Evaluation of Two Magnetic Bead-Based Viral Nucleic Acid Purification Kits and Three Real-Time RT-PCR Reagent Systems in Two TaqMan® Assays for Equine Arteritis Virus Detection in Semen

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This study showed that under specifically defined conditions with respect to nucleic acid extraction method and testing reagents, a previously described real-time RT-PCR assay (T1 assay) provides sensitivity equal to or higher than virus isolation for the detection of equine arteritis virus in semen.
Equine arteritis virus (EAV) is the causative agent of equine viral arteritis (EVA) which is a respiratory and reproductive disease of horses and other equid species (5, 6). Most EAV infections are asymptomatic or subclinical in nature, but occasional outbreaks occur which are characterized by influenza-like signs in adult horses, abortion and pneumonia or pneumo-enteritis in young foals (7, 9, 12, 15). Approximately 10-70% of EAV infected stallions can become persistently infected and will continue to shed the virus in semen (15). Carrier stallions are the natural reservoir of EAV; they ensure the virus is maintained in equine populations between breeding seasons (15, 17). The continued growth in international trade in horses and semen has served as a significant means of dissemination of EAV strains around the world (1, 8, 14-17). Identification of the carrier stallion is therefore of critical epidemiological importance in the prevention and control of EAV infection (3, 4, 13-15). Virus isolation (VI) is currently the World Organization for Animal Health (OIE)-approved gold standard for the detection of EAV in semen and is the prescribed test for international trade (18). In a recent study, we found rRT-PCR to be less sensitive (93.4%) than the OIE-prescribed VI test (gold standard) for the detection of EAV RNA in equine seminal plasma (10). The objective of the current study was to see if we could increase the diagnostic sensitivity of two previously described one-tube rRT-PCR assays (T1 and T2) (2, 11) using one-step rRT-PCR reagents from three different commercial kits (QuantiFast™ Probe RT-PCR + ROX Viral Kit [Qiagen, Valencia, CA], TaqMan® One-Step RT-PCR Master Mix Reagents Kit and TaqMan®EZ RT-PCR Core Reagents Kit [Applied Biosystems, Foster City, CA]) in combination with RNA extraction with magnetic bead-based nucleic acid extraction kits (MagMAX™-96 Viral RNA Isolation Kit (Applied Biosystems, Foster City, CA) and BioSprint 96 One-For-All Vet Kit (Qiagen, Valencia, CA). In order to compare the two assays in a standard and normalized manner, the commercial kits and rRT-PCR cycle...
parameters used in this study followed the manufacturer’s recommendations (Supplemental Table 1). First, the analytical sensitivity of these two assays was evaluated using 10-fold dilutions of in vitro transcribed (IVT) RNA ($10^{10}$-$10^1$ molecules) using three different commercial kits. Surprisingly, the analytical sensitivity of the two rRT-PCR assays varied significantly depending on which of the three commercial one-step rRT-PCR reagent kits was used (Supplemental Figure 1). The T1 assay detect one IVT RNA molecule using any of the three one-step rRT-PCR reagent kits. The T2 assay could only detect 100 IVT RNA molecules using the TaqMan One-Step RT-PCR reagent kit. However, using QuantiFast and TaqMan EZ RT-PCR reagent kits, the T2 assay detect 10 IVT RNA molecules. Thus, the T1 assay had greater analytical sensitivity and PCR efficiency when used with QuantiFast or TaqMan EZ One-Step RT-PCR reagent kits (Wilcoxon $P<0.001$). Of the three real-time RT-PCR reagent kits, the TaqMan One-Step rRT-PCR had the lowest sensitivity and therefore, it was not further evaluated in this study.

The analytical sensitivity of T1 and T2 rRT-PCR assays was further investigated using viral RNA extracted from 10-fold dilutions ($10^0$-$10^{10}$) of tissue culture fluid (TCF) containing the virulent Bucyrus (VB) strain of EAV with two different commercial kits (MagMAX and BioSprint) in combination with two different commercial one-step rRT-PCR amplification kits (C1-C8; Supplemental Figure 2). The T1 assay detected $\geq 1$ PFU/ml of virus when RNA was extracted with MagMAX and used in combination with either of the one-step rRT-PCR kits (C1 and C2; Supplemental Figure 2). This assay with the TaqMan EZ rRT-PCR reagent kit had similar sensitivity to that obtained when RNA was extracted with the BioSprint kit (C6). Thus, C1, C2 and C6 combinations were more sensitive as compared to the other combinations (C3, C4, C5, C7 and C8) which were only able to detect $\geq 10$ PFU/ml or $\geq 100$ PFU/ml of virus. These data further confirmed that the T1 assay had higher sensitivity when compared to the T2 assay. Furthermore, these data also clearly confirmed that not only the
reagent conditions, but also the primers and probes used in these two assays could influence assay sensitivity. Overall, these data indicated that C1, C2, and C6 combinations provided the highest sensitivity for detection of EAV RNA when extracted from 10-fold dilutions of TCF-containing virus.

