Analytical validation of cardiac troponin I assays in horses

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Abstract. Human cardiac troponin I (cTnI) assays have been used in equine medicine, often without prior analytical validation for equine use. In the absence of appropriate validation, the clinical significance of assay results is uncertain and can lead to misdiagnosis. We followed the American Society for Veterinary Clinical Pathology guidelines and investigated linearity, precision, limit of quantification (LoQ), and comparative recovery for 6 commercial cTnI assays developed for use in human medicine. Clinically acceptable linearity was observed in assays A–D, whereas assay E did not detect equine cTnI in any sample. Comparative recovery revealed 1–3-fold differences between assay results, and low analyte recoveries (2.2–3.4%) were observed in assay F. Precision was investigated in assays A and B, and found to be within acceptable limits. The LoQ was 1.53 ng/L for assay A, and 0.031 µg/L for assay B. Assays A and B performed within clinically acceptable limits and were deemed suitable for use in equine medicine. Assays C and D did not undergo full validation but had acceptable linearity, which demonstrates their potential for use in equine medicine. Assays E and F are unsuitable for use in horses given issues with detection of equine cTnI. The variability in results between assays indicates that reference intervals and cutoffs for diagnostic decision-making are assay specific and should be established prior to adoption by diagnostic laboratories.

Key words: Biomarkers; cardiac troponin I; horses; myocardium.

Introduction

Cardiac troponins are globular proteins that, along with tropomyosin and actin, form part of the thin filament of the contractile apparatus in striated muscle. The troponin complex is made up of 3 polypeptide subunits: troponin I, T, and C that regulate the calcium-dependent actin–myosin interaction necessary for muscle contraction.8 Troponins I and T have cardiac-specific isoforms that are distinguishable from those of skeletal muscle, allowing differentiation between myocardial and skeletal muscle damage. The analytical specificity of cardiac troponins and their release during cardiac injury has led to the development of commercial immunoassays and their adoption as markers of myocardial injury.

Cardiac troponin I (cTnI) assays are important for the detection of myocardial damage and have been in widespread use in human medicine since the 1990s.2,6,13 In 2000, cardiac troponins were adopted as the biomarkers of choice for detection of myocardial infarction in human patients by joint consensus of the European Society of Cardiology and the American College of Cardiology.7 Use of cTnI assays in veterinary medicine followed shortly after their introduction, and studies have documented their use in several non-human species, including the horse.9,10,17,21 However, the incorporation of cTnI for use in animals has relied exclusively on the adoption of assays designed for and validated in humans, often without performing analytical validation in the species of interest. This is potentially problematic, because although the amino acid sequence is well conserved between humans and other domestic species, amino acid substitutions and deletions do exist at target epitopes.20 In addition, species-specific physiology, disease states, and composition of the test matrix (plasma or serum) may have unforeseen effects on assay performance. In order to identify and quantify potential sources of error, the American Society for Veterinary Clinical Pathology (ASVCP) recommends that analytical validation be performed before adoption of a new test or method for routine laboratory use. In the absence of analytical validation and subsequent epidemiologic (clinical) validation, the clinician or researcher is unaware of the reliability of the test and therefore of the predictive value of a given result.

To date, there are several published studies describing the clinical use of cTnI assays in the horse, but only 2 detailing full or partial analytical validation.15,16,22,25,26 The results of these studies were consistent with the hypothesis that com-
Commerical cTnI assays are capable of detecting myocardial damage in horses. However, because the accuracy of these assays depends on their analytical performance, validation should be performed prior to assessing their suitability as laboratory tests. The objective of our study was to analytically validate commercial human cTnI assays for use in the horse.

Materials and methods

Animal use and study protocol were approved by the University of Guelph’s Animal Care Committee, Animal Utilization Protocol 1339.

