Performance of a Revised Cardiac Troponin Method That Minimizes Interferences from Heterophilic Antibodies

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Background: Recent guidelines for use of cardiac troponin to detect cardiac damage and for cardiovascular risk stratification have made increasingly sensitive troponin assays important. Troponin assays continue to be plagued by interferences caused by heterophilic antibodies (HAs). We evaluated the performance of a revised cardiac troponin I (cTnI) assay designed to have increased analytical sensitivity and to minimize the effect of HAs.

Methods: The revised Dade Behring Dimension® cTnI assay was evaluated according to NCCLS EP5-A at five institutions. Plasma samples from 14,309 patients were assayed by the original Dimension cTnI assay. To identify samples that may have interfering HAs, samples with values >1.4 µg/L were reanalyzed on the Dade Behring Stratus® CS cTnI assay. Samples with possible interfering antibodies were also analyzed before and after selective absorbance studies on the revised Dade Behring Dimension cTnI assay.

Results: The limit of quantification in the revised method was 0.1 µg/L with imprecision (CV) of 11–17% at 0.1 µg/L. Values correlated well with the Stratus CS cTnI method: revised = 1.06(original) + 0.01; r = 0.98, SYx = 0.25 µg/L. Falsely increased results consistent with myocardial infarction by the original Dimension cTnI assay and presumably attributable to HAs were identified in 0.17% of all patients with samples submitted for cTnI analysis. The revised Dimension cTnI assay eliminated the interference in 17 of 25 samples identified and greatly decreased the interference in the other 8.

Conclusions: The revised Dimension cTnI method greatly minimizes the effect of interfering HAs. It also exhibits analytical performance characteristics consistent with recent guidelines for use of this assay to detect cardiac damage.

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Measurement of cardiac troponins is important for the diagnosis of myocardial infarction and for risk stratification (1–5). In light of recent European Society of Cardiology/American College of Cardiology (ESC/ACC) and National Academy of Clinical Biochemistry (NACB) recommendations for the use of troponins (1, 2) and reports of falsely increased cardiac troponin I (cTnI) values attributable to heterophilic antibodies (HAs) (6–8), modifications to cTnI methods that increase sensitivity and decrease interferences will be important. HAs can cause interference in immunoassays (6–11), but the estimated incidence varies tremendously, from 0.2% to 40% (9–11). In the clinical laboratory, HAs that cross-react with immunoglobulins of different species are of particular inter-
est because they can cross-link the solid phase and soluble antibodies in two-site “sandwich” immunoassays. Because many in vitro diagnostic immunoassays use murine monoclonal antibodies, heterophilic human anti-mouse immunoglobulin antibodies (HAMAs) are of particular concern. Development of HAMAs can follow the use of murine monoclonal antibodies for therapeutic and diagnostic imaging purposes (12). Other proposed etiologies for developing interfering HAs include incidental or occupational exposure to foreign animal proteins (10) and microbial antigens that induce broadly reactive HAs that bind immunoglobulins from other species (8, 13). HAs with anti-immunoglobulin activity can also be found in patients with autoimmune diseases such as rheumatoid arthritis (7, 9).

The presence of HA interferences in immunoassays has led manufacturers to indicate this possibility in package inserts and to add “blocking” agents to their immunoassay formulations (14, 15). Despite these measures, the problem persists (6–8, 16–21). Here we examine the incidence of interfering antibodies in the original Dimension cTnI assay and evaluate a revised Dimension cTnI assay designed to be more sensitive and to minimize the effect of interfering HAs.

Materials and Methods

cTnI Measurements

The revised Dimension RxL cTnI method (Dade Behring) is a two-site sandwich immunoassay that uses the same two monoclonal antibodies specific for the stable central region of the molecule (22) used in the original Dimension RxL cTnI and the Dade Behring Stratus CS methods (23, 24). The same calibrators are used except that the revised Dimension cTnI method uses a human serum-based matrix, whereas the original method used a buffered bovine serum albumin matrix. Calibrator value assignment is based on comparisons with a set of master calibrators containing purified human troponin complex. The revised method incorporates a new, proprietary conjugate reagent formulation, an increased concentration of irrelevant mouse immunoglobulin to minimize HAMA interference, and a smaller sample volume of 50 μL. All assays were used according to the manufacturer’s suggestions. The five evaluation sites were Washington University (St. Louis, MO; site A); CHU Hôpital Henri Mondor (Créteil, France; site B); Azienda Ospedaliera di Padova (Padova, Italy; site C); University of Alberta (Edmonton, Canada; site D); and Dade Behring Research Laboratory (Newark, DE; site E). All sites obtained approval from their institutional review boards.