A total of 409 semen samples from stallions located in the USA, Canada and various European countries received between January 2007 and August 2009 by the OIE equine viral arteritis reference laboratory at the Maxwell H. Gluck Equine Research Center, University of Kentucky, Lexington, Kentucky were tested in the study. Isolation of EAV from equine semen samples was attempted in high passage rabbit kidney-13 (RK-13) cell lines according to the OIE described protocol (18). Of the 409 semen samples tested, 80 (80/409) were positive for EAV by VI in RK-13 cells (Supplemental Table 2). The virus titers in semen samples varied from $0.5 \times 10^1$ to $>10^5$ PFU/ml of seminal plasma. MagMAX and BioSprint kits were used to extract nucleic acid from the 409 equine semen samples which was subsequently tested by the T1 assay using both QuantiFast and TaqMan EZ reagent kits (combinations C1, C2, C5 and C6; Figure 1). Of the 409 semen samples tested, 83, 74, 82 and 62 were positive for the presence of EAV RNA using C1, C2, C5 and C6 combinations, respectively. Statistical analysis using the McNemar test showed that the respective sensitivities of the C1, C2 and C5 combinations were not significantly different ($P>0.05$) when compared with VI in RK-13 cells. However, the C6 combination was significantly less sensitive ($P<0.001$) than VI. Three of seven sequential semen samples from one stallion collected between January 9 and February 20, 2008 were VI negative but positive by rRT-PCR (C1,C2, C5 and C6; Table 1). The three semen samples that gave discrepant results between VI and rRT-PCR were repeated independently and the results were reconfirmed. In summary, the T1 real-time RT-PCR assay (2) provides sensitivity equal to or higher than VI for the detection of equine arteritis virus in semen when viral RNA extraction using magnetic
bead-based nucleic acid extraction method (MagMAX™, Applied Biosystems) is combined with reagents from one-step QuantiFast RT-PCR kit (Qiagen; Figure 1 [C1 combination]).

Based on the published literature, this is the first time that an rRT-PCR assay to detect viral nucleic acid has been optimized using a number of commercial reagent kits for nucleic acid extraction and amplification. The findings of this study serve to emphasize that the choice of nucleic acid extraction kit, as well as the choice of the rRT-PCR reagent kit can have a major influence on the overall diagnostic sensitivity and robustness of the assay. This study also showed that under specifically defined conditions with respect to test reagents and testing procedures, the one-step rRT-PCR assay described by Balasuriya et al., (2002; (2) [T1]) has sensitivity equal to or higher than VI in RK-13 cells for the detection of EAV nucleic acid in semen samples. In light of our findings, the rRT-PCR protocol developed in this study could be considered equivalent or an alternative to VI for EAV detection at least in samples of raw semen.

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REFERENCES


FIGURE AND TABLE LEGENDS

FIG 1. Schematic of EAV detection results for 409 equine semen samples tested by VI in high passage RK-13 cells and by rRT-PCR using various test combinations (C1, C2, C5 and C6).

TABLE 1. Comparison of the results of VI and rRT-PCR from testing sequential semen samples collected from a carrier stallion.

SUPPLEMENTAL FIG 1. (A) Flowchart of various combinations of the two rRT-PCR assays (T1 and T2) and the three one-step rRT-PCR kits (QuantiFast kit, TaqMan One-Step kit and TaqMan EZ kit) used to determine the analytical sensitivity by testing 10-fold dilutions of the IVT RNA. The serial dilutions of IVT RNA were tested in duplicate and repeated three times in an independent series using three commercial rRT-PCR kits with both assays. (B) Analytical sensitivities of various combinations: average Ct values for dilutions containing 100, 10 and 1 copy of IVT RNA. The efficiencies, slopes and R² values based on testing serial dilutions are given in the three right hand columns.