Preparation of test substrates and matrix

Serum, myocardium, and skeletal muscle were collected from a single donor horse. Inclusion criteria included the following: apparently healthy, <2-y-old, and no history of race training. Physical examination was performed prior to tissue collection to exclude clinically apparent systemic illness or cardiac dysfunction. Prior to anesthesia, a 14-gauge intravenous catheter was placed in a jugular vein using aseptic technique, and a 4.1-mL extension set attached. The filly was pre-medicated with xylazine (Rompun 100 mg/mL injectable, Bayer, Shawnee Mission, KS), general anesthesia induced with intravenous (IV) ketamine hydrochloride (Ketalar 10 mg/mL injection, Pfizer, New York, NY) and diazepam (Sandoz Canada, Boucherville, QC, Canada), followed by maintenance with isoflurane inhalant (BIMEDA-MTC Animal Health, Cambridge, ON, Canada). Following induction, a second IV catheter was placed in the contralateral jugular vein, and whole blood was collected into 10 sterile 1-L blood collection bags. The horse was then euthanized with pentobarbital sodium IV (Merck Animal Health, Kirkland, QC, Canada) and immediately transferred to a postmortem suite.

A gross postmortem examination was performed, and the heart extracted within 20 min of euthanasia to reduce degradation of cardiac troponin complex. The heart was then frozen at −80°C. A portion of the triceps brachii was also extracted and frozen. Whole blood was allowed to clot for 1 h at room temperature, and then for 12 h at 4°C to allow full maturation of the clot and maximum serum yield. All steps were carried out at room temperature unless otherwise stated.

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Isolation of crude cardiac troponin complex

Isolation of crude cardiac troponin complex from harvested myocardium was achieved using a protocol described previously. Modifications to the protocol were made to accommodate the decreased volume of tissue used, and are listed in the summary below. All steps were carried out at room temperature unless otherwise stated. Briefly, myocardium from the left ventricular free wall was homogenized, using a micro-homogenizer (Omni International, Kennesaw, GA), in 4°C buffer solution (1% Triton X-100, 50 mM KCl, and 5 mM Tris, pH 8) and centrifuged at 11,000 × g for 15 min at 4°C. The pellet was resuspended in buffer solution, and the process was repeated 8 times. The pellet was transferred to a Buchner funnel and washed with chilled 95% ethanol under suction 3 times. The process was repeated using diethyl ether in place of ethanol, and the resultant etherized sample was allowed to dry overnight at 4°C. The etherized substrate was resuspended in 200:1 volume/weight high-salt solution (1 M KCl, 20 mM Tes, pH 7, and 15 mM BME), rather than 20:1. The rationale for this change was that a smaller mass of myocardium was used in our study, and the sample volume would have been too low in a 20:1 solution to accurately test and adjust pH. The substrate was allowed to extract in the high-salt solution overnight at 4°C. The sample was centrifuged at 11,000 × g for 20 min at 4°C and the pellet discarded. Ammonium sulfate (1.62 M) was added to the supernatant and the pH adjusted to 7.0. The sample was centrifuged at 11,000 × g for 20 min at 4°C and the pellet discarded. Ammonium sulfate (0.55 M) was added to the supernatant, and the sample was centrifuged at 11,000 × g for 15 min at 4°C. The resultant pellet was stored in polypropylene microcentrifuge tubes (Fisherbrand Snapcap 1.5 mL microcentrifuge tubes, Fisher Scientific, Pittsburgh, PA) at −20°C until use. Because the substrate of interest was the crude cardiac troponin complex, further purification through dialysis and chromatography was not attempted.

Immunoblotting

Western blot analysis was performed using a modified protocol described previously. The gels were loaded with homogenized murine myocardium and equine skeletal muscle in addition to the crude equine troponin isolate. The samples were run on 12% SDS-polyacrylamide gels and the proteins transferred to a nitrocellulose membrane. The membrane was incubated in a 1:1,000 solution of anti-TnI antibodies (Troponin I [C-4]: sc-133117, Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C. The membrane was then incubated in a 1:5,000 solution of secondary antibodies (Antimouse IgG [Fab specific] peroxidase antibody produced in goats, Sigma-Aldrich, St. Louis, MO) conjugated with horseradish peroxidase. Protein bands were detected using Western Lighting Plus-ECL (PerkinElmer, Waltham, MA). Immunoblot images were captured, and were viewed on ImageJ (https://imagej.nih.gov/ij/).

