Troponin assays in the assessment of the equine myocardium

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Summary

In 2000, troponin assays were adopted as the test of choice for detection of myocardial injury in man. This decision was made after extensive testing and followed a 60 year search for a biomarker of myocardial damage with sufficient analytical sensitivity and specificity. This has led to proliferation of assays for use in human medicine, each requiring extensive testing and validation before it could be made available on the open market for human use. The search for ever-more analytically sensitive assays and for a standard reference material continues. The adoption of troponin testing in veterinary medicine followed shortly after its development for use in man, providing a much-needed means of detecting and monitoring myocardial damage in horses. However, application of these tests in veterinary medicine has been exclusive to cases where there is no mandated requirement for test validation in veterinary medicine and, while many of these assays have been shown to be capable of detecting equine troponin, the wide diversity of available tests, lack of validation, absence of protocols for their use and lack of standardisation make their application problematic. The objective of this review article is to address this issue, offering guidance where data are available and encouraging caution where there are none. Ultimately, the overall goal of this review is to examine critically the use of troponin assays in the horse and to promote the accurate and appropriate interpretation of valid results.

Keywords: horse; cardiac biomarkers; equine myocardial disease; test validation; sudden death

Introduction

Clinically apparent disease of the myocardium arises infrequently in the horse, and when damage is present it is often subclinical. When significant arrhythmias are identified during systemic disorders, such as colitis, colic or toxicity, myocardial involvement may be suspected, though the immediate cause may be homeostatic disturbance, not primary myocardial disease, and the possibility of the latter may be overlooked [1–4]. Viral infections, such as equine herpesvirus, African horse sickness and equine viral arteritis, as well as intoxications associated with consumption of ionophores, oleaner and blister beetle-contaminated hay, can have a direct effect on equine myocardium, causing inflammation, degeneration or necrosis [3,5–10]. Again, clinical manifestations are primarily those of rhythm disturbance, except in severe cases where overt heart failure may develop. Of importance, but often overlooked, is involvement of the myocardium in a range of systemic conditions. When an animal has to deal with systemic disease, the heart must sustain increased workload in the face of homeostatic disturbances and toxicities, and may also be subject to direct insults such as exposure to cardiotoxic agents. This more subtle or occult (background or secondary), yet potentially cumulative, myocardial damage may be of particular significance in a performance animal, such as the horse. In terms of histopathology, the myocardium is not a very expressive tissue, and confirmation of damage, at least at the level of light microscopy, when tissue is available, may be difficult. Earlier confirmation would clearly be more desirable. The clinician must constantly be alert to the possibility of myocardial damage. The presence of myocardial damage or incipient myocardial damage is not difficult to anticipate, but confirmation and assessment of the degree and course of damage are necessary to ascertain prognosis and clinical progress, and this requires a suitable test.

Cardiovascular disease in man, a significant source of morbidity and mortality, encompasses a broad range of illnesses and clinical circumstances. Among these is the phenomenon of sudden cardiac death, most often attributed to coronary artery disease and myocardial infarction [11]. In those cases where no definitive cause can be assigned at post mortem examination, differential diagnoses include ion channel abnormalities and cardiac rhythm disturbances. In contrast to man, coronary artery disease and myocardial infarction are rare in horses, though functional variations in heart sound and rhythm are common. Cardiovascular disease is thus infrequently confirmed as a primary cause of death in horses, even in sudden death cases, where cardiovascular involvement may be overlooked in favour of more visible findings. Sudden death in horses is also a rare event, usually occurring in the context of intense exercise, when numerous other competing factors are at play [12,13].

