-polymerase chain reaction (RT-PCR) assay using four different primer pairs for the detection of equine arteritis virus (EAV) RNA in semen and tissue samples was evaluated. A fragment encoding the leader sequence of the EAV genome was most successfully amplified. The specificity and sensitivity of RT-PCR was assessed by virus isolation in cell culture, restriction analysis, dot blot hybridisation and nested PCR. To this end, 23 semen samples from seropositive stallions and 11 tissue samples from 4 aborted foals were tested. Compared to the virus isolation test in cell culture, the sensitivity of the molecular methods proved to be higher. In the RT-PCR, dot blot hybridisation and nested PCR tests, semen samples from 11 stallions and tissue samples from all 4 foals were found positive, while the virus could be isolated in cell culture from only 4 semen samples and tissue samples from 1 aborted foal. The sensitivity of the dot blot hybridisation test was superior to that of the RT-PCR test. The nested PCR test proved to be the most sensitive one, because 3 semen samples were recognised as positive by this method only. Considering the sensitivity, and rapidity and reliability, RT-PCR followed by dot blot hybridisation or nested PCR represents the best method for diagnosis of EAV and should be included in the official diagnostic regimes.

Key words: equine arteritis virus; RT-PCR; dot blot hybridisation; nested PCR; semen samples; diagnostics

Introduction

EAV (genus *Arterivirus*) is present in horse populations throughout the world. Within the last 10 years, the reported occurrence of EVA has increased (Timoney and McCollum, 1990; Kölbl *et al.*, 1991; Eichhorn *et al.*, 1995; Wood *et al.*, 1995). Although the infection often remains subclinical, it can become a serious threat to pregnant mares. Abortion or delivery of moribund foals may represent consequences of the infection during pregnancy (Doll *et al.*, 1957; Golnik *et al.*, 1986; Lange-Herbst *et al.*, 1995). Prevention and control programmes detecting stallions carrying the virus have been developed to minimise the spread of infection in breeding animals and to reduce the risk of virus-related abortions (Timoney and McCollum, 1996).

The main routes of EAV transmission includ direct contact during viraemia and contaminated semen during natural or artificial insemination. Therefore, control programmes include serological testing of stallions; in case of a seropositive result, the virus isolation in cell culture has to be performed from semen. However, the virus

Abbreviations: AMV = avian myeloblastosis virus; BSA = bovine serum albumin; CPE = cytopathic effect; CSFV = classical swine fever virus; DEAE-dextran = diethyl aminoethyl-dextran; DEPC = diethyl pyrocarbonate; DMSO = dimethylsulfoxide; EAV = equine arteritis virus; EDTA = ethylenediamine tetraacetate; EHV = equine herpes virus; FCS = foetal calf serum; GTC = guanidine thiocyanate; MEM = Minimal Essential Medium, PAGE = polyacrylamide gel electrophoresis; PCR = polymerase chain reaction; PPRSV = porcine reproductive and respiratory syndrome virus, RT-PCR = reverse transcription-PCR

isolation is laborious and time consuming, and its success strongly depends on the handling of samples (Huntington *et al.*, 1990; Timoney, 1992). In addition, semen samples may have a toxic effect on cell cultures (Vernazza *et al.*, 1996; Legeay *et al.*, 1997). One way to overcome this problem is the detection of EAV RNA by RT-PCR.

However, the successful use of RT-PCR depends mainly on the quality of the RNA preparation and the efficiency of the method chosen for the detection of the PCR products. Particular problems may occur in isolating RNA from semen samples, because inhibitory factors can lead to false negative results in the RT-PCR (Lugaro *et al.*, 1988; Dyer *et al.*, 1996).

In the present work, conditions for successful preparation of RNA from EAV-contaminated semen samples have been established. RT-PCR assays targeting different regions of the EAV genome were evaluated and compared with the virus isolation, restriction analysis, dot blot hybridisation and nested PCR as subsequent confirmatory methods.

Materials and Methods

Specimens consisted of semen samples from 23 serologically positive stallions and various foetal and placental tissues from 4 cases of abortion. The semen samples originated from a specific programme for the detection of the carrier state in a breeding stallion population. They were collected during the breeding season using an artificial vagina and were kept at -70°C until examination. The tissue samples originated from bacteriological and equine herpes virus (EMV) surveillance in two different regions in Germany.