Preparation of cTnI-spiked serum

Low cTnI equine serum was used as a matrix for the spiked samples. In the absence of a gold standard, the cTnI concentration in this serum was measured by the high-sensitivity assay A, which has a limit-of-blank (LoB) of 0.6 ng/L.12
Affinity of assay A for equine cTnI was later confirmed through analytical validation. The crude troponin complex was suspended in this matrix and mixed using a vortex spinner. The concentration of cTnI in the spiked serum was ascertained using assay D given the availability of the assay at the time. Serial dilutions were produced from this spiked serum to make up various concentrations of cTnI for use in the validation process. The samples were then frozen at −80°C until analysis.

**Measurement of cTnI**

Hospital laboratories and manufacturers were recruited for participation in the analytical validation of commercial cTnI assays. The number of validation experiments performed depended on both the performance of the assays and the continued participation of these industry partners. Six assays currently being used in veterinary and human hospital laboratories were included in our study (assays A–F; Table 1). Samples were prepared by the researchers and shipped to the laboratories on dry ice. Analyzing laboratories were blinded as to the concentration of the samples.

**Linearity experiment**

Linearity was assessed in assays A–E. The methodology described in guidelines published by the ASVCP (Principles of Quality Assurance and Standards for Veterinary Clinical Pathology, http://www.asvcp.org/pubs/qas/index.cfm) was employed in the assessment of assay linearity. Five concentrations of cTnI-spiked serum, spanning the full range of the tests, as reported by the manufacturer, were prepared. Three replicates of each concentration were run on assays A–E. The values for each concentration were plotted against the expected value and the plot visibly inspected for outliers and linearity. Curve fitting was performed with linear regression using Origin 8.6 software (Origin Lab, Northampton, MA).

**Comparison of results**

Recovery was compared between assays by running aliquots of 5 concentrations of cardiac troponin–spiked serum on assays A–F. For each concentration, 3 replicates were run and the mean calculated. Results for each assay were compared to a high-sensitivity referent (assay A) and recovery expressed as a percentage of the referent. In lieu of a gold standard, assay A was chosen as a referent based on its high manufacturer-stated sensitivity, given that we had no a priori knowledge of greater accuracy or performance at the time of sample analysis.

**Short- and long-term precision**

Short-term precision was determined for assays A and B. As recommended in the ASVCP guidelines, 2 concentrations of cTnI were used: a low concentration close to the limit of quantification (LoQ), and a second concentration in the presumed pathologic range based on the human literature. Twenty replicates of each sample were tested within a single 8-h control period. Long-term precision was determined for assay B, using the same samples. Assay A was not available for long-term precision testing given financial constraints. Twenty replicates were run over a 20-d period for both concentrations. The mean concentration, standard deviation (SD), and coefficient of variation (CV) were calculated using Excel 2010 (Microsoft, Redmond, WA).

**Limit of quantification**

A spiked serum sample of low cTnI concentration was used to determine the LoQ for assays A and B. Serial dilutions of the spiked serum were prepared using isotonic saline, and 20 replicates of each dilution were tested within a single control period. The mean concentration, SD, and CV were calculated for each dilution using Excel 2010. The greatest dilution (lowest concentration) with a CV of <20% was accepted as the LoQ.