Studies investigating sudden death in apparently healthy racehorses have found signs at post mortem examination consistent with cardiac involvement, including pulmonary congestion and haemorrhage, pulmonary oedema and rupture of pulmonary or thoracic vessels [12;14–16]. In almost half of the cases described in those studies, however, the cause of death was not apparent. It can take several days for even microscopic changes to become apparent in myocardium [17]. Histopathology can thus be unrewarding. Given the peracute nature of sudden death, fatal rhythm disturbance must be considered in cases where there is no clear, definitive cause. This hypothesis may be supported by results of a recent study, which recorded electrocardiogram (ECG) traces in Standardbred racehorses during scheduled races [18]. Clinically significant, complex ventricular rhythm disturbances during the cool-down period were found in 16% of race events. These disturbances involved 18% of the horses monitored during the study. Although none of the affected horses died and the presence of the arrhythmias was not clinically apparent, they occurred at a point during recovery from intense exercise that has been associated with a peak in sudden deaths [15,19]. This suggests the possibility that such disturbances in rhythm may have the potential to become malignant and raises the question of whether these arrhythmias are a physiological response to intense exercise or whether they are pathological and associated with myocardial injury. If exercise-associated arrhythmias are pathological, then myocardial damage may precede rhythm disturbance. Alternatively, damage may follow rhythm disturbance. Detection of underlying myocardial injury would clearly require a suitable test.

Numerous imaging, biochemical and other diagnostic tests are available to clinicians for the detection of myocardial damage and dysfunction in man, but for logistical or biological reasons they are not currently used in equine medicine. Body surface ECG has been used in horses for many decades and is a reliable tool for detection of heart rate and rhythm abnormalities. However, body surface ECG recordings represent only a small fraction of ventricular myocardium in the horse because the structure of the conduction system leads to significant mirror-image cancellation of electrical activity [20,21]. As a result, the body surface ECG...
is insensitive to changes in chamber size and to myocardial injury without concomitant rhythm disturbance. Echocardiography is in use in many referral centres for detection of structural and functional cardiac abnormalities and is sensitive and specific for changes in chamber size, wall thickness and left ventricular function [22,23]. The extensive shoulder musculature in the horse coupled with the cranial orientation of the right ventricle place constraints on imaging of the right ventricle, however, that limits detection of changes in that chamber. In addition, although useful for gross changes in structure and function, echocardiography is not sensitive to myocardial compromise at the cellular level. Imaging modalities such as cardiac magnetic resonance imaging could, in theory, detect occult myocardial damage in horses. However, the cost, limited availability of equipment, logistical difficulties and necessity for general anaesthesia during these procedures make such tests impracticable as screening tools at this time.

Biochemical markers have long been used to diagnose myocardial damage in both human and veterinary medicine [24–27]. Biomarker assays are often inexpensive and are easily accessible to ambulatory practitioners. Serum enzymes, such as lactate dehydrogenase isoenzymes 1 and 2, alanine transaminase and aspartate aminotransferase, have been used to diagnose myocardial infarctions in human patients since the early 1950s [24,25]. Serum elevations of these enzymes are sensitive indicators of cell necrosis and enzyme leakage. As they are present in many tissues, however, elevation in serum levels is not specific to cardiac damage. Elevations are seen in cases of skeletal muscle damage, hepatic disease, endocrine disorders and pulmonary conditions [24,25]. In addition, it often takes hours to days for levels to exceed normal values, making them of limited value in emergency room settings where rapid intervention is necessary. The creatine kinase-myocardial band enzyme is highly sensitive for myocardial damage and can be detected in the circulation soon (4–6 h) after injury [28,29]. In otherwise healthy individuals, creatine kinase-myocardial band is also highly specific for cardiac damage. However, in some cases of concurrent disease, such as chronic neumocirculur disease, creatine kinase-myocardial band may be expressed in skeletal muscle, leading to false positives [30,31]. These findings make creatine kinase-myocardial band a less-than-ideal choice for detection of myocardial injury, especially in the face of intense exercise [32]. The current standard in human medicine is the cardiac troponin assay which, as of 2000, was declared the preferred biomarker for diagnosis of acute myocardial infarction by joint consensus of the European Society of Cardiology and the American College of Cardiology [17].

What are cardiac troponins?

Troponins are globular proteins found in striated muscle that regulate the calcium-dependant myosin-actin interaction necessary for muscle contraction. Along with tropomyosin and actin, they are components of the thin filament within the myofilbril. Three proteins make up the troponin complex, namely troponins I, T and C. These subunits form complexes in a 1:1:1 ratio in the contractile apparatus of myocytes [33]. Troponin T binds the troponin complex to tropomyosin and actin, while troponin I inhibits activity of the actomyosin ATPase, preventing myofilament interaction. When the troponin C subunit binds Ca2+, changes in protein conformation interfere with troponin complex to tropomyosin and actin, while troponin I inhibits activity of the actomyosin ATPase, preventing myofilament interaction. When the troponin C subunit binds Ca2+, changes in protein conformation interfere with troponin complex assembly. This second rise is more prolonged due to ongoing cellular degeneration, and in some cases from ongoing myocardial insult.

