

## Evaluation of Two Magnetic-Bead-Based Viral Nucleic Acid Purification Kits and Three Real-Time Reverse Transcription-PCR Reagent Systems in Two TaqMan Assays for Equine Arteritis Virus Detection in Semen<sup>∇†</sup>

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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 reverse transcription-PCR (rRT-PCR) assay (T1 assay) provides sensitivity equal to or higher than that of 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 which are characterized by influenza-like signs in adult horses and abortion and pneumonia or pneumoenteritis in young foals occur (7, 9, 12, 15). Approximately 10 to 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 that 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 real-time reverse transcription-PCR (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 reagent kit, and TaqMan EZ RT-PCR core reagent kit [both from 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 manufacturers' recommendations (see Table S1 in the supplemental material). First, the analytical sensitivities of these two assays were evaluated using 10-fold dilutions of *in vitro*-transcribed (IVT) RNA ( $10^{10}$  to  $10^{-1}$  RNA molecules) by using three different commercial kits. Surprisingly, the analytical sensitivities of the two rRT-PCR assays varied significantly depending on which of the three commercial one-step rRT-PCR reagent kits was used (see Fig. S1 in the supplemental material). The T1 assay could detect one IVT RNA molecule by using any of the three one-step rRT-PCR reagent kits. The T2 assay could detect only 100 IVT RNA molecules by using the TaqMan one-step RT-PCR reagent kit. However, using QuantiFast and TaqMan EZ RT-PCR reagent kits, the T2 assay could 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 sensitivities of T1 and T2 rRT-PCR assays were further investigated using viral RNA extracted from 10-fold dilutions ( $10^0$  to  $10^{-10}$ ) of tissue culture fluid (TCF) con-

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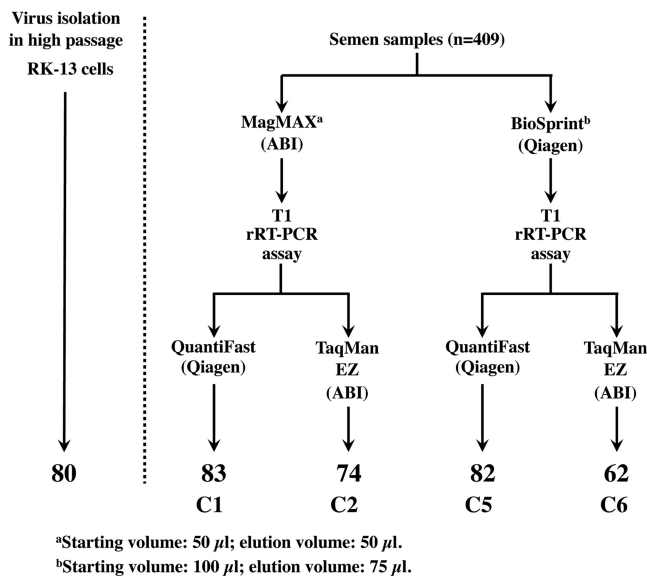


FIG. 1. Schematic of EAV detection results for 409 equine semen samples tested by VI in high-passage RK-13 cells and by rRT-PCR using various test combinations (C1, C2, C5, and C6).

taining 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 to C8; see Fig. S2 in the supplemental material). 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; Fig. S2). 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, the C1, C2, and C6 combinations were more sensitive than the other combinations (C3, C4, C5, C7, and C8), which were able to detect only  $\geq 10$  PFU/ml or  $\geq 100$  PFU/ml of virus. These data further confirmed that the T1 assay had higher sensitivity than 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 the 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 United States, 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, KY, 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 were positive for EAV by VI in RK-13 cells (see Table S2 in the supplemental material). The virus titers in semen samples ranged 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, and it was subsequently tested by the T1 assay using both Quantifast and TaqMan EZ reagent kits (combinations C1, C2, C5, and C6; Fig. 1). Of the 409 semen samples tested, 83, 74, 82, and 62 were positive for the presence of EAV RNA by 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 ( $P > 0.05$ ) from that of 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 9 January and 20 February 2008 were VI negative but positive by rRT-PCR (C1, C2, C5, and C6; Table 1). Analyses for 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 that of VI for the detection of equine arteritis virus in semen when viral RNA extraction using a magnetic-bead-based nucleic acid extraction method (MagMAX; Applied Biosystems) is combined with reagents from the one-step Quantifast RT-PCR kit (Qiagen) (C1 combination; Fig. 1).

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

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

Semen collection date (mo/day/yr)	Semen sample identification no.	$C_T$ values obtained with combination of <sup>a</sup> :				Virus isolation	
		MagMAX kit (ABI)		BioSprint kit (Qiagen)		Virus isolated	Virus titer (PFU/ml) for high-passage RK-13 cells (passage number)
		Quantifast kit (Qiagen) (C1)	TaqMan EZ kit (ABI) (C2)	Quantifast kit (Qiagen) (C5)	TaqMan EZ kit (ABI) (C6)		
01/09/2008	S-4168	33.16	—	—	—	+	$1 \times 10^1$ (P404)
01/15/2008	S-4178	31.40	—	—	—	+	$5 \times 10^1$ (P405)
01/28/2008	S-4256	38.25	33.37	35.50	35.42	—	—
02/08/2008	S-4257	37.83	34.60	37.14	35.33	—	—
02/11/2008	S-4258	36.25	32.98	34.93	36.57	—	—
02/13/2008	S-4259	35.34	—	38.07	—	+	$1.5 \times 10^1$ (P402)
02/20/2008	S-4260	33.57	37.35	34.91	—	+	$7.5 \times 10^1$ (P401)

<sup>a</sup>  $C_T$ , cycle threshold; —, negative.

Balasuriya et al. in 2002 (2) (the T1 assay) has sensitivity equal to or higher than that of 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 to or an alternative to VI for EAV detection, at least in samples of raw semen.

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