Performance Characteristics of the Revised Dimension cTnI Assay

Within-run and total reproducibility were determined using aliquots of MAS Tru-Liquid-TM Quality Control (three concentrations) and serum pools. Analysis was performed in duplicate, once a day for 20 days according to the NCCLS EP5-A protocol, and analyzed by ANOVA. Each site calculated their own statistics, and site E calculated the statistics for combined data. Different lots of quality-control material were used at the evaluation sites. To determine precision at lower cTnI concentrations, a precision profile was performed at site C. Eight dilutions of a patient serum sample, with an initial concentration of 0.3 μg/L, were made with the zero calibrator, aliquoted, and frozen. Aliquots from each sample were tested in duplicate for 10 days over a 20-day period.

The minimum detection limit, defined as the lowest concentration that can be differentiated from zero, was determined separately at sites A, B, C, and E by assaying 20 replicates of the revised cTnI zero calibrator at the beginning and end of the 12- to 16-week evaluation period.

Assay range and linearity were determined using pooled serum and plasma samples with increased cTnI concentrations and the Level 5 revised Dimension cTnI calibrator (concentration, 34.4 μg/L). Samples were diluted with the Level 1 calibrator (concentration, 0 μg/L) at sites A, B, C, and E. Each dilution was assayed in triplicate and compared with expected results by linear regression. The lower limit of linearity was determined similarly using a plasma sample containing 1.0 μg/L cTnI. Calibration stability was determined by assaying the five calibrators in triplicate once a week for 9 weeks at site A. Paired serum and lithium-heparin-plasma samples from 135 patients, obtained at sites A (n = 54), B (n = 51), and C (n = 30), were assayed to examine the relationship between serum and plasma cTnI values. The effect of sample quality was examined at site E according to NCCLS guideline EP7 using a sample containing 1.5 μg/L cTnI and up to the indicated concentrations of the following: bilirubin (600 mg/L), hemoglobin (10 g/L), and triglycerides (30 g/L).

Reference Interval and Method Comparison

The reference interval for the revised Dimension cTnI method was determined using serum and plasma samples from 342 volunteers or patients (176 males and 166 females; age range, 18–83 years) with no history or evidence of cardiac disease at sites A (n = 121), B (n = 171), and C (n = 50). Values were ranked nonparametrically to estimate the central 95% region and the upper 99 percentile (25).

Results from a total of 234 serum or plasma samples with cTnI values from <0.4 to 209.7 μg/L on the original Dimension cTnI assay were compared with Stratus CS results by linear regression. Results from a total of 618 serum or plasma samples with cTnI values from <0.1 to 220.0 μg/L on the revised Dimension cTnI assay were compared with results from the Stratus CS across all five evaluation sites.

Identification and Characterization of Interfering Antibodies

Plasma samples from 14,309 patients were submitted for routine cTnI analysis by the original Dimension cTnI.
assay between August 14, 2000, and April 11, 2001, at Barnes-Jewish Hospital. Samples with values ≥1.4 μg/L were selected for repeat testing by the Stratus CS cTnI method, and those with Stratus CS values ≤0.1 μg/L were considered clinically discrepant and chosen for further investigation. We chose 1.4 μg/L as the “cutoff” because this was the value used at our institution (site A) and stated in the original Dimension cTnI assay package insert as being consistent with myocardial infarction. The lower limit for reporting values from the Stratus CS method at site A is 0.1 μg/L. To further investigate the discrepant samples, 150 μL of plasma was mixed with 50 μL of Sepharose-conjugated protein A (Pharmacia), Sepharose-conjugated irrelevant murine IgG1/IgG2a antibodies (murine anti-parathyroid hormone receptor monoclonal antibodies) (26), or unconjugated Sepharose 4B for 16–18 h, with gentle rotation, at room temperature. Discrepant samples were also mixed with 50 μL of saline (control) for 2 h, with gentle rotation, at room temperature. After adsorption and removal of the Sepharose beads, samples were analyzed on the original Dimension cTnI assay.

Results

The within-run imprecision (CV) was 1.0–8.4% and day-to-day total CV was 3.4–15% at sites that performed full precision studies (Table 1). CVs ≥6.0% were observed only for samples with cTnI values <0.7 μg/L. The imprecision profile at low cTnI concentrations showed a CV of 10% at 0.14 μg/L and 20% at 0.08 μg/L (Fig. 1A). The minimum detection limit (mean plus 2 SD of zero calibrator) was 0.02 μg/L.

