In vitro Detection of Equine Arteritis Virus from Seminal Plasma for Identification of Carrier Stallions

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ABSTRACT. Equine arteritis virus (EAV) was readily isolated in RK-13 cell monolayers by plaque assay from seminal plasma of experimental carrier stallions when they contained high titers of virus regardless of the presence of non-viral cytotoxicity in the seminal plasma. The cytotoxicity interfered with virus isolation from seminal plasma which contained virus at titers less than 10 PFU/ml. However, it was possible to detect the virus in seminal plasma pretreated with RT-PCR from crude seminal plasma which contained virus at titers of more than 10^2.7 PFU/ml. In vitro detection of EAV by virus isolation supplemented with RT-PCR using seminal plasma was proved to be an effective alternative to the standard test mating as a diagnostic method for carrier stallions.—KEY WORDS: carrier stallion, equine arteritis virus, seminal plasma.

NOTE Virology

It is a requirement that sero-positive stallions must undergo test mating at the time of international movement or trading since it is considered the most reliable method for the detection of equine arteritis virus (EAV) shedding stallions. However, in vitro identification of carrier stallions of equine viral arteritis (EVA) has been reported by the method of virus isolation in cell culture [20, 21], as well as by polymerase chain reaction (PCR) [1, 2, 8, 17], using seminal plasma collected from naturally infected carrier stallions. Even though the Office International des Epizooties recommended either tests of virus isolation or test mating for screening stallions in the updated International Animal Health Code [11], the methods for identifying carrier stallions are not uniformly agreed among trading countries which use their own measures or combinations of measures. We experimentally established EAV shedding stallions and confirmed scientifically that the identification of carrier stallions can be proved by in vitro virus detection from seminal plasma and is an effective alternative to the standard test mating in Japan.

Four pony stallions of mixed breeds, aged 5 to 8 years, were intranasally inoculated with 5 ml of the 84 KY-A1 strain of EAV at titers of 10^3.3 PFU/ml with a passage history of 9 times in RK-13 cells [7]. As shown in Table 1, two stallions (Nos. 1 and 3) received testosterone enanthate (Enarmon Depot; Teikoku Zoki Pharmaceutical Corp., Tokyo, Japan) in oil administered subcutaneously twice a week from one week pre-infection until 8 weeks post inoculation (PI) with EAV, at a total dose of 613 µg/kg of bodyweight per week, by a modified method of Little et al. [13]. In order to induce estrus in mares for the purpose of semen collection from stallions, two pony mares of mixed breeds, aged 6 and 9 years, were administered progesterin and estradiol as described previously [5]. The animals were kept in separate stall boxes in a physiological containment stable during the experiment from October to December. Seminal plasma was collected weekly by using an artificial vagina (Fujihira Industry Co., Tokyo, Japan) in accordance with the method described by Timoney et al. [20, 21] and processed for virus isolation as described previously [20]. Seminal plasma was pretreated when non-viral cytotoxicity in RK-13 cell monolayers inoculated with the seminal plasma appeared. It was diluted 1:10, 1:100 and 1:1000 in tissue culture maintenance medium containing 2% fetal bovine serum (2% FS-MEM), and polyethylene glycol (PEG #6000) [6] was added to a final concentration of 10% to each dilution and allowed to stand overnight at 4°C with gentle stirring. The mixtures were then centrifuged at 5000 rpm for 30 min and the supernatants were discarded. Each precipitate was suspended in 2% FS-MEM to a volume one-tenth that of the original dilutions and homogenated. The suspensions were then centrifuged at 5000 rpm for 30 min and the supernatants were used as inocula. Viral plaques formed in RK-13 cell monolayers were recovered and subcultured into newly prepared RK-13 cell monolayers. Identification of EAV was conducted with an indirect immunofluorescence test using a murine monoclonal antibody to the nucleocapsid protein of EAV as described previously [22]. RT-PCR was performed essentially by the same method described previously by Sugita et al. [18].

All 4 stallions showed clinical signs of EVA associated with pyrexia. The infection was confirmed by the virus isolation from buffy coat [4] by one week PI and the development of SN antibody [4] by two weeks PI. Similar experimental studies on EVA in stallions have been...
previously reported [7, 9, 16]. However, it was demonstrated in the present study that numbers of spermatozoa of seminal plasma collected from the stallions, except for one stallion (No. 3), sharply decreased 4 to 5 weeks PI, and merely 1.0 to 5.0% of preinfection numbers remained at 8 weeks PI (Table 1). The numbers of spermatozoa seemed to be more greatly reduced in stallions not administered testosterone than in those administered testosterone. Serum concentrations of testosterone [19] ranged from 2097 to 6674 pg/ml in the two stallions which received testosterone administration during the examination from October to December, but from 778 to 2064 pg/ml in the control stallions (data not shown). Two stallions were given testosterone by design in an attempt to establish efficiently an EV A carrier state based on the findings that the carrier state in stallions was dependent on testosterone [13] and serum concentrations of testosterone decreased at the time when the amount of daylight became less [10]. Nevertheless, a specific correlation between serum concentration of testosterone levels and constant shedding of EA V in seminal plasma was not observed.