Bucyrus and *Vienna* strains of EAV, field EAV isolates Giessen 1 and Giessen 2, and two EAV-positive semen samples (both kindly supplied by Dr. Herbst, University of Giessen, Germany) were included as reference materials. Other equine viruses (EHV 1 and EHV 4) and RNA viruses of other species (porcine reproductive and respiratory syndrome virus, PRRSV; classical swine fever virus, CSFV; bovine viral diarrhoea virus, BVDV) were used as negative controls.

Preparation of RNA was performed by three different methods.

The method 1 using guanidine thiocyanate (GTC). Virus-infected cells and virions pelleted from cell culture supernatants by ultracentrifugation were processed according to the method of Chomczynski and Sacci (1987). In order to increase the RNA yield, the samples were supplemented with 30 ng/µl carrier RNA (RNase-free, Boehringer Mannheim) before the phenol-chloroform extraction, and with 20 µg/µl glycogen (from oyster, mol.biol. grade, Calbiochem) before the both precipitation steps. Additionally, for the preparation of RNA from semen samples and tissue suspensions, the concentration of GTC in the GTC solution was increased from 4 mol/l to 6 mol/l. The final pellet was resuspended in diethylpyrocarbonate-treated distilled water (DEPC-H_2O) at a concentration of 0.5 – 1.0 µg of total RNA per 6 µl (semen samples) or 2 µg/6 µl (tissue samples).

Tissue samples (about 50 µg) were deep-frozen, crushed in a mortar on ice and lysed in 500 µl of 6 mol/l GTC solution for 30 mins. Following centrifugation, 150 µl of DEPC-H_2O were added to 300 µl of the aqueous phase. Then the RNA extraction was continued as described above.

The method 2 using commercial kits: the QIAamp Viral RNA Kit (originally developed for hepatitits C virus detection) for the processing of seminal plasma and the RNeasy Mini Kit for RNA extraction from tissue samples. The kits (both QIAGEN) were used according to the instructions of the producer. In addition, seminal samples were first lysed with GTC, extracted with phenol-chloroform and precipitated with isopropanol. The RNA precipitate was further processed by use of the RNeasy Mini Kit.

The method 3 using a pre-treatment with proteinase K and chelating resin. Semen samples were pre-treated with proteinase K and Chelex 100 (both Sigma) in final concentration of 100 µg/ml and 5%, respectively, for 1 hr at 56°C (Osterrieder *et al.*, 1994). For this purpose, a stock solution of 1 mg/ml proteinase K in 10 mmol/l Tris pH 7.5, 5 mmol/l ethylenediamine tetraacetate (EDTA) and 0.5% sodium dodecyl sulfate (SDS), was used. Proteinase K in the samples was inactivated at 95°C for 8 mins. The rest of the method was already described under the Methods 1 or 2.

EAV (Bucyrus strain) originating from tissue culture mixed with a pool of EAV-negative semen samples was used as positive control. RNA preparations were stored at -70°C until used in RT-PCR.

