Nucleic acid extraction methods for detection of EHV-1 from blood and nasopharyngeal secretions

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THE sensitivity and reliability of PCR for routine diagnostic and research purposes relies upon efficient procedures of extraction and purification of nucleic acids. The use of novel amplification methods in human and veterinary medicine, such as real-time PCR, has increased the diagnostic sensitivity of molecular assays, reduced analytical costs and shortened the time needed for tests (Kaltenboeck and Wang 2005, Pusterla and others 2006a). In equine medicine, this has translated into better care and faster implementation of isolation measures to prevent outbreaks of infectious disease. The benefits of molecular diagnostics in the prevention of disease outbreaks has been shown in the management of equine herpesvirus type 1 (EHV-1) outbreaks at riding schools, racetracks and veterinary hospitals throughout North America and Europe (Slater and others 2006). Correct preparation of diagnostic samples is essential for optimal recovery of DNA for further molecular amplification. Most veterinary diagnostic and research laboratories rely on well-established manual protocols for the extraction of nucleic acids. However, a high throughput of samples is often needed while maintaining a high nucleic acid recovery. There is scope for improving automation and the sensitivity of nucleic acid amplification technology testing. Newer robotic systems minimise the risk of contamination, and reduce the extraction time and costs (Smit and others 2000, Kessler and others 2001), but there are no reports of the validation of such automated extraction platforms for the veterinary field. This study evaluated a novel automated high-throughput protocol for extracting nucleic acid from samples of anticoagulated blood and nasopharyngeal swabs collected from healthy horses and horses naturally exposed to EHV-1, by comparing it with a manual extraction method.

A total of 20 anticoagulated blood samples and 20 nasopharyngeal swabs were collected from 10 healthy adult horses belonging to the Center for Equine Health herd at the University of California, Davis. All procedures were approved by the Institutional Animal Care and Use Committee of the University of California. In addition, 12 anticoagulated whole blood samples and 15 nasopharyngeal swabs were collected from 15 horses exposed to EHV-1 during confirmed outbreaks of myeloencephalopathic EHV-1. Anticoagulated blood (5 ml per tube) was collected from the jugular vein using evacuated blood tubes (Vacutainer; Becton Dickinson). Nasopharyngeal swabs (Fox Converting) were advanced through the ventral meatus of the right and left nostrils to the pharynx and allowed to soak for 10 seconds. The swabs were placed in conical centrifuge tubes containing 5 ml of viral transport medium (minimal essential medium with 0·125 per cent gentamicin and 0·1 per cent amphotericin B). All samples were placed on ice after collection and processed for DNA extraction within one to three hours of collection.

From each of the blood samples collected from the healthy horses, two aliquots of 100 µl were collected for each of the extraction protocols. Only one aliquot of 100 µl blood from the EHV-1-infected horses was used for each of the extraction protocols. Each swab was vortexed for 10 seconds, inverted and centrifuged at 16,000 *g* for five minutes to retrieve a cell pellet. After removing the swab and supernatant, each pellet was resuspended into 400 µl phosphate-buffered saline solution (PBS) and the sample was divided into two equal aliquots of 180 µl for each of the extraction protocols. The blood and nasopharyngeal samples were then extracted using either the automated nucleic acid extraction CAS-1820 X-tractor Gene (Corbett Life Science), or the manual nucleic acid extraction DNeasy Tissue Kit (Qiagen) according to the manufacturer's recommendations.

For the automated extraction, 180 µl of each sample (for blood, 100 µl of blood plus 80 µl of PBS; for nasopharyngeal secretions, 180 µl of resuspended pellet in PBS) was added to a deep-well plate (Masterblock; Greiner Bio-One). After sample loading, the system was fully automated as follows: 100 µl of liquid sample digest buffer (Xtractor Gene Liquid Sample Reagent Pack; Sigma) was added to the sample, mixed five times and incubated for 10 minutes at room temperature; 360 µl of lysis buffer including 25 per cent propanol (Sigma) was added, mixed six times and incubated for five minutes at room temperature; 530 µl of the lysed samples were loaded on to the capture plate (96-well low skirt 800 µl GF/B filter; Whatman) and vacuumed for two minutes at 30 kPa; 500 µl of lysis buffer with 25 per cent propanol was added and vacuumed for five minutes at 30 kPa; 300 µl of ethanol wash buffer (Sigma) was added and vacuumed for three minutes at 30 kPa; the samples were dried for five minutes at 30 kPa, eluted with 100 µl of water at 21°C (DEPC-Treated Water; Ambion), incubated for two minutes and vacuumed for one minute at 45 kPa.