**Pre-analytical storage**

Post-exercise serum samples from 9 Standardbred racehorses were used to assess the effect of pre-analytical storage temperature on assay results. Samples were collected from horses competing in a regularly scheduled 1.6-km race at Mohawk Racetrack in southern Ontario. In agreement with industry partners (track management, horsemen, and the
Ontario Racing Commission), participation in the study was made a condition of race entry. For each horse, whole blood samples were collected via jugular venipuncture into 2 red-top vacutainer tubes. The first tube was allowed to clot for 45 min at room temperature, and the serum was then separated and divided into 2 aliquots. One aliquot was immediately stored at −80°C and used as the control group. The second aliquot was placed in a −20°C freezer for 30 d and then stored at −80°C until analysis. The second tube was divided into 2 aliquots before clot formation. The aliquots were stored either at room temperature (24°C) or 4°C for 24 h, and then centrifuged at 3000 × g for 15 min. Serum was then separated and stored at −80°C until immediately before analysis. All samples were analyzed using assay A because it had the highest analytical sensitivity of assays tested. The difference between each test group and the control group stored at −80°C was assessed using a mixed linear regression model with horse ID as a random intercept. Model fit was assessed by visual inspection of standardized residuals and best linear unbiased predictions (BLUPS). Statistical analysis was performed (STATA 13, Stata, College Station, TX).

Results

Preparation of test substrate and matrix

Physical examination of the donor filly revealed bilateral angular limb deformities of the forelimbs. All other findings were within normal limits. Postmortem examination documented moderate-to-severe, bilateral carpal valgus, and varus of the metacarpal–phalangeal joints. No other clinically significant abnormalities were found. The serum harvested from this filly was tested using assay A and found to have a cTnI concentration above the LoB (0.6 ng/L) of 0.88 ± 0.32 ng/L (mean ± SD).

Western blot analysis

A protein band of ~24 kDa that is consistent with presence of cTnI was detected in the lanes loaded with the crude equine cardiac troponin isolate (Fig. 1). A protein band of ~24 kDa was detected in the lane loaded with murine myocardium.

Linearity

Results for assay E were under the detection limit of the test for all sample concentrations. Linearity results for assays A–D are shown in Figure 2. Least squared linear regression revealed $R^2$ values of 0.965–0.996 (mean: 0.977 ± 0.006 SD) for these assays.

Comparison of results

Recoveries for assays A–D and F compared to assay A were 0.9–323% (mean: 145 ± 29; Table 2). Results were below the detection limit for 2 samples using assay F and were not included in the analysis. A result for the highest concentration sample was not obtained for assay D because of a shipping error.

Short- and long-term precision

The short-term or “within-run” precision for assay A was 1.6 ± 0.2 ng/L (CV: 13.6%) and 2,003 ± 56 ng/L (CV: 2.8%). Short-term precision for assay B was 0.058 ± 0.003 µg/L (CV: 5.7%) and 26.2 ± 0.2 µg/L (CV: 0.8%). Long-term or “between-run” precision for assay B was 0.044 ± 0.009 µg/L (CV: 20.9%) and 25.6 ± 0.32 µg/L (CV: 1.2%).

Limit of quantification

The LoQ for assay A, calculated during a single 8-h control period, was 1.53 ± 0.27 ng/L (CV: 17.6%). Using the same procedure, the LoQ for assay B was 0.031 ± 0.002 µg/L (CV: 7.5%).

Pre-analytical storage

Samples were thawed prior to analysis, and run in a single 8-h control period using assay A. Mean cTnI concentrations, in ng/L, for the −80°C, −20°C, 4°C, and 24°C groups were 5.11 ± 1.64, 4.62 ± 1.41, 4.75 ± 1.59, and 4.22 ± 0.49, respectively. A univariable mixed linear model was run with cTnI concentration as the dependent variable and storage temperature as the independent variable. A random intercept for horse ID was included to account for clustering at the horse level (intra-class coefficient: 0.95). No statistically significant difference was found between storage groups ($p = 0.74$).
Discussion