Regression analysis of observed and expected values from dilutions of a patient sample (site B) containing 32.10 μg/L cTnI yielded: observed = [0.99(SD, ±0.01) × expected] + 0.29(±0.22); S_y|x = 0.31 μg/L; r = 0.99. Comparable results were obtained at sites A, C, and E, using purified water, saline, and the zero calibrator as the diluent. A comparison of observed vs expected values for dilutions of a plasma sample containing 1.0 μg/L showed that the lower limit of linearity was at least 0.1 μg/L (data not shown). Together, the linearity studies and precision profile produce a linear range for quantification of 0.1–30.0 μg/L. Linear regression analysis of values obtained for the five calibrators, run as unknowns over a period of 9 weeks at site A, showed no significant slope change (P >0.05) at any concentration.

Comparison of values from 135 paired serum and plasma samples showed excellent correlation without significant bias: plasma = [1.00(±0.01) × serum] + 0.03(±0.04); S_y|x = 0.43 μg/L, r = 0.99; Fig. 2A. Less than 10% interference in observed values was observed in

| Table 1. Precision data for the revised Dimension cTnI assay from three different sites. |
|-------------------|------------------|-------------|-----------------|-----------------|-------------------|-----------------|-----------------|-----------------|
|                   | Serum pool 1| Serum pool 2| Serum pool 3| Serum pool 4| OC 1\*\*| OC 1\*\*| OC 2| OC 3 | 
| Site A             | Mean, μg/L | 0.48        | 1.50        | 17.63          | 40.05          | 0.35            | 5.28            | 14.52           |
|                    | Within-run CV, % | 2.6          | 2.3          | 1.4            | 1.9            | 2.7             | 1.0             | 1.0             |
|                    | Total CV, %   | 7.7          | 5.8          | 3.9            | 4.5            | 7.7             | 4.2             | 4.9             |
| Site B             | Mean, μg/L | 0.11        | 0.30        | 1.30           | 17.25          | 0.68            | 12.74           | 19.47           |
|                    | Within-run CV, % | 8.4          | 3.1          | 2.3            | 1.9            | 2.7             | 2.4             | 1.9             |
|                    | Total CV, %   | 11           | 5.6          | 4.0            | 3.4            | 9.8             | 5.8             | 5.8             |
| Site E             | Mean, μg/L | 0.08        | 0.47        | 1.44           | 27.71          | 0.32            | ND              | 17.26           |
|                    | Within-run CV, % | 7.3          | 2.9          | 2.6            | 1.9            | 3.7             | ND              | 2.2             |
|                    | Total CV, %   | 15           | 6.2          | 5.2            | 3.6            | 7.1             | ND              | 5.8             |

\*Different serum pools were used at each site.
\*\*Sites A and E used lot A of quality-control material, whereas site B used lot B of quality-control material.
\* QC, quality control; ND, not done.
samples containing high concentrations of bilirubin (600 mg/L), hemoglobin (10 g/L), or triglycerides (30 g/L). The upper reference values for the revised Dimension cTnI assay were <0.05 μg/L (97.5 percentile) and <0.07 μg/L (99 percentile).

Values from 234 samples measured by the original Dimension cTnI assay showed good correlation with values by the Stratus CS assay across the entire analyte range (r = 0.98; S_{\text{bias}} = 1.29 μg/L), including a subset of 218 samples with values <10 μg/L (r = 0.96; S_{\text{bias}} = 0.91 μg/L), but exhibited a proportional bias for all samples (slope, 1.30) and for samples with values <10 μg/L (slope, 1.35) relative to the Stratus CS assay.

Values for 618 samples from the revised Dimension cTnI assay showed excellent agreement with values from the Stratus CS assay (slope, 1.00; r = 0.99; S_{\text{bias}} = 0.84 μg/L), as well as for a subset of 500 samples with values <5 μg/L (slope, 1.06; r = 0.98; S_{\text{bias}} = 0.25 μg/L; Fig. 2B). There was consistency in the individual regression analyses at the five testing sites, with slopes ranging from 0.96 to 1.05, intercepts ranging from 0.02 to 0.24, and all r values ≥0.99. No statistical outliers were observed at any site.

Of the 14 309 patients with cTnI testing ordered, 1596 patients had at least one cTnI ≥1.4 μg/L by the original Dimension assay, and of these, 25 produced a cTnI value ≤0.1 μg/L on the Stratus CS assay. Assuming that these 25 discrepant values were attributable to an interfering antibody gives an incidence of 0.17% of all patients with samples submitted for cTnI analysis and 1.6% of patients with a cTnI value ≥1.4 μg/L. These 25 patients (13 males and 12 females) ranged in age from 30 to 84 years (mean, 60.9 years). Thirteen patients had documented histories of infectious disease, 13 had endocrine disease, 13 had recent surgery, 9 had cardiac disease, 8 had renal disease, 7 had pulmonary disease, 7 had gastrointestinal disease, 6 had cancer, and 2 had connective tissue disease.