For the manual extraction, the following protocol was followed: 180 µl of each sample was incubated with 200 µl of Buffer AL and 20 µl proteinase K for 10 minutes at 56°C in a 1·5 ml microfuge tube; 200 µl of 100 per cent ethanol was mixed with each sample, applied to the column and centrifuged for one minute at 16,000 *g*; the flow-through was discarded and 500 µl of Buffer AW1 was added to the column and centrifuged for one minute at 16,000 *g*; the flow-through was discarded and 500 µl of Buffer AW2 was added to the column and centrifuged for three minutes at 16,000 *g*; the column was moved to a new 1·5 ml tube, 100 µl of water (Ambion) at 90°C was added, and the column was centrifuged for three minutes at 16,000 *g*. In order to minimise contamination, all pipetting steps were performed under laminar flow.

The amount of time elapsed during the extraction of a comparable number of samples by the automated protocol (the time from loading the deep-well plate to the end of the automated extraction) and the manual protocol (the time from adding the Buffer AL and the proteinase K to the sample to the elution of DNA) was measured with a stopwatch. The test turnaround time, including hands-on time, for both protocols was determined for one, 24 and 96 samples.

The DNA yield from the samples of blood and nasopharyngeal secretions using both protocols was quantified by spectrophotometric measurement of the absorbance at 260 nm using a microplate spectrophotometer (BoTek).

To determine the DNA quality, all the samples were analysed for the detection of the universal 18S rRNA housekeeping gene using a commercially available assay (Eukaryotic 18S RNA; Applied Biosystems). To evaluate the presence of EHV-1, all the samples were assayed for the presence of the glycoprotein B (gB) gene of EHV-1 using a previously reported real-time TaqMan PCR assay (Pusterla and others 2006b). The PCR for both assays contained 400nM of each primer, 80nM of the TaqMan probe and commercially available PCR mastermix (TaqMan Universal PCR Mastermix; Applied Biosystems) containing 10mM Tris-HCl (pH 8·3), 50mM potassium chloride, 5mM magnesium chloride, 2·5mM dNTPs, 0·625 U AmpliTaq Gold DNA polymerase per reaction, 0·25 U AmpErase UNG per reaction and 1 µl of the DNA sample in a final volume of 12 µl. The samples were amplified in a combined thermocycler/fluorometer (ABI PRISM 7900 Sequence Detection System; Applied Biosystems) for two minutes at 50°C, 10 minutes at 95°C, and then 40 cycles of 15 seconds at 95°C and 60 seconds

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at 60°C. Fluorescent signals were collected during the annealing temperature, and cycle threshold (C_t) values extracted with a threshold of 0·1 and baseline values of 3 to 15.

The mean (sd) C_t value for the detection of the universal 18S rRNA gene and gB gene of EHV-1 were calculated for both extraction systems. The statistical difference ($P<0·05$) in the extraction efficiency and amount of extracted DNA between the two protocols was determined using the Mann-Whitney test.

DNA was extracted successfully from all 52 blood aliquots using both extraction systems. The mean (sd) C_t value of the universal 18S rRNA gene for the automated protocol was 21·1 (0·4) and for the manual protocol it was 22·2 (0·5). The difference in C_t values of the universal 18S rRNA gene between the automated and manual extraction systems was significant ($P=0·01$). All the blood aliquots extracted with the automated system yielded stronger PCR signals for the universal 18S rRNA gene than those extracted with the manual protocol. The differences in C_t values of the universal 18S rRNA gene ranged from 0·45 to 2·36. The total DNA extraction yield using the automated system (mean [sd] 5·56 [3·98] µg DNA/100 µl of blood) and the manual protocol (2·44 [1·21] µg DNA/100 µl of blood) was statistically different ($P=0·001$).

Both extraction protocols were successful in yielding DNA from all the nasopharyngeal swabs. The mean (sd) C_t value of the universal 18S rRNA gene was 22·4 (1·0) for the automated protocol and 21·3 (0·9) for the manual protocol. The difference in C_t values of the universal 18S rRNA gene between the two protocols was not statistically significant. Nineteen samples extracted with the manual protocol yielded stronger signals than with the automated system, with differences in C_t values ranging from 0·08 to 1·39. Sixteen samples extracted with the automated system yielded stronger signals than the manual system, with differences in C_t values ranging from 0·14 to 0·7. The difference between the total amounts of DNA yielded with the automated system (mean [sd] 3·32 [3·21] µg DNA/180 µl of resuspended cell pellet) and the manual protocol (4·20 [1·98] µg DNA/180 µl of resuspended cell pellet) were not significant.

EHV-1 genomic DNA was detected in 12 blood samples extracted with the automated system and six blood samples extracted with the manual protocol. The C_t values of EHV-1 from the six blood samples that tested positive by both extraction systems were not significantly different. However, the difference in C_t values of the 18S rRNA housekeeping gene for all blood samples was statistically significant between the automated (mean [sd] 21·4 [0·6] and manual (22·6 [0·7]) extraction systems ($P=0·01$). EHV-1 was detected in 15 nasopharyngeal secretions extracted with the automated system and 13 extracted with the manual protocol. The C_t values of EHV-1 from the 13 nasopharyngeal samples that tested positive by both extraction systems were not statistically different. The difference in C_t values of the 18S rRNA housekeeping gene for all 15 positive samples was not statistically significant between the automated (mean [sd] 22·9 [1·5]) and manual (22·7 [1·8]) extraction systems. None of the blood and nasopharyngeal samples collected from the healthy horses tested PCR positive.