RT-PCR test was optimised with both EAV reference strains. To check its specificity, RNA preparations from mock-infected RK-13 cells and cells infected with EHV 1, EHV 4, PRRSV and CSFV were used. Four different primer pairs corresponding to different virus genome sequences were tested in RT-PCR: EAV 1 (located at the 3'-end of ORF 1B, nt 9130 – 9665), EAV 2 (located in ORF 4, nt 10,683 – 11,165), EAV 3 (located in the leader sequence, nt 18 – 186), and EAV 4 (located at the 3'-end of ORF 1B, nt 9246 – 9524). The primers (St. Laurent *et al* (1994); Chirnside and Spaan (1990)) were custom-ordered from Pharmacia. The following conditions were selected for RT-PCR: viral RNA sample (6 µl) was heated at 70°C for 10 mins in the presence of 10 mmol/l Tris, 30 mmol/l KCl, 4.6 mmol/l $MgCl_2$, 1% bovine serum albumin (BSA), 1% dimethylsulfoxide (DMSO), 25 mmol/l dNTPs and 10 pmoles of a reverse primer in a final volume of 30 µl. After slow cooling to room temperature, the sample was placed on ice, 15 U of RNasin and 15 U of avian myeloblastosis virus (AMV) reverse transcriptase (Promega) were added and the mixture was incubated at 42°C for 75 mins and then at 95°C for 5 mins. Ten µl of the RT reaction product and 39 µl of a PCR buffer (15 mmol/l Tris, 67 mmol/l KCl, 2.6 mmol/l $MgCl_2$, 0.5% BSA, 25 mmol/l dNTPs, and 10 pmoles of the corresponding primers) were mixed and heated to 90°C for 5 mins. To secure a semi-hot start of the amplification, the mixture was slowly cooled to 54°C. At this temperature, 2.5 U of Taq DNA polymerase (Promega) and 35 µl of mineral oil (Sigma) were added to the sample. The amplification was carried out using 30 cycles of 1 min at 95°C, 1 min at 50°C, 2 mins at 72°C, and a final elongation step of 8 mins at 72°C. Each RT-PCR run included DEPC-H_2O as negative control. The RT-PCR was performed in an automated thermal Mini Cycler (MJ Res. Inc.). Finally, 10 µl of the reaction product was electrophoresed on a 1.5% agarose gel and stained with ethidium bromide.

Nested PCR test. Appropriate oligonucleotides were designed from the leader sequence of EAV Bucyrus strain, clone PB 586 (de Vries, 1990) using the Vector NTI Programme (Version 4.0, InforMax, Inc.). The primers prepared by Pharmacia and Gibco BRL had the following sequences: 5'-TCT CGG TAA ATC CTA GAG GGC TTT CCT C-3' (*lead 1*) and 5'-CCG TCA AGC CAC AAG ATG -3' (*lead 2*). The nested PCR was performed with 1 µl of the first round amplification product in a 50 µl volume including the same buffer as above. It consisted of one denaturation cycle (95°C, 3 mins) and 30 amplification cycles (95°C for 30 secs, 55°C for 30 secs, 72°C for 30 secs, the final elongation step extended to 2 mins in the last cycle). The resulting product comprised 106 bp (nt 58 – 163) of the leader sequence

DNA dot blot hybridisation test. The primer *lead 1* was also used as a probe for hybridisation after labelling with digoxygenin. Dot blot hybridisation was done according to the instructions of the manufacturer (DIG Labelling and Detection System, Boehringer Mannheim). Briefly, 1 µl aliquots of the 1:2 and a 1:10 dilutions of the PCR products were denatured at 100°C for 10 mins, dotted onto a nylon membrane (positively charged) and fixed by UV crosslinking. After prehybridisation for at least 1 hr, the membrane was left to react with the DIG-labelled probe overnight. This step was followed by washing steps of different stringency, incubation with anti-DIG AP (Fab fragments of an antibody against digoxigenin conjugated to alkaline phosphatase) and colour detection with NBT/BCIP as substrate. The colour development was monitored each hour and stopped when positive and weakly positive control samples were clearly visible, usually after 3 hrs. Temperatures of the hybridisation and washing steps had to be optimised. The highest specificity was achieved at 68°C for hybridisation and at 37°C for the second washing step. PCR products of negative control RNAs (mock-infected RK-13 cells and cells infected with BVDV, EHV 1, PRRSV, and CSFV) were used in the verification of the test specificity.

Restriction analysis PCR products were cleaved with *Hinf*I into 2 fragments of 81 and 88 bp. For cleavage, 20 µl of the amplified product diluted 1:10 with TE buffer (10 mmol/l Tris, 1 mmol/l EDTA) was purified as described (Sambrook *et al.*, 1989) and dissolved in 22.2 µl of TE buffer. Then 2.5 µl of 10x SuRE/Cut Buffer H and 0.3 µl of *Hinf*I (3 U) were added (both from Boehringer Mannheim). The sample was incubated for 1 hr at 37°C and the reaction was terminated by addition of 0.5 ml of 0.5 mol/l EDTA or 5 µl of a DNA sample buffer. The cleavage products were visualised after polacrylamide gel electrophoresis (PAGE).