Cardiac troponin I has a half-life of 0.47 h in horses, based on a study using i.v. infusions of exogenous CtnI [38]. These results differ from a study on rats and dogs, which found CtnI half-lives of 0.8 and 1.85 h, respectively [39]. Cardiac troponin I is cleared through glomerular filtration in the kidneys and conforms to a first-order elimination model, where clearance rate is dependent on plasma concentration, in all species studied [38–40]. Renal dysfunction has the potential to interfere with elimination of CtnI; however, while compromise does occur in patients with kidney failure, the result of decreased clearance or myocardial damage has not been confirmed [41,42].

Troponin I as a cardiac biomarker

The potential of troponin I to serve as a marker of myocardial damage was first recognised in the late 1980s, when researchers were looking for an ideal cardiac biomarker [43,44]. The impetus behind this search was the need for a sensitive and specific diagnostic indicator of myocardial damage that could be used in an emergency room setting. As mentioned earlier, methods of detecting myocardial damage at the time included serum levels of creatine kinase-myocardial band and lactate dehydrogenase isoenzymes 1 and 2. These isoenzymes are fairly sensitive to myocardial necrosis but are not unique to cardiomycocytes, and therefore serum elevations are not specific for cardiac damage [30,31,45]. This lack of specificity becomes problematic when trying to detect cardiac damage in the face of neuromuscular disorders, myopathies, intense exercise, chronic renal failure or endocrine disorders. A more analytically specific marker of myocardial damage was needed for accurate diagnosis of cardiac disease.

An ideal biomarker for myocardial damage needs to fulfil certain criteria in order to be used in clinical medicine with confidence [46]. It must be specific for cardiac tissue, which is achievable only if it has a distinct cardiac isoform not present in other tissues. 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 cardiac isoform of troponin I is not expressed in either healthy or diseased skeletal muscle, unlike cardiac troponin T, which is expressed in skeletal muscle in cases of chronic muscular disease, such as polymyositis, muscular dystrophy and rhabdomyolysis [47,48]. Troponin C is found in the same isoform in both cardiac and slow-twitch skeletal muscle, making changes in its activity less specific for myocardial injury [49].

An ideal biomarker must be present at only low levels in the serum of healthy individuals and should be rapidly released into the bloodstream after injury. Low normal levels allow clear cut-points between normal and abnormal to be established, thereby increasing diagnostic confidence on the part of the clinician. Low levels can be a drawback, however, where available tests lack sensitivity and have relatively high minimum detection, with most of the reference population being below the detection limit of the assay. This makes it impossible to detect small changes that may nonetheless be of possible clinical significance. Studies in man have shown that CtnI is present at levels at or below the detection limit of most commercial assays in clinically healthy subjects. The 99th percentile upper reference limit for CtnI was defined in a population of apparently healthy Standardbred racehorses, using the Statrus CS CtnI immunoassay, and found to be 0.06 ng/ml [50]. Most animals in that study had CtnI levels below the 0.04 ng/ml detection limit of the assay. Newer-generation ‘high’ or ‘ultra’-sensitivity assays are now in development from several manufacturers and, while not yet available for commercial use, have been used in research studies, including a study by Nostell et al., involving endotoxin in ponies [51]. Some ambiguity exists concerning use of the term ‘high sensitivity’, because it has been used inappropriately to denote newer-generation tests with lower detection limits. As of 2012, an assay must meet 2 criteria to be designated ‘high sensitivity’, i.e. the total imprecision at the 99th percentile must be ±10%, and a measurable concentration of CtnI should be obtainable by the assay in ≥50% of healthy individuals [52]. No studies have been done on the troponin I release curve post injury in horses. However, release and
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clearance curves have been established for man and laboratory animals, and suggest that detectable increases in serum cTnI occur within 1–4 h after myocardial insult, with peak levels occurring within 6–24 h, depending on species [29,53,54]. The insult in these studies ranged from acute infarction to experimentally induced toxic damage. The observed release time may have been impacted by the type and severity of myocardial injury, and should be taken into account when interpreting these studies.

