

RT-PCR AS EFFECTIVE DETECTION METHOD OF EQUINE ARTERITIS VIRUS IN THE SEMEN OF NATURALLY INFECTED STALLIONS

MAGDALENA LARSKA AND JERZY ROLA

Department of Virology,
National Veterinary Research Institute,
24-100 Pulawy, Poland
e-mail: m.larska@piwet.pulawy.pl

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The aim of the study was to present the usefulness of one tube reverse transcriptase polymerase chain reaction assay (RT-PCR) in comparison to virus isolation test (VI) in the detection of equine arteritis virus. The group of 82 semen samples taken from different stallions was examined. Equine arteritis virus was isolated in RK-13 cell monolayers from the seminal plasma of 11 animals. The same results were obtained by one tube RT-PCR assay. The study showed that RT-PCR assay provides a sensitive, accurate, convenient and less time-consuming way for equine viral arteritis diagnosis in close tube system which minimises the risk of cross-contamination.

Key words: stallion, semen, equine arteritis virus, PCR, isolation.

Equine arteritis virus (EAV) is the causative agent of equine viral arteritis (EVA) in horses and donkeys widely spread all over the world. EAV was first isolated in 1953 on a Standardbred farm near Bucyrus, Ohio (7). It is a member of the *Arteriviridae* family of the order *Nidovirales* that includes porcine reproductive and respiratory syndrome virus (PRRSV), simian haemorrhagic fever virus (SHFV) and lactate dehydrogenase-elevating virus (LDV) of mice (3, 6). EAV is enveloped, positive-stranded RNA virus, which has five structural proteins. The nucleocapsid protein N, the non-glycosylated protein M, and the glycosylated protein G_L are the major antigens of the virus (14). EVA spreads through routine transport of horses and horse semen. It is transmitted through the respiratory and reproductive systems. Clinical signs in horses vary, and a severe infection can lead to abortions in pregnant mares and neonatal foal death (5, 9, 20). Control and prevention programmes were developed to reduce the spread of EAV in the breeding and to minimise the risk of virus-related abortions and neonatal deaths. The main concern of the veterinary services and the horse breeders is the possibility of virus infection by natural or artificial insemination of mares by the EAV carrier stallions (17). After the acute phase of the disease stallions become persistently infected with EAV which is localised in the accessory genital glands and may be shed by semen (10, 11). Stallions, shedders of EAV are a reservoir of the virus within the equine population. The EVA diagnostics is based on serological testing of stallions with virus neutralisation test and in the case of

a positive result on virus isolation (VI) from semen in the cell culture. VI is sensitive and effective method but also laborious and time consuming one. There is also evidence that higher passage levels of RK-13 cells can be less suited to virus isolation. Other problem is the quality of the collected semen and its cytotoxicity. All these factors make virus isolation difficult and by this reason diagnostic laboratories need an alternative method for virus detection. The objective of the study was to adapt one tube RT-PCR assay to routine detection of EAV in horse semen.

Material and Methods

Samples. In the study, 82 samples of frozen semen collected from different stallions from the national stud situated in the eastern part of Poland were used. There were sampled 59 half Anglo-Arabs, 11 Polish Draft horses, 5 half-breds, 3 Wielkopolski horses, 1 pure bred Arabian horse, 1 English Thoroughbred horse, 1 half-Arabian horse and 1 half-Thoroughbred. As the positive control sample Bucyrus EAV reference strain was used. The negative control was the semen of a seronegative stallion.

Preparatory treatment of samples. All the samples of semen were frozen at -20°C and transported chilled in freezer packs to the laboratory. They were then pretreated by short-term sonication (3 x 15-second cycles) followed by centrifugation at $1000 \times g$ for 10 min at 4°C to sediment spermatozoa and to obtain semen plasma (1).

Virus Isolation (VI). Virus isolation procedure was basically the same as used by Timoney *et al.* (18, 19) with some slight modifications. The monolayers of rabbit kidney-13 cells (RK-13, ECACC No 00021715) in 24-well plate were inoculated by 10-fold dilutions (from 10^{-1} to 10^{-3}) of seminal plasma. After adsorption for 1 h at 37°C monolayers were overlaid with Eagle MEM containing 2% foetal calf serum and antibiotics. The plates were incubated at 37°C in 5% CO_2 atmosphere and examined microscopically daily for 5-7 d for viral CPE. If no CPE was observed after 5-7 d, the plates were frozen in -20°C and next after thawing the second passage into fresh RK-13 cell monolayers was made. After the incubation for further 5 d, the samples that did not produce any CPE were regarded as negative and those with CPE were considered to be positive.

RNA extraction. Total viral RNA was obtained by purification of seminal plasma using TRI Reagent (Sigma) according to the company procedure. Briefly, 1 ml of the TRI reagent was added to 0.5 ml of semen plasma in the microtube, mixed and left to stand in RT for 5 min. Next, the mixture was supplemented with 0.2 ml of chloroform, shaken vigorously for 15 s and stored for 3 min in RT. Subsequently, the microtubes were centrifuged at $12\ 000 \times g$ for 15 min at 4°C . The RNA in the upper aqueous phase was transferred into fresh microtube and precipitated in the presence of 0.7 ml of isopropanol by centrifugation at $12\ 000 \times g$ for 10 min at 4°C . The paellet was then washed with 1 ml of 75% ethanol, air-dried and resuspended in 50 μl of DEPC water. Then the tubes were incubated in 57°C for 5 min and stored frozen in -70°C .