Virus isolation in cell culture. The EAV isolation was performed in monolayer cultures of RK-13 cells. The cells were cultivated in Eagle's Minimal Essential Medium (MEM) supplemented with 10% foetal calf serum (FCS) (growth medium) or 2% FCS (maintenance medium). The virus was propagated by infection of fresh cultures with freeze-thawed infected cultures. To prepare an inoculum from tissues, a 10% suspension was made in the maintenance medium and clarified at 4000 rpm for 20 mins. The isolation of EAV from seminal plasma was done according to the method of Klopries (1992). In addition, 50 µg/ml diethylaminoethyl-dextran (DEAE-dextran, Pharmacia) was used to support virus adsorption. After inoculation, the cell cultures were observed for cytopathic effect (CPE) for 7 days. If no CPE appeared, further 3 – 7 blind passages were performed. The identity of EAV isolates was confirmed by neutralisation test using polyclonal horse antisera.

Results

RT-PCR assay

After setting up optimal assay conditions for EAV sequence amplification, RNA isolated from cell cultures infected with EAV Bucyrus strain was found positive in RT-PCR using the four different primer pairs (Table 1). However, the EAV 2 primer pair failed to detect RNA isolated from cells infected with EAV Vienna strain, and the EAV 1 and EAV 2 primer pairs did not reveal specific sequences in RNAs from cells infected with the Giessen 1 and Giessen 2 field isolates. Likewise, positive results were obtained only using the EAV 3 and EAV 4 primer pairs with two semen samples of virologically positive stallions as well as with the brain of an aborted foal. All subsequent investigations of field samples were carried out using the EAV 3 primer pair. To confirm the specificity of the test, 9 positive semen samples were assayed also with the EAV 4 primer pair. This assay was negative for 2 samples. Another 6 samples yielded weaker signals in the agarose gel with the EAV 4 primer pair in comparison to the EAV 2 primer pair.

Using EAV 1, EAV 3 and EAV 4 primer pairs, RNAs from mock-infected RK-13 cells and cells infected with other viruses gave no false positive results.

Detection of EAV in semen and tissue samples

Eleven of 23 semen samples originating from seropositive stallions were classified as EAV-positive (Table 2), while 12 samples were negative by all the methods used. Analysis of specimens from the aborted foals demonstrated that EAV RNA was detectable in samples originating from all 4 animals (Table 3). However, there were clear differences in the detectability of EAV in different tissue samples.

Comparison of the methods used for detection of EAV

The results showed that the sensitivity of virus isolation is lower in comparison with molecular-biological methods. The virus isolation test successfully recognised only 5 (4 stallions and 1 aborted foal) of 15 (11 stallions and 4 foals) positive animals. The higher sensitivity of the RT-PCR assay could be further increased by the dot blot hybridisation test. A total of 19 samples were found positive by dot blot hybridisation, while only 12 of these 19 samples were RT-PCR-positive. The highest sensitivity, however, was obtained with the nested PCR. Three of the semen samples which were found negative in the other tests reacted positively in the nested PCR. Examples of results achieved by the molecular-biological methods are presented in Fig. 1. None of the samples evaluated as positive by virus isolation were negative by any of the molecular-biological methods.

Table 1. RT-PCR with RNAs of different origin using different primer pairs

RNA origin	Result of RT-PCR with primer pair EAV 1	EAV 2	EAV 3	EAV 4
EAV Bucyrus strain, RK-13 cells	+	+	+	+
EAV Vienna strain, RK-13 cells	+	–	+	+
EAV Giessen isolate 1, RK-13 cells	–	–	+	+
EAV Giessen isolate 2, RK-13 cells	–	–	+	+
Brain of aborted foetus	–	–	+	+
Brain of aborted foetus after cultivation [a]	–	–	+	+
Semen sample No. 1	–	–	+	+
Semen sample No. 2	–	ND	+	–

(+), (–) = positive, negative. ND = not done.
[a]The same brain.