The extraction of DNA is a key preliminary step for molecular analysis. In selecting the most appropriate protocol, some of the issues to consider include the type of sample and volume required, yield, purity, ease of operation, throughput, cost, and whether the protocol involves the use of hazardous reagents or can be automated. Both extraction protocols used in this study are solid-phase extraction methods that take advantage of the reversible binding of DNA to silica. With these systems, the DNA is precipitated by the addition of alcohol, after cell lysis and protein digestion of the initial sample. The sample is then allowed to pass through a silica-impregnated filter, which binds and purifies the DNA from debris present in the alcohol mixture. Filtration is achieved by placing the sample under a vacuum, as in the automated system used in this study, or by centrifugation, as in the manual protocol. The bound DNA is then washed and subsequently eluted using nuclease-free water. In this study, the automated system yielded more total DNA from anticoagulated blood than the manual system, while both extraction systems performed similarly on nasopharyngeal secretions. The expected ranges of DNA yield provided by the manufacturer of the manual (1 to 5 µg) and automated (1 to 10 µg) systems are comparable, which is in agreement with the results of this study. If larger amounts of DNA are required for molecular analysis, the automated extraction system has the flexibility to use different DNA capture plates with binding capacities of up to 40 µg.

More important than the high recovery of DNA from the extraction protocol is the quality of the extracted samples, which should allow sensitive and accurate detection of the target gene (in this study, the gB gene of EHV-1). This study used the universal eukaryotic 18S rRNA gene as the housekeeping gene to determine whether the extracted DNA specimens were free of amplification inhibitors. Real-time PCRs targeting the 18S rRNA gene from anticoagulated blood samples and nasopharyngeal swabs gave consistent amplification levels for both extraction systems. The automated extraction system performed significantly better on whole blood than the manual system; however, similar amplification efficiencies were obtained using the two protocols when the nasopharyngeal secretions were tested.

The biological application of both extraction protocols was evaluated using field samples from horses naturally exposed to EHV-1. The detection of EHV-1 in nasopharyngeal secretions prepared with the two extraction systems was similar, with 13 PCR-positive samples after extraction with the manual protocol and 15 positive samples after extraction with the automated system. The minor discrepancy between the 15 nasopharyngeal samples is most likely related either to the dilution of extracted EHV-1 DNA (total extracted DNA was resuspended in 100 µl of sterile water) or to the small amount of DNA (1 µl) used for each PCR. The use of triplicates for each clinical sample and/or larger volumes of DNA (up to 5 µl) per reaction may have increased the number of EHV-1 positive results for samples extracted using the manual protocol. However, for diagnostic purposes, such an approach would be unrealistic. The detection of EHV-1 in blood showed great differences between the two extraction protocols, with only half of the samples testing PCR positive after manual extraction. This difference is likely to be related to the more efficient extraction of DNA by the automated system, as exemplified by the significantly stronger C_t values. These results have important diagnostic implications for the routine molecular detection of EHV-1, and highlight the advantage of using automated extraction systems, especially when using anticoagulated blood as the diagnostic sample.

The costs of reagents per single extraction, based on the manufacturer's listed kit price plus material (pipettes and tubes), were 16 per cent higher for the manual system (US $2.02) than for the automated system (US $1.74). One major advantage of the automated system is the flexibility to extract up to 96 samples at a time. The manual extraction protocol was limited by centrifuge space, which allowed the processing of a maximum of 24 samples at the same time. The hands-on and turnaround times were directly related to the number of samples processed for both extraction systems. While the automated system required 50 minutes for one single extraction, the manual system was able to extract the same sample in 23 minutes. However, for 24 samples, the automated and manual protocols required 63 and 115 minutes, respectively. A hypothetical run of 96 samples would have taken 97 for the automated system and 460 minutes for the manual system.

A major concern for any DNA extraction protocol is the cross-contamination of negative samples as a consequence of aerosolisation of positive samples. For manual protocols, cross-contamination is reduced by performing all the steps

under laminar flow and keeping the number of simultaneous extractions as small as possible. Specimen contamination during the manual process was not tested in this study, but it is a potential concern, given the dependency of the efficiency of the manual protocol on the skill of the technician. Several studies have determined that contamination of samples during automated nucleic acid extraction remains a theoretical rather than a real concern, since faulty robotics and robotic errors rarely occur, and most of the systems, including the one tested in this study, are equipped with a filtering system (Smit and others 2000, Kessler and others 2001, Knepp and others 2003, Gärtner and others 2004, Beuselinck and others 2005).

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