SUPPLEMENTAL FIG 2. (A) Flowchart of various combinations of the two rRT-PCR assays (T1 and T2) and the two one-step rRT-PCR kits (QuantiFast kit and TaqMan EZ kit) used to...
determine the analytical sensitivity using 10-fold dilutions (10^0-10^-10) of TCF containing the EAV VB strain. RNA purification was performed according to the respective manufacturer’s instructions using a KingFisher® 96 (Thermo Fisher Scientific, Inc., Waltham, MA) automatic nucleic acid extraction machine. In the case of the MagMAX™-96 Viral RNA Isolation Kit, the recommended starting sample volume was 50 µl and the RNA elution buffer volume was 50 µl, whereas for the BioSprint 96 One-For-All Vet Kit, the recommended starting sample volume was 100 µl and the RNA elution buffer volume was 75 µl. Purified viral RNA from serial dilutions of TCF was tested in duplicate and repeated three times in an independent series using two commercial rRT-PCR kits (QuantiFast and TaqMan EZ) in both T1 and T2 assays. (B) Analytical sensitivities of various combinations: average Ct values for 10^5, 10^6 and 10^7 dilutions containing 100 PFU/ml, 10 PFU/ml and 1 PFU/ml. The efficiencies, slopes and R² values based on testing serial dilutions are given in the three right hand columns.

SUPPLEMENTAL TABLE 1. Reagents, primers and probes concentrations, and thermocycling conditions used for the detection of EAV nucleic acid by rRT-PCR using the QuantiFast kit (A), the TaqMan One-Step kit (B) and the TaqMan EZ kit (C).

SUPPLEMENTAL TABLE 2. Real-time RT-PCR and VI results. Ct values obtained when 83 semen samples were tested with T1 rRT-PCR assay using various combinations (C1, C2, C5 and C6) of reagent kits (two RNA purification kits [MagMAX and BioSprint kits] and two one-step rRT-PCR kits [QuantiFast and TaqMan EZ kits]). Virus isolation attempted in high passage (P399-P409) RK-13 cells.
TABLE 1. Comparison of the results of VI and rRT-PCR from testing sequential semen samples collected from a carrier stallion.

<table>
<thead>
<tr>
<th>Semen collection date</th>
<th>Semen sample Id</th>
<th>c1</th>
<th>c2</th>
<th>c3</th>
<th>c4</th>
<th>c5</th>
<th>c6</th>
<th>Virus isolation / Virus titer (PFU/ml)</th>
<th>High passage RK-13 cells (passage number)</th>
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<tr>
<td>01/09/2008</td>
<td>S-4168</td>
<td>33.16</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>1 x 10^1 (P404)</td>
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<td>31.40</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>33.37</td>
<td>35.50</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>37.83</td>
<td>34.60</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
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<td>36.25</td>
<td>32.98</td>
<td>34.93</td>
<td>36.57</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>-</td>
<td>38.07</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>1.5 x 10^1 (P402)</td>
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<tr>
<td>02/20/2008</td>
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<td>33.57</td>
<td>37.35</td>
<td>34.91</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>7.5 x 10^1 (P401)</td>
</tr>
</tbody>
</table>

* - Negative

The table shows the comparison of the results of VI and rRT-PCR from testing sequential semen samples collected from a carrier stallion. The table includes the semen collection date, semen sample Id, and various Ct values obtained with combinations of MagMAX kit (ABI), BioSprint kit (Qiagen), QuantiFast kit (Qiagen), TaqMan EZ kit (ABI), and TaqMan EZ kit (ABI). The table also includes the positive or negative status of virus isolation and virus titer (PFU/ml), and the high passage RK-13 cells (passage number).
Virus isolation in high passage
RK-13 cells

Semen samples (n=409)

MagMAX\textsuperscript{a} (ABI)

T1 rRT-PCR assay

QuantiFast (Qiagen)

TaqMan EZ (ABI)

83
C1

82
C5

BioSprint\textsuperscript{b} (Qiagen)

T1 rRT-PCR assay

QuantiFast (Qiagen)

TaqMan EZ (ABI)

74
C2

62
C6

\textsuperscript{a}Starting volume: 50 \textmu l; elution volume: 50 \textmu l.

\textsuperscript{b}Starting volume: 100 \textmu l; elution volume: 75 \textmu l.