Table 3. Detection of EAV in various organs of aborted foals by virus isolation, RT-PCR and dot blot hybridisation

Animal No	Organ	Virus isolation	RT-PCR	Dot blot hybridisation
1	Brain	+	+	+
	Lung	+	+	+
	Spleen	+	+	+
2	Lung	–	+	+
	Placenta	–	+	+
	Kidney	–	–	+
3	Lung	–	–	+[a]
	Spleen	–	–	+[a]
4	Lung	–	+	+
	Spleen	–	–	+
	Liver	–	–	+

[a]Only after further concentration of the RNA preparation.

RNA preparation

Our preliminary investigation (data not shown) showed that the RNA preparation from EAV-positive semen samples by the method 1 may result in false negative RT-PCR results. In this study we show that the pretreatment of samples with proteinase K and Chelex 100 may overcome this problem. Serial 10-fold dilutions of cell culture virus were prepared in the maintenance medium as well as in a pool of four EAV-negative semen samples. When the RNA was prepared by the method 1, the virus diluted in seminal plasma was RT-PCR- negative. However, when the pretreatment was included in this procedure, the RT-PCR gave positive results for the virus diluted both in the medium and seminal plasma.

RNA extraction from semen samples using the QIAamp Viral RNA Kit was found generally suitable. However, it became obvious that an additional concentration of the extracted RNA by precipitation and resuspension in a smaller volume was helpful. EAV RNA could be detected in 8 out of 11 positive semen samples by use of the commercial kit.

Table 2. Detection of EAV in semen samples by different methods

Sample[a] No.	RT-PCR	*Hinf*I cleavage	Dot blot hybridisation	Nested PCR	Virus isolation
1	+	+	+	+	+
2	+	ND	+	+	+
3	+	+	+	+	+
4	+	+	+	ND	–
5	+	+	+	ND	–
6	+	ND	+	+	–
7	–	ND	+	+[b]	+
8	–	ND	–	+	–
9	–	ND	–	+	–
10	–	ND	–	+	–
11	–	ND	+	+	–

[a]All the samples were regarded as EAV-positive.
[b]Positive only after further concentration of the RNA preparation.
(+), (–) = positive, negative. ND = not done.

Additional positive results were obtained in one case after further RNA concentration and in two cases after combining the methods 1 and 2.

Successful preparation of RNA from tissue samples was dependent on the amount of the virus in the sample or on the condition of the sample. The positive RNA samples from the animal No. 1 (Table 3), which induced EAV-specific CPE in the first passage, originated directly from both tissue

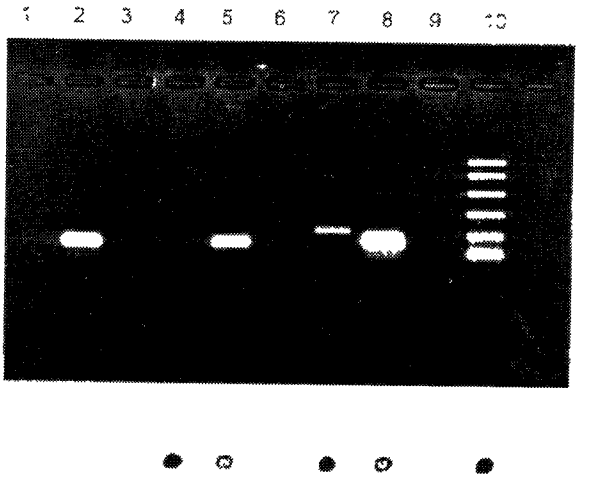

Fig. 1
Three semen samples tested by RT-PCR, nested PCR and dot blot hybridisation
Upper part. Gel electrophoresis of RT-PCR products. samples No. 8 (lane 1), No 11 (lane 4) and No. 6 (lane 7). Gel electrophoresis of nested PCR products: samples No. 8 (lane 2), No 11 (lane 5) and No. 6 (lane 8). DNA ladder (lane 10).
Lower part Dot blot hybridisation. Samples No. 8 (lanes 1 (dilution 1:2) and 2 (1.10)), No 11 (lanes 4 (1:2) and 5 (1:10)) and No. 6 (lanes 7 (1:2) and 8 (1 10)) Positive control (lane 10)

suspension and tissue pieces. In samples from which EAV could not be isolated, the detection of EAV RNA proved to be successful only after direct treatment of tissue pieces. The quality of RNA obtained using the RNeasy Mini Kit (the method 2) was not superior to that prepared by the method 1. On the other hand, in two cases, further RNA concentration was necessary for successful EAV RNA detection.