In certain cases of myocardial injury, particularly those in which damage is minor or subclinical, suspicion of cardiac disease may not be aroused for several days after the initial insult. If the biomarker of choice does not persist for long in circulation then clinicians may not be able to confirm their diagnosis or may potentially misdiagnose the condition. Adequate persistence in circulation is therefore important when selecting an ideal biomarker. Unlike creatine kinase-myocardial band, which returns to baseline within 48–72 h, cTnI persists in the blood for 6–8 days post injury, allowing delayed diagnosis [43]. As with release times, these numbers may vary based on the type and severity of myocardial injury and whether it is a single event or an ongoing pathological process.

In addition to diagnosis, an ideal cardiac biomarker can provide prognostic information if serum levels strongly correlate with lesion size or severity of disease. A 2006 study by O'Brien et al. found that cTnI levels correlated closely with the size and severity of myocardial lesions in groups of laboratory mice, rats and dogs with experimentally induced cardiac toxicity [55]. Likewise, a positive correlation between peak cTnI levels and infarct size on cardiac magnetic resonance imaging was observed in a study using a canine model of acute myocardial infarction [55]. Although few studies investigating the correlation between cTnI levels and severity of cardiac disease have been published in nonlaboratory animals, there are numerous examples in the human literature. In human patients presenting with acute chest pain, peak cTnI levels were positively correlated with worse outcomes, including mortality at 30 days and at 6 months, revascularisation and progression to chronic cardiac disease [56]. Even low troponin levels suggested a worse prognosis over undetectable levels. A similar association was found in patients with acute heart failure, with mortality increasing with increasing cTnI levels [57]. A study in dogs presented for cardiovascular examination found that cTnI levels at the initial consultation were negatively correlated with survival time [58]. Dogs that responded well to treatment had significantly lower cTnI levels on recheck appointment; the same was not true for dogs that responded poorly. Mean survival time between the 2 groups differed significantly, with dogs that had a reduction in cTnI levels on follow-up living longer on average than dogs that did not show this improvement. These findings suggest that cTnI can be used as a prognostic indicator in nonhuman species with cardiac disease.

Cardiac troponin I fulfils all of the criteria for an ideal marker of myocardial injury. For this reason, it has become the preferred biomarker in human medicine and is an established tool for diagnosis and surveillance of cardiac disease.

Use of troponin in horses

Although all currently available commercial troponin I assays were designed for human patients, there are numerous reports of their use in horses with cardiac disease. Published studies have found increases in troponin I levels above presumed reference levels in horses with pioplamiosis, atypical myopathy, ruptured aortic jet lesions, structural cardiac disease and neoplasia, as well as after transvenous electrical cardioversion [1,7,59–62]. Serum levels of cTnI showed a statistically significant increase after i.v. infusion of endotoxin in ponies in one study [51]. The elevations were followed by ventricular arrhythmias in all animals. These findings are indicative of myocardial dysfunction resulting in rhythm disturbance. In a study by Divers et al., horses were given various doses of sodium monensin via nasogastric tube in conjunction with either cold water or saline [7]. Cardiac troponin I levels were measured throughout the study and were found to be increased in horses exhibiting clinical signs of cardiac disease or failure. The highest cTnI levels were observed in the animals that progressed to fulminant heart failure. No increase was observed in horses that were given low doses of monensin and that did not exhibit clinical signs of myocardial damage.

Several studies have been published exploring the effect of exercise on cTnI levels in horses [50,63,64]. The results have been variable, with 2 studies finding mild-to-moderate increases in cTnI after racing or an endurance event, and one finding no significant difference. The lack of agreement could be due to differences in methodology, exercise intensity or duration, population studied, sample times or assays used. Standard-sensitivity cTnI assays have minimal detection limits below the resting level of healthy subjects, which means that mild-to-moderate elevations in cTnI levels could be missed by some assays [50,65,66]. Further studies using a standardised approach, based on knowledge of the troponin I release curve and assay sensitivity and precision, are necessary to determine the significance of exercise-associated troponin I release.