The range of cTnI values on the original Dimension cTnI assay for these 25 patients was 1.6–6.0 μg/L (mean, 2.8 μg/L). Seventeen of the 25 (68%) discrepant patient samples produced a cTnI value <0.1 μg/L on the revised Dimension cTnI assay. However, eight of the discrepant samples still produced detectable cTnI concentrations (0.1–0.4 μg/L) on the revised method. Three of these patients had a history of congestive heart failure, past myocardial infarction, or coronary bypass surgery, whereas the others had no documented history or risk factors for cardiac disease. A sample from the patient previously described by Covinsky et al. (8) as producing a falsely increased cTnI value of 225 μg/L attributable to a monoclonal HAMA also gave a result of <0.1 μg/L by the revised Dimension cTnI method.

Sufficient sample was available to perform adsorption studies on 10 of the 25 discrepant patient samples (Table 2). Three other patient samples (samples 26–28), identified by clinicians as possibly discrepant before August 14, 2000, were also studied but not used to calculate incidence. Complete to partial correction of the discrepant values by the Dimension cTnI assay was accomplished by adsorption with either HBR or murine IgG1/IgG2a- Sepharose in all 13 samples, confirming the presence of an interfering antibody with HAMA activity. The four samples that failed to fully correct with either HBR or murine IgG1/IgG2a-Sepharose (samples 4, 7, 11, and 28) may contain interfering antibodies with specificity to other assay components other than mouse immunoglobulin. Incubation with protein A-Sepharose markedly reduced or corrected the interference in 6 of 12 patient samples (samples 1, 2, 3, 5, 26, and 27), suggesting that the HAs were primarily IgG. The remaining six samples (samples 4, 7, 9, 11, 13, and 28) were minimally affected or unaffected by protein A-Sepharose.
Table 2. Characterization of the interfering antibodies in discrepant samples measured for cTnI by the original and revised Dimension cTnI assay vs the Stratus CS assay.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Patient</th>
<th>Original Dimension</th>
<th>Stratus CS</th>
<th>Revised Dimension</th>
<th>HBR</th>
<th>IgG1/IgG2a-Sepharose</th>
<th>Protein A-Sepharose</th>
<th>Unconjugated Sepharose</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>3.60</td>
<td>&lt;0.10</td>
<td>0.29</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>0.45</td>
<td>2.20</td>
</tr>
<tr>
<td>2</td>
<td>4.17</td>
<td>0.10</td>
<td>0.30</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>4.20</td>
</tr>
<tr>
<td>3</td>
<td>2.00</td>
<td>&lt;0.10</td>
<td>0.37</td>
<td>0.20</td>
<td>0.24</td>
<td>0.75</td>
<td>1.40</td>
</tr>
<tr>
<td>4</td>
<td>2.80</td>
<td>&lt;0.10</td>
<td>0.12</td>
<td>0.88</td>
<td>0.10</td>
<td>1.89</td>
<td>1.70</td>
</tr>
<tr>
<td>5</td>
<td>3.62</td>
<td>&lt;0.10</td>
<td>0.11</td>
<td>&lt;0.10</td>
<td>1.99</td>
<td>0.17</td>
<td>3.90</td>
</tr>
<tr>
<td>7</td>
<td>3.30</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>1.20</td>
<td>1.20</td>
</tr>
<tr>
<td>9</td>
<td>3.00</td>
<td>&lt;0.10</td>
<td>0.21</td>
<td>0.90</td>
<td>0.60</td>
<td>2.56</td>
<td>2.20</td>
</tr>
<tr>
<td>11</td>
<td>2.70</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>0.71</td>
<td>1.94</td>
<td>2.40</td>
</tr>
<tr>
<td>13</td>
<td>3.20</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>0.45</td>
<td>0.95</td>
<td>1.23</td>
</tr>
<tr>
<td>26\textsuperscript{a}</td>
<td>2.40</td>
<td>QNS\textsuperscript{c}</td>
<td>QNS</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>0.71</td>
<td>QNS</td>
</tr>
<tr>
<td>27\textsuperscript{a}</td>
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<td>QNS</td>
<td>QNS</td>
<td>0.15</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>QNS</td>
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<tr>
<td>28\textsuperscript{a}</td>
<td>3.70</td>
<td>QNS</td>
<td>QNS</td>
<td>1.24</td>
<td>&lt;0.10</td>
<td>1.45</td>
<td>QNS</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Values shown are in µg/L. Values obtained in the adsorption studies for the indicated substances were measured on the original Dimension cTnI assay.\textsuperscript{b} Samples identified before August 14, 2000.\textsuperscript{c} QNS, quantity not sufficient.