Discussion

Although the virus isolation in cell culture is the currently used and official method for the detection of EAV, it is limited by several factors. The EAV RNA amplification by RT-PCR holds promise as a diagnostic method which appears to be suitable for overcoming disadvantages of the virus isolation (Chirnside, 1992; Belak *et al.*, 1994; Herbst *et al.*, 1995). However, a suitable method for isolation of RNA from semen and organ samples appears to be crucial for obtaining reliable results in RT-PCR assay.

By optimising the RNA preparation from such samples, the detectability of RNA in semen samples could be improved by their pretreatment with proteinase K and Chelex 100. In this way, polyvalent cations which may disturb the enzymatic reaction are removed. Furthermore, to reduce labour and danger of contamination caused by additional handling, commercial RNA preparation kits were used. The QIAamp Viral RNA Kit was tested with regard to its suitability for EAV RNA preparation from semen samples. The method using this kit was rapid and standardised to ensure the isolation of high quality RNA. However, the amount of EAV RNA was not always sufficient for RT-PCR. In these cases, concentration of the extracted RNA by ethanol precipitation was performed. Alternatively, a complete new RNA preparation by the methods 1 or 3 was done. Finally, it could be shown that an improved quality of RNA could be obtained by combining the advantages of RNA extraction/ ethanol precipitation with the solid phase principle of the QIamp Viral RNA Kit as described by Bonham and Danielpour (1996) for the detection of mRNA's from rat prostatic cells.

So far, a similar sensitivity has been demonstrated for virus isolation and molecular-biological methods of viral RNA detection (Chirnside and Spaan, 1990; Belak *et al.*, 1994; St.Laurent *et al.*, 1994; Cierpisz and Golnik, 1996). In the present study, the RT-PCR assay proved to have a higher sensitivity than the virus isolation test. Among 11 positive semen samples and samples from 4 aborted foals altogether 5 samples were found positive in the virus isolation and 9 in the RT-PCR. The higher sensitivity was achieved by both the improved RNA preparation methods and the optimisation of reaction parameters for RNA reverse transcription and amplification. For the latter, the slow cooling after the annealing steps and the introduction of a semi-hot start to initiate the amplification were essential.

At the beginning of the investigation, the primers selected by St. Laurent *et al.* (1994) were used because of their high sensitivity for field EAV isolates. Surprisingly, the detection of EAV RNA from virus isolates from three regions of Germany was not possible with these primers. The RT-PCR remained negative also for cell culture passages of these isolates. The reason for this could reside in alterations on the molecular level, which have to be checked by sequencing.

The EAV 3 and EAV 4 primer pairs used in the comparative study exhibited different sensitivities. The best results were obtained with primers derived from the leader sequence, while the amplification of the ORF 1B region with the EAV 4 primer pair was less efficient.

To clear ambiguous RT-PCR results and to confirm unambiguous ones the restriction analysis, dot blot hybridisation with DIG-labelled oligonucleotide probe, and nested PCR were used.

The restriction analysis of PCR products is known to be an easy and relatively rapid method for providing evidence of specificity. Unfortunately, only samples with unambiguous signal in the gel werefound positive, while those with doubtful signal were not recognised.

On the other hand, the dot blot hybridisation test not only confirmed the RT-PCR specificity but also showed a higher sensitivity. In further experiments not involved in the present study, out of 22 semen samples tested 10 were dot blot hybridisation positive but only 6 were RT-PCR- positive (not shown). Therefore, the dot blot hybridisation test with a specific DIG probe seems to be helpful and feasible tool for checking doubtful RT-PCR results.

In the meantime, the nested PCR has been developed as the most sensitive molecular-biological method for the detection of viral nucleic acid for a series of viral diseases of animals. For EAV it was described by Belak *et al.* (1994) and Sekiguchi *et al.* (1995). In accordance with their results, the highest detection rate of EAV-positive animals was achieved with the nested PCR assay. Thus 3 of 11 semen samples reacting negatively by other methods were found positive. These findings were validated by including negative controls as well as by separate preparation and amplification of individual samples. An additional partial confirmation of these results was obtained by one of these 3 semen samples, which after 5 passages became positive in the virus isolation test.