RK-13 cell monolayers were inoculated with the seminal plasma samples of 4 stallions diluted from 1:10 to 1:1000. As shown in Table 2, the samples taken from two stallions Nos. 1 and 3 before infection with EA V demonstrated cytotoxicity at dilutions of 1:10 and no cytotoxicity, respectively. However, the cytotoxicity appeared to rise in 1:100 and 1:10 dilutions of seminal plasma of each of the stallions administered testosterone repeatedly following 4 weeks PI. In contrast, seminal plasma samples of stallion No. 2 were not cytotoxic and those of stallion No. 4 revealed cytotoxicity at 1:10 dilutions all the time irrespective of EA V infection. The results of virus isolation in RK-13 cell monolayers inoculated with seminal plasma samples of 4 stallions collected every week from 0 to 8 weeks PI are shown in Table 3. EA V was isolated from all of the samples of 4 stallions 4 weeks PI at titers ranging from 10 to 10^6.1 PFU/ml, and two stallions Nos. 1 and 4 persisted in shedding virus until 8 weeks PI, but virus titers decreased apparently in stallion No. 2 by 6 to 8 weeks PI. No virus was detected in the first examination from any samples of stallion No. 3 due to cytotoxicity of seminal plasma at 1:10 dilutions. Nevertheless, it was successfully detected from the sample collected 4 weeks PI at titers of 10 PFU/ml after treatment with PEG (#6000). PEG did not affect virus titers [6] and the pretreatment of seminal plasma with PEG was neither complicated nor laborious. Isolation of EA V was not always successful in cell cultures accompanied with cytopathic effects (CPE) when inoculated with clinical specimens such as buffy coat [3, 14, 15], nasal secretions [3, 14] and urine [3], but was constantly fulfilled by plaque assay in the present study at the first passage of RK-13 cell monolayers 4 to 5 days after inoculation with seminal plasma. This is a result similar to the results reported by Timoney et al. [20, 21]. A relatively wide range of cell susceptibilities to EA V was reported [12], and RK-13 cells have been frequently made use of in virus isolation for EA V from seminal plasma by many workers [1, 8, 13, 16, 17, 20, 21]. Virus isolation in RK-13 cells inoculated with seminal plasma may be a much better method than those using inoculations of other clinical materials, or the plaque assay may be a more practical

**Table 1.** Spermatozoa numbers in seminal plasma samples collected weekly from 4 stallions following inoculation with the 84KY-A1 strain of EA V

<table>
<thead>
<tr>
<th>Horse No.</th>
<th>0</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a)</td>
<td>4.0 × 10^8</td>
<td>9.8 × 10^7</td>
<td>3.5 × 10^7</td>
<td>2.3 × 10^7</td>
<td>2.1 × 10^7</td>
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<td>2</td>
<td>2.9 × 10^8</td>
<td>1.3 × 10^8</td>
<td>4.8 × 10^7</td>
<td>1.0 × 10^7</td>
<td>4.4 × 10^6</td>
<td>2.9 × 10^6</td>
</tr>
<tr>
<td>3a)</td>
<td>5.4 × 10^8</td>
<td>9.0 × 10^7</td>
<td>7.1 × 10^7</td>
<td>3.7 × 10^7</td>
<td>7.2 × 10^7</td>
<td>1.2 × 10^8</td>
</tr>
<tr>
<td>4</td>
<td>2.1 × 10^8</td>
<td>1.8 × 10^8</td>
<td>3.8 × 10^7</td>
<td>3.7 × 10^7</td>
<td>1.1 × 10^7</td>
<td>7.8 × 10^6</td>
</tr>
</tbody>
</table>

a) Testosterone administered.
b) Per ml of seminal plasma.

**Table 2.** Cytotoxicity of serial 10 fold dilutions of seminal plasma samples collected weekly from 4 stallions following inoculation with the 84 KY-A1 strain of EA V

<table>
<thead>
<tr>
<th>Horse No.</th>
<th>0</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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<td>10^-1</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10^-2</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<td>–</td>
<td>–</td>
<td>+</td>
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</tr>
<tr>
<td>10^-2</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10^-3</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10^-2</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<td>–</td>
<td>–</td>
<td>+</td>
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</tr>
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</table>

a) Testosterone administered.
method for defining virus isolation than the method observing CPE.

The results of RT-PCR analysis were consistent with those of the virus isolation when seminal plasma contained virus at titers of more than 10^{1.7} PFU/ml even if crude seminal plasma samples were used without any pretreatment. In contrast to the negative results for the samples which contained virus at titers of 10^{1.1} and 10^{2.2} PFU/ml of seminal plasma, a weak positive reaction was solely observed in one sample which contained virus at titers of 10 PFU/ml (Table 3). The contradiction led to the suggestion that results of RT-PCR may not be only dependent on the content of viable virus, but also influenced by inhibitory factors of seminal plasma [17]. Our RT-PCR assay was less sensitive than virus isolation, but it was simply and rapidly performed, within 2 days [18], and would be rather useful for supplementary use when cytotoxicity appeared in cell cultures inoculated with seminal plasma.

The sensitivity of the test mating for the detection of EAV from seminal plasma has not been thoroughly evaluated, but our previous experimental data indicated that virus suspension at titers of more than 8 × 10^6 PFU was evaluated, but our previous experimental data indicated that EA V from seminal plasma has not been thoroughly investigated. A positive reaction was revealed would be more sensitive for the detection of EA V than test mating. Furthermore, in vitro virus isolation in cell cultures supplemented with RT-PCR assay should be an effective alternative to test mating as a rapid, simple and economic system for routine diagnosis of carrier stallions.

REFERENCES