RT-PCR assay. RT-PCR test was carried out using Access RT-PCR System kit (Promega) which enables running reverse transcription and polymerase chain reaction in one tube. Two external primers, selected from the nucleocapsid gene of EAV such as: upstream OEVA 14a with the sequence 5'TCG ATG GCG TCA AGA

CGA TCA C3' and downstream OEVA 15 with the sequence: 5'GGT TCC TGG GTG GCT AAT AAC TAC TTC AAC3' were used according to Belak *et al.* (2). After amplification 395 bp products were obtained. RT-PCR was performed in 0.2 ml PCR microtubes in the volume of 50 µl. The components of the reaction were as follows: 10 µl AMV/Tfl 5 x Reaction Buffer, 1 µl 10 mM dNTP Mix, 50 pM of primer OEVA 14a and OEVA 15, 2 µl 25 mM MgSO₄, 5 U AVM Reverse Transcriptase, 5 U Tfl DNA Polymerase, 2 µl RNA extraction product, and 29 µl of nuclease-free water. The reverse transcription and PCR amplification were carried out in a T-personal thermocycler (Whatman Biometra).

RT-PCR sensitivity. Sensitivity of RT-PCR was assessed against the reference Bucyrus strain of EAV. The titre of the strain was 10^{6.5} TCID₅₀. There were prepared serial 10-fold dilutions contained from 10 000 TCID₅₀ to 0.01 TCID₅₀ in 500 µl of seminal plasma of EAV seronegative stallion. RNA extraction of each dilution and RT-PCR were performed as described above. The highest dilution with positive reaction was determined.

Electrophoretical analysis. Ten µl of RT-PCR products were analysed in 1.5% agarose gel in 1x TAE buffer (pH 8.0), stained with ethidium bromide and compared with molecular mass markers: pUC19 DNA/MspI (HpaII) and φ X174 DNA/BsuRI (*Hae*III, MBI Fermentas).

Results

The overall and breed-specific results of the stallions tested with virus isolation test and RT-PCR method are summarised in Table 1. From 82 samples examined 11 were found positive in VI test. Positive results were obtained within 1-2 passages in RK-13 cells.

Table 1
Comparison of virus isolation test and RT-PCR results
with the distribution among horse breeds

| Horse breed | Virus isolation | | RT-PCR | |
|----------------------|-----------------|----------|----------|----------|
| | Negative | Positive | Negative | Positive |
| Half Anglo-Arab | 51 | 8 | 51 | 8 |
| Polish Draft | 9 | 2 | 9 | 2 |
| Half-bred | 5 | 0 | 5 | 0 |
| Wielkopolski horse | 3 | 0 | 3 | 0 |
| Arab | 1 | 0 | 1 | 0 |
| English Thoroughbred | 1 | 0 | 1 | 0 |
| Half Arab | 1 | 0 | 1 | 0 |
| Half Thoroughbred | 0 | 1 | 0 | 1 |
| Summary | 71 | 11 | 71 | 11 |

The same samples reacted positively in RT-PCR. The amplified products appeared in the agarose gel as clear bands of the size 395 bp in all positive samples (Fig. 1, lanes 1-11). Their layout could be compared with the band of positive control – the product of RT-PCR of Bucyrus EAV reference strain (Fig. 1, lane 13). Non specific amplification was not observed. In addition, no PCR product was obtained from negative control (Fig. 1, lane 14) and from the semen samples negative in VI (Fig. 1, lane 12).

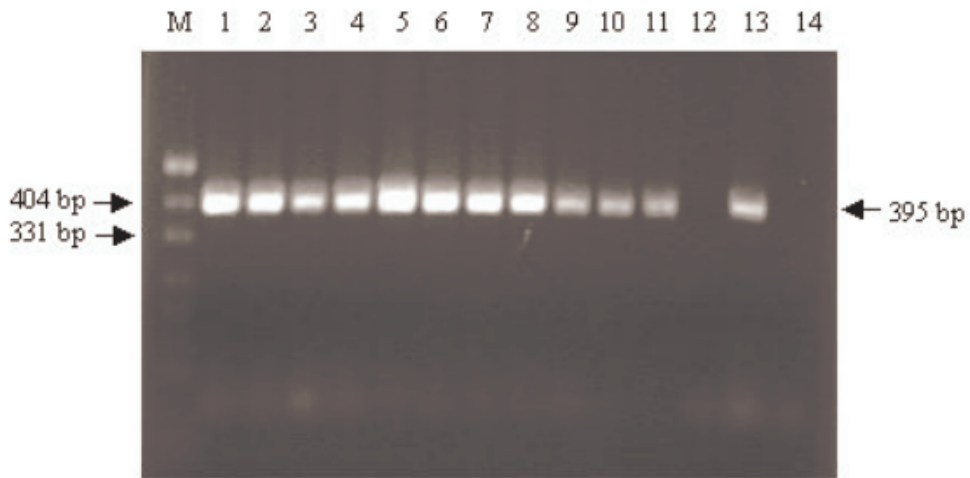


Fig. 1. Electrophoresis of RT-PCR products. Lane M: marker pUC19 DNA/MspI (HpaII, MBI Fermentas), lanes 1-11: semen samples showing positive reaction, lane 12: semen sample producing negative result, lane 13: positive control (Bucyrus EAV reference strain), lane 14: negative control (semen of seronegative stallion).