The sensitivity of the nested PCR assay as performed in this work exceeds that of the virus isolation test in contrast to the data published earlier. The sensitivity and rapidity make the nested PCR the method of choice. However, a disadvantage of carry-over contamination should be kept in mind. A reduction of this risk can be achieved by a

stepwise use of the presented methods, in which the dot blot hybridisation test is employed for the validation of the RT-PCR results, and only the so far negative samples should be further subjected to the nested PCR assay.

In conclusion, the data presented here underline the higher sensitivity of the RT-PCR assay compared to the virus isolation test in detecting EAV. RT-PCR results can be further improved by subsequent dot blot hybridisation or nested PCR tests. Besides, these methods allow the detection of EAV within 1 – 4 days in dependence on the method used. These results as well as the results of other authors (Chirnside and Spaan, 1990; St.-Laurent *et al.*, 1994; Herbst *et al.*, 1996; Cancelotti *et al.*, 1996) emphasize the necessity to improve the current official procedures used in diagnostic of EAV.

References

Anonymous (1996): Community Legislation on Equide. *Directive 90/426/EEC*

Belak S, Ballagi-Pordany A, Timoney PJ, McCollum WH, Little TV, Hyllseth B, Klingeborn B (1994): Evaluation of a nested PCR assay for the detection of equine arteritis virus. In Nakajima H, Plowright W (Eds): *Proc. VII. Int. Conf. Equine Infect. Dis* , Tokyo.

Bonham MJ, Danielpour D (1996): Improved Purification and Yields of RNA by IRNeasy. *BioTechniques* **21**(1), 57–60.

Cancelotti FM, Ramina A, Dalla Valle L, De Mas S, Tisato E, Zuin A (1996): Application of a biotechnological methodology (RT-PCR) to the diagnosis of equine arteritis virus in horse semen. *I. Eur Symp. Equine Viral Arter.*, Utrecht.

Chirnside ED (1992): Equine Arteritis Virus: An Overwiew. *Br. Vet. J* **148**, 181–197.

Chirnside ED, Spaan JM (1990): Reverse transcription and cDNA amplification by the polymerase chain reaction of equine arteritis virus (EAV). *J Virol. Methods* **30**, 133–140.

Chomczynski PY, Sacci N (1987). Single-step method of RNA isolation by acid guanidin thiocyanate-phenol-chloroform extraction. *Anal Biochem.* **162**, 156–159.

Cierpisz JK, Golnik WW (1996): The comparison between virus isolation and polymerase chain reaction in detecting equine arteritis virus in the semen of seropositive stallions. *I. Eur. Symp. Equine Viral Arter.*, Utrecht.

den Boon JA, Snijder EJ, Chirnside ED, de Vries AA, Horzinek MC, Spaan WJ (1991): Equine arteritis virus is not a togavirus but belongs to the coronaviruslike superfamily. *J. Virol.* **65** (5), 2910–1920.

de Vries AAF, Chirnside ED, Bredenbeek PJ, Gravestein LA, Horzinek MC, Spaan WJ (1990): All subgenomic mRNAs of equine arteritis virus contain a common leader sequence. *Nucleic Acids Res.* **18** (11), 3241–3247.

de Vries AAF, Horzinek MC, Rottier PJM, de Groot RJ (1997): The genome organization of the Nidovirales: similarities and differences between Arteri-, Toro- and Coronaviruses. *Semin Virol.* **8**, 33–47.

Doll ER, Bryans JT, McCollum WH, Crowe MEW (1957): Isolation of a filtrable agent causing arteritis and abortion by mares. *Cornell Vet* **47**, 3–41.

Dyer JR, Gilliam BL, Eron JJ, Grosso L, Cohen MS, Fiscus SA (1996). Quantitation of HIV-1 RNA in cell free seminal plasma. *J. Virol. Methods* **60**, 161–170.