Our study relied on cTnl-spiked serum for the majority of the analytical validation procedures. Efforts were made to select a healthy donor animal of racehorse breeding that had not participated in race training, as this could potentially result in elevated cTnl concentrations. Use of myocardium and serum from a single animal is a potential limitation of our study, as these tissues may not account for normal variation in troponin I amino acid sequence in the equine population. However, to our knowledge, there are no reports of sequence variation in the absence of myocardial disease in either the human or equine literature. The decision to use a single donor was based on ethical concerns, such as limiting animal usage, as well as economic constraints. Western blot analysis of the troponin complex isolate from this filly revealed a protein band with a weight consistent with cTnl (molecular weight: 24 kDa) based on protein weight markers and murine controls. Cross-reactivity with other proteins is possible, especially with skeletal troponin I. However, the exclusive use of myocardium for cTnl isolation, combined with the molecular weight of the resulting protein band should eliminate this possibility. The results indicate that the protocol was successful in isolating cTnl from the myocardium.

Examination of linearity data for assays A–D revealed a linear relationship between the observed and expected cTnl values ($R^2 = 0.965–0.996$). These results suggest that these assays are capable of detecting equine cTnl, and there is a strong linear relationship between the “real” and observed concentration of cTnl throughout the reportable range of}$

![Figure 2. Linearity results for assays A–D. A dilution series of cardiac troponin I (cTnl)-spiked serum was prepared, and 3 replicates of 5 concentrations were run on each assay. Results are stated in µg/L and represented by solid dots. Dashed line represents fitted line using linear regression. Assays A–D showed good linearity based on visual inspection and linear regression.](#)

<table>
<thead>
<tr>
<th>Assay A</th>
<th>Assay B</th>
<th>Assay C</th>
<th>Assay D</th>
<th>Assay F</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 (24.6)</td>
<td>290 (71.45)</td>
<td>91.3 (22.47)</td>
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<tr>
<td>100 (17.98)</td>
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<td>100 (11.6)</td>
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<td>268 (31.12)</td>
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<td>100 (4.49)</td>
<td>283 (12.69)</td>
<td>60.6 (2.72)</td>
<td>212 (9.53)</td>
<td>(&lt;0.2)</td>
</tr>
<tr>
<td>100 (0.022)</td>
<td>68.2 (0.015)</td>
<td>0.9 (0.0002)</td>
<td>182 (0.04)</td>
<td>(&lt;0.2)</td>
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</tbody>
</table>

Actual levels recorded by each assay are presented in µg/L in parentheses. Percentage of recovery is not stated for the 2 lowest dilution runs on assay F because the results were below the limit of detection for that assay. A result for the highest dilution was not obtained for assay D because of a shipping error, indicated by a dash (—).
each assay. Although visual examination of the line graphs (Fig. 1) shows that linearity was not perfect, performance of each assay was acceptable clinically.

Assay recovery results, expressed in Table 2 as comparative recovery, varied widely among the assays tested, with assay F having the lowest recovery and assay B the highest. Similar discrepancies in results between assays has been observed in other studies using human and canine patient samples, where differences of 2–5 fold between assay results were found.\(^1\) Given that there is no gold standard, it is difficult to determine which assay most accurately measures cTnI concentration. The current lack of standardization between assays is likely the result of differences in antibody configuration and epitope targets, and the absence of a standard reference material.\(^7\)\(^,\)\(^23\) Some assays may be better at detecting cTnI fragments in serum, or cTnI that is in complex with other troponin subunits. Therefore, cTnI reference intervals should be developed for each hospital population, using a single assay, and comparisons of results between assays for clinical purposes should be avoided.