The sensitivity of RT-PCR is shown in Fig. 2. Detection limit of RT-PCR in our experiment corresponded to 10 TCID₅₀ of the reference strain Bucyrus.

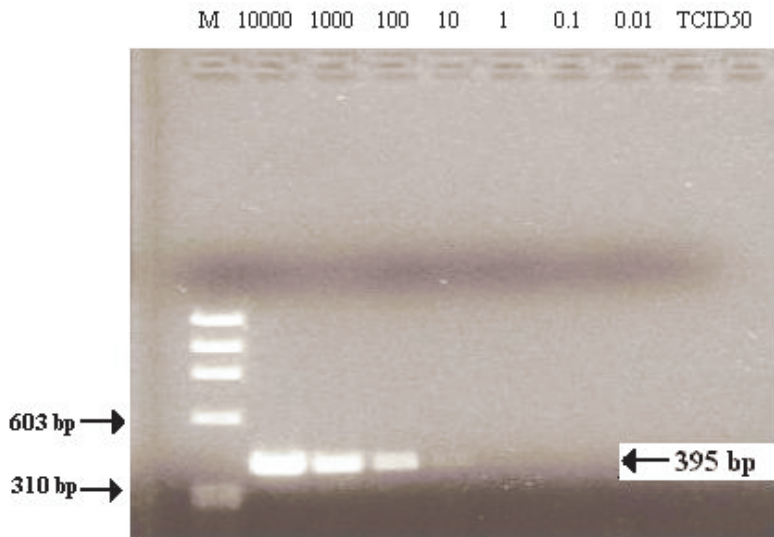


Fig. 2. Sensitivity of RT-PCR. Lane M – marker ϕ X174 DNA/BsuRI (HaeIII).

Discussion

Virus isolation in cell culture is one of the official methods recommended by OIE for the detection of EAV in horse semen for international trade. A proper handling of samples during collection and submission to the laboratory, cytotoxicity of semen and susceptibility of cells for virus infection are main factors which influence results of the test. The aim of the study was to adapt RT-PCR as the alternative method of horse semen testing in routine diagnosis of EAV.

Virological examination of semen samples confirmed carrier state of 11 stallions out of 82 tested. Eight positive results were found in Anglo-Arab stallions, 2 in Polish Draft and 1 in Half Thoroughbred. According to Timoney *et al.* (16) after the acute phase of infection 30-50% of stallions can become persistently infected and shed virus with semen. The authors have not observed clear differences in the rate of virus persistence between horse breeds. In our experiment most positive results were found in Anglo-Arab stallions but this group was the largest one among tested. The obtained results for breed distribution can not be considered conclusive until a bigger, representative group for horse population is tested.

Exactly the same results (13.4% positives in the studied group) were obtained in RT-PCR. Clear amplification products were observed in all samples, which were positive in VI test. The sensitivity of RT-PCR was determined as 10 TCID₅₀ in 500 µl of seminal plasma. It is comparable to the results obtained by other authors (4, 12, 15). First time, a single RT-PCR method to detect EAV was described by Chirnside and Spaan (4). Using primers located within the EAV leader sequence they could detect 600 PFU of virus in 1 ml of seminal plasma. St-Laurent *et al.* (15) with primers derived from ORF 1b detected 20 ID₅₀ of virus in 100 µl of semen. Development of nested PCR increased sensitivity of the method. Belak *et al.* (2) using primers selected from ORF 7 achieved sensitivity less than 1 PFU in 100 µl of semen. Sekiguchi *et al.* (8) and Gilbert *et al.* (13) reported similar augmentation of viral RNA detection sensitivity. Although nested PCR may be found more accurate, the introduction of reamplification with internal primers lengthens the process, introduces cross-contamination problems and may result in non-specific products.

Making direct comparison between virus isolation and RT-PCR we need to take under consideration all the features such as sensitivity and technical data. First of all, the PCR method allows recognising all specific viral RNA fragments included in the sample, therefore the incorrect transport conditions that damage viral particles do not influence efficiency the assay. However, it can not differentiate between infectious and non-infectious virus and the test confirms both death and live EAV. Virus isolation test allows detecting only live virus although it is time consuming (1-3 weeks) what may be an obstacle if there is a need of rapid diagnosis. In summary, RT-PCR method is quick, sensitive and less laborious, making possible to diagnose also the reduced presence of EAV in the semen of persistently infected stallions. It appears to be suitable for overcoming disadvantages of the virus isolation. RT-PCR generates also products which can be used further for nucleotide sequencing of genes coding the antigenic determinants of EAV in horse population.

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