Eichhorn W, Heitmann M, Kaaden OR (1995): Equine viral arteritis with abortions: serological and virological evidence in Germany. *J. Vet. Med* B **42**, 573–574.

Golnik W, Moraillon A, Golnik J (1986): Identification and antigenic comparison of equine arteritis virus isolated from an outbreak of epidemic abortion of mares. *J. Vet. Med.* B **33**, 413–417.

Herbst W, Krämer C, Vieler E (1995): Zur Bedeutung des equinen Arteritisvirus bei Trächtigkeitsverlusten des Pferdes in Deutschland. *21 Kongr Dtsch Vet.-Med. Ges.*, Bad Nauheim.

Herbst W, Lange-Herbst H, Vieler E (1996): Survey of antibodies against EAV in german horses from 1985 to 1995 and detection of EAV in aborted foals. *I Eur. Symp. Equine Viral Arter.*, Utrecht.

Huntington PJ, Ellis PM, Forman AJ, Timoney PJ (1990): Equine viral arteritis. *Austr. Veter. J.* **67**(12), 429–431.

Klopries M (1992): Diagnose der Pferdearteritis. *AVID Mitteilung I/ 1992*, Anlage 15, S.1–6.

Kölbl S, Schuller W, Pabst J (1991): Serologische Untersuchungen zur aktuellen Verseuchung österreichischer Pferde mit dem Virus der equinen Arteritis. *Dtsch. Tierärztl. Wschr.* **98** (2), 43–45.

Lange-Herbst H, Herbst W, Vieler E (1995): Occurence of equine virus arteritis in Germany. *Proc IV Int Symp. Positive Strand RNA Viruses*, Utrecht.

Legeay O, Bounaix S, Denis M, Arnauld C, Huret E, Carlolet R, Albina E, Jestin A (1997). Development of a RT-PCR test coupled with a microplate colorimetric assay for the detection of a swine arterivirus (PRRSV) in boar semen. *J. Virol Methods* **68**, 65–80.

Lugaro G, Campagnari F, Moretti R, Caselatto MM (1988): Inhibition of DNA polymerization and DNA transcription to RNA by seminal plasma peptides. *Biochem. Biophys Acta* **950**, 420–428.

Sambrook J, Fritsch EF, Maniatis T (1989): *Molecular cloning A Laboratory Manual* 2nd ed. Cold Spring Harbor, NY.

Osterrieder N, Hübert PH, Brandmüller Ch, Kaaden OR (1994): A touchdown PCR for the differentiation of EHV-1 field strains from the modified live vaccine strain RacH. *J. Virol Methods* **50**, 129–136.

Sekiguchi K, Sugita S, Fukunaga Y, Kondo T, Wada M, Kamada M, Yamaguchi S (1995): Detection of equine arteritis virus (EAV) by polymerase chain reaction (PCR) and differentiation of EAV strains by restriction enzym analysis of PCR products. *Arch. Virol.* **140**,1483 1491.

St.-Laurent G, Morin G, Archambault D (1994): Detection of Equine Arteritis Virus following Amplification of Structural and Nonstructural Viral Genes by Reverse Transcriptase-PCR. *J. Clin. Microbiol.* **32** (3), 658–665.

Timoney PJ (1992): Equine Viral Arteritis. In Castro AE , Heuschele WP (Eds): *Veterinary Diagnostic Virology. A Practitioner's Guide.* Masby-Year Book, Inc.

Timoney PJ, McCollum WH (1990): Equine arteritis virus: current clinical and economic significance. *Proc. 36th Ann. Conf. Am. Assoc. Equine Pract.*, Lexington, pp. 403–409.

Timoney PJ, McCollum WH (1996): Equine viral arteritis, an emergent disease of international significance. *I. Eur. Symp. Equine Viral Arter.,* Utrecht.

Vernazza PL, Eron JJ, Fiscus SA (1996): Sensitive method for the detection of infectious HIV in semen of seropositiv individuals. *J. Virol. Methods* **56**, 33–40.

Wood JLN, Chirnside ED, Mumford JA, Higgins AJ (1995) First recorded outbreak of equine arteritis virus in the United Kingdom. *Vet. Record* **15**, 381–385.