These case reports and studies suggest that at least some commercially available cTnI assays could be useful diagnostic tools in equine medicine. However, caution must be employed before basing clinical decisions on assays designed for another species and, with one exception [50], not validated for use in the horse. Issues referenced in this review and associated with use of cTnI assays should all be taken into account when interpreting results.

Issues associated with measuring troponin I

Epitopes

Tests used to detect and measure proteins and polypeptides most often use immunological strategies (for example, enzyme-linked immunosorbent assays, immunofluorescence) and use carefully selected antibodies. The similarity in amino acid sequences in cTnI between man and horses means that protein/polypeptide sites (epitopes) targeted by test (assay) antibodies are likely to be well conserved [34]. This homology has been used to justify the use of human cTnI assays in horses without appropriate laboratory validation. However, good homology does not guarantee that epitopes will be accessible to antibodies, because small changes in amino acid sequence can affect polypeptide folding and potentially obstruct binding sites. For instance, even single amino acid substitutions or deletions can alter the antibody affinity for an epitope, affecting the analytical sensitivity and precision of the test. The American Society for Veterinary Clinical Pathology recommends that studies of long- and short-term precision, functional sensitivity, linearity, analyte recovery and interference be performed before considering inclusion of a test in the diagnostic laboratory [67]. Without this information, it is impossible to determine the predictive value of assay results for confirming myocardial injury.

Standardisation

There are many commercial cTnI assays on the market, made by several manufacturers, with each assay being designed to run on dedicated, proprietary analysers. These tests also use unique, proprietary antibodies. A major theme, not only in veterinary medicine but also in human medicine, is the lack of standardisation among these assays. The consequence is our inability to compare cTnI values from different analysers, creating the potential for confusion and misinterpretation of results. A recent study compared the results of 7 different cTnI assays run on patient samples, and found a 5- to 7-fold difference in values from the same sample [68]. This lack of agreement is due to a number of factors, starting with the large number of different assays available. These assays each use different antibodies for capture and detection of cTnI that target various epitopes on the N- or C-terminus of the protein. Antibodies are monoclonal (from a single identical cell line), polyclonal (from multiple cell lines) or a mixture thereof, and they vary in the indicator molecule used. This diversity of antibodies and epitopes naturally leads to a lack of concordance and to variation in the ability of assays to detect different forms of cTnI released into the circulation.

Standard reference material

The lack of a standard reference material for calibration is a problem that arises from the range of cTnI assays on the market. Ideally, this standard
would mimic the composition of cTnI forms released into the circulation after myocardial damage [69]. Unfortunately, attempts to produce such a reference material have failed so far, and there has been no standardisation or harmonisation of cTnI assays [70].

**Free and complex troponin I**

Cardiac troponin I is released into the circulation in several forms after myocardial injury [69,71,72]. In addition to free troponin I subunits, which make up only a small percentage of the troponin I released, cTnI is often released in tertiary or binary complex with troponin T and C, the most common form being a complex of C and I subunits [69,72]. The existence of these complexes may have a profound effect on assay results, because the presence of troponin C or T could restrict access to target epitopes for antibody binding. Conversely, some assays use antibodies that preferentially bind to cTnI in complex with other troponin components, leading to more variation in assay results [72].

**Target degradation**

The N- and C-termini of cTnI are susceptible to degradation by proteases, both at the site of myocardial damage and in serum [71,73]. During myocardial necrosis, proteolytic enzymes are released from lysosomes and act on contractile proteins within hours after the initial insult. A study by Katrukha et al. found that fragmentation of cTnI occurred within 2 h of tissue necrosis and was most profound in free cTnI rather than in complex [71]. This fragmentation was most apparent in the regions at the extreme end of the C- and N-terminus, whereas amino acid residues in the more central regions were largely preserved, probably due to protection from proteases. The effect of degradation on assay antibody immunoreactivity was dependent on the region targeted by the test.

Increasing discordance in test results between assays with time was observed [71]. These findings indicate that observable peak release time and elimination curves will vary with the assay used. Therefore, interpretation of results, and thus clinical diagnosis and case management, are highly dependent on the assay used.