Discussion

The revised Dimension cTnI method was evaluated at five sites to assess performance with respect to recent guidelines from the NACB, ESC, ACC, and American Heart Association (1–3). NACB recommends the use of two decision limits for cTnI, a low limit to identify patients with small amounts of myocardial injury (97.5 percentile cTnI value) and a higher limit consistent with injury to the extent that it meets WHO criteria for diagnosis of acute myocardial infarction (AMI). The ESC and ACC recommend that the cutoff limit should be set at the 99 percentile of results from a population of healthy individuals. These guidelines place the cutoff value for the revised Dimension cTnI assay at 0.1 µg/L. This takes into account the precision profiles at 0.07 µg/L (CV ~17%) and 0.14 µg/L (CV = 10%). At site A, values are rounded to the nearest tenth for reporting so that a cutoff of 0.1 µg/L is used. Analytical sensitivity, or the lowest detectable concentration, for the revised method is 0.02 µg/L, well below the lower cutoff. The AMI cutoff range determined by ROC analysis remained at 0.6–1.5 µg/L, as determined with the original Dade Stratus cTnI assay. The ESC and ACC recommend a CV <10% at the 99 percentile and AMI cutoff. Total precision for the revised method approaches this goal, with CVs of 11–17% at 0.1 µg/L, and exceeds the goal, with CVs of 4–8%, at the ROC-determined cutoff for AMI.

The revised method shows excellent linearity across the analytical range (0.1–30.0 µg/L), calibration stability that is adequate for routine clinical use, and equivalent cTnI values for either heparinized plasma or serum samples, a finding that contrasts with other troponin methods (27–29). Finally, results obtained by the revised Dimension cTnI assay show good agreement with values from the Stratus CS assay, in contrast to the original Dimension method, which produced higher values than the Stratus method at the time of this evaluation.

We determined that the incidence of falsely increased cTnI results attributable to HAs that were consistent with the ROC cutoff for AMI on the original Dimension cTnI assay was 0.17% of all patients with cTnI samples submitted and 1.6% of all cTnI samples with values >1.4 µg/L. This incidence corresponds to lower estimates of interfering HAs described in the literature. However, the incidence might be higher if samples between 0.4 µg/L (our lowest reported value on the original Dimension method) and 1.3 µg/L were also examined in light of the new ESC/ACC and NACB criteria for myocardial damage (1–3).

Samples available for further study produced either undetectable or greatly decreased cTnI values after adsorption with HBR or IgG1/IgG2a-Sepharose, suggesting the presence of interfering HAs with HAMA activity, leading to falsely increased cTnI values. Seven samples still produced a significant, albeit reduced, cTnI value after adsorption with HBR, suggesting that some of the heterophilic activity may have been directed against epitopes on other assay components, such as the enzyme-antibody conjugate. Eight of these samples still produced values between 0.01 and 0.4 µg/L in the revised method, suggesting that the modifications may not fully eliminate the interferences. Alternatively, samples may contain HAs and also contain low amounts of cTnI. However, we did not identify any samples with original Dimension values >1.4 µg/L and Stratus CS values >0.1–1.0 µg/L, which would have suggested the presence of both HA interference and low cTnI concentrations. Taken together, our findings demonstrate that the revised Dimension method is very effective in minimizing false-positive results attributable to HAs or HAMAs.
Six of 12 samples showed correction after adsorption with protein A, suggesting that ~50% of these HAs were IgG and the remaining likely contained interfering IgM antibodies, although IgG3 and IgA, which do not bind protein A, cannot be ruled out. This is consistent with other studies documenting both IgG and IgM HAMAs (8, 9, 30). Interestingly, four of the samples containing IgM interfering antibodies showed the least correction with HBR or IgG1/IgG2a-Sepharose (samples 4, 7, 11, and 28). It is possible that these IgM antibodies were induced by recent infection by microorganisms and that they expressed broadly reactive specificity to nonimmunoglobulin components.

In conclusion, although the incidence of interfering HAs is low, clinically discrepant results in immunoassays continue to occur. Because of this, it is important that manufacturers continue to address this issue. One example is the revised Dimension cTnI assay, which reduces the effects of interfering HAs that were recently described for the original version of this method. The revised method also showed good analytical performance and is consistent with recent guidelines put forth by the NACB, ESC, and ACC.

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