Despite using many of the same epitopes as assays with acceptable performance, assay E did not detect equine cTnI in any of the samples. These results may reflect the way in which these epitope targets are used. Both assay E and C employ antibodies against amino acid residues 87–91 in the cTnI polypeptide.\(^4\) Examination of the amino acid sequence of equine cTnI, compared to that of the human protein, reveals a single substitution of glutamic acid for alanine at residue 91. \(^24\)\(^–\)\(^40\) Assay C utilizes this epitope along with amino acids 24–40 as targets for its 2 capture antibodies, whereas this is the only epitope target of assay E’s detection antibody. Similarly, assay F uses the residue 87–91 epitope as the target for its capture antibody.\(^4\) The comparative recovery of assay F in our study was much lower (2.2–3.4\%) than other assays tested, indicating a poor analytical sensitivity for equine cTnI. These examples suggest that the amino acid substitution decreases or negates the affinity of assay antibodies for this epitope and impacts the accuracy of assays that rely solely on this affinity for antigen capture or detection. This lack of detection precluded further validation, and assays E and F were deemed unsuitable for use in equine medicine.

ASVCP guidelines recommend that a test must have a CV of \(\leq 20\%\) within the reportable range to be considered clinically acceptable. Assessment of short-term precision in assays A and B found a CV of \(<15\%\) at both a high and low cTnI concentration, indicating acceptable performance. Results for the long-term precision experiment in assay B showed a low CV (1.2\%) for the high-concentration sample, but a CV slightly above the acceptable cutoff for the low concentration (20.9\%). These results emphasize the need for a well-regulated quality control program and for a strict procedural protocol in order to minimize sources of analytical variation and to maximize precision.

A LoQ of 0.031µg/L was found for assay B, which is in agreement with the LoQ in human samples,\(^4\) suggesting that the analytical sensitivity of this assay is similar in humans and horses. Assay A had a LoQ of 1.53 ng/L, which is an order of magnitude lower than assay B. This higher sensitivity allows the clinician to detect changes in cTnI concentration that are below the detection limit of most commercial assays, potentially leading to fewer false negatives, both in diagnosis and clinical progression. In addition, higher sensitivity tests will provide information on biological variability, and a better understanding of basal cTnI concentrations in sub-populations, such as performance animals.

Analysis of the impact of pre-analytical storage conditions on cTnI results revealed no difference between groups. These findings are consistent with those of human studies that found minimal changes in cTnI concentration on the basis of pre-analytical storage.\(^3\)\(^,\)\(^19\)\(^,\)\(^27\) Storage groups in our study were designed to mirror common conditions in equine practice in which serum cannot be immediately separated or frozen. Results suggest that short-term storage under these conditions does not significantly impact results. However, the sample size was small (\(n = 9\)), and these samples were taken from clinically healthy horses with low cTnI concentrations. Studies should be performed using larger study populations, and include horses with myocardial disease to further assess the impact of pre-analytical storage.

The objective of our study was to perform analytical validation on commercial cTnI assays. The extent of analytical validation was dependent on the continued participation of third-party laboratories, which was not always feasible, and therefore some of the assays did not go through full analytical validation. However, assays A and B were fully validated (with exception of long-term precision for assay A) and determined to have clinically acceptable performance when used on equine samples. Precision can evaluate the impact of environmental conditions, assay calibration and quality control, and sample on repeatability of results. The impact of changes in environment, assay calibration, and quality control has a greater impact on long-term than short-term precision because analysis takes place over multiple control periods. Given that the majority of variance in long-term precision studies stems from environmental changes and quality control rather than sample, long-term precision may not be necessary for cross-species validation of established assays. Although not fully validated in our study, results of the linearity experiment suggest that assays C and D may be suitable for use in equine medicine. Full analytical validation should be performed before including these assays in the diagnostic laboratory. Population-specific reference intervals should be established for each assay and diagnosis cutoffs determined. In human medicine, the 99th percentile of the reference population is typically accepted as the cutoff for myocardial injury.\(^2\)\(^,\)\(^24\) These guidelines were validated by clinical studies that investigated the diagnostic sensitivity and specificity at different cutoffs, in patients with possible myocardial infarctions.\(^11\)\(^,\)\(^14\) Further studies into appropriate cutoffs in equine patients should be performed to assess case characteristics and optimize diagnostic accuracy.
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Declaration of conflicting interests
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