**Chemical modifications**

Chemical modification, such as phosphorylation of serine groups, oxidation and reduction, and N-terminus acetylation, can occur both intracellularly and in the circulation [73,74]. A study from Labugger et al. found that some assay antibodies changed their immunoreactivity to cTnI after dephosphorylation of serum, while other assays were not affected [73]. This suggests that only epitope sequences that include phosphorylation sites would be susceptible to variability and imprecision by this means. A study comparing diseased and healthy myocardium in man found that healthy tissue contained higher concentrations of phosphorylated cTnI [75]. These results suggest that assay performance may differ in cases of chronic cardiac disease. Little is known about the effects of oxidation and reduction on assay results, though the potential exists for such modification also to interfere with findings.

**Standard sample protocols**

A final consideration is the lack of a standard laboratory protocol for collection of patient samples. The diversity of cTnI assays on the market means that the preferred sample type (serum vs. plasma, as well as type of anticoagulant) varies by assay. Some assays are able to process either plasma or serum, whereas others can also use whole blood. The variety of options seems like a convenience when taken at face value. However, sample additives, such as anticoagulants, as well as byproducts of clot formation, can have an effect on results. Several studies have documented lower recoveries for haemapped samples compared with serum in both healthy subjects and those with cardiac disease [76–78], though these results were not consistent across the literature, and some studies found no difference [79]. It is likely that that these discrepancies are in part due to antibody differences in the assays used, because heparin is thought to interfere in some cases by binding and restricting access to target epitopes [77].

**Anticoagulants**

Ethylene diaminetetraacetic acid can also affect cTnI results by chelation of circulating calcium ions, because maintenance of troponin complexes is calcium dependent [80]. Free troponin I released into the circulation because of low Ca\(^{2+}\) is vulnerable to degradation by proteases. This could have profound effects on measurable troponin levels, because free troponin I may have a shorter half-life than that in complex. Test antibodies may also vary in their affinity for free cTnI [71].

Given that anticoagulants have been shown to affect assay results, the logical choice would be to use serum. However, coagulation products, such as fibrin, have the potential to give spurious results, because they are prone to nonspecific binding by assay antibodies [81,82]. This can cause falsely elevated levels and could give rise to clinical misdiagnoses. However, these false positives are considered uncommon and seem to be the consequence of incomplete clot formation and centrifugation [81,82].

Blood samples taken in field conditions are often exposed to extreme temperatures, inappropriate handling and incomplete centrifugation. Therefore, care should be taken to establish consistent sampling protocols to ensure accurate assay results. Further studies examining the extent of fibrin and anticoagulant interference in man and in the horse are required in order to determine the most appropriate protocol and sample to be used in clinical settings.

**Conclusion and future work**

Cardiac troponin I is a sensitive and specific marker of myocardial damage, and its measurement is the test of choice in human medicine. Several case reports and observational studies have documented its potential use as a diagnostic tool in equine medicine. However, it is not possible to offer guidance on the use of assays in clinical settings at this time. Work still needs to be done to validate commercial assays to ensure that they are reliable and accurate when used in the horse and to establish sample-collection protocols. Given that a number of different assays exist on the market, none of them standardised, care must be taken to establish laboratory protocols and reference intervals that are specific to each assay. Future studies should include the establishment of the equine cTnI release curve in normal, exercising animals and the determination of the most appropriate times to sample potentially diseased animals. These data would facilitate accurate diagnosis of myocardial damage and minimise false-positive and false-negative results. Further studies using high-sensitivity assays at peak release times are needed to investigate fully the relationship between exercise and cTnI levels and to assess any relationship between cTnI release, rhythm disturbances and myocardial damage.

**Authors’ declaration of interests**

No competing interests have been declared.

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**Authorship**

T.M. Rossi did much of the literature review and interpretation of published data for this review paper. The text of this manuscript was written primarily...
by T.M. Rossi. W.G. Pyle did some of the literature review for this paper. He contributed by giving insight into the interpretation of currently published data and edited the manuscript. D.L. Pearl was instrumental in interpreting data in the context of test validation and disease screening and edited the manuscript. M.G. Maxie did some of the literature review for the article and interpreted the data and also edited the manuscript. P.W. Physick-Sheard did some of the literature review and interpretation of published data and wrote some of the manuscript. In addition, he edited the manuscript. Final approval of the manuscript was given by T.M. Rossi in conjunction with the other authors.

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