Evaluation of a No-Pretreatment Cyclosporin A Assay on the Dade Behring Dimension RxL Clinical Chemistry Analyzer

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Background: Monitoring whole-blood concentrations of cyclosporin A (CsA) is common practice in the management of solid organ and bone marrow transplant recipients. In a multicenter study we evaluated a new, direct (no pretreatment) CsA assay on the Dade Behring Dimension RxLTM system and compared results with those from the Abbott TDx CsA immunoassay and a HPLC method.

Methods: Whole-blood samples from heart (n = 111; 35 patients), liver (n = 201; 44 patients), kidney (n = 279; 65 patients), and miscellaneous organ (n = 77; 12 lung, 12 bone marrow, 5 kidney/pancreas, and 1 pancreas patient) recipients were obtained from patient populations of the participating institutions. Routine clinical monitoring of CsA was performed using either the TDx method or HPLC.

Results: The minimum detectable concentration of CsA averaged 9.4 g/L, and the lower limit of quantification was 30 g/L. The method was linear from 30 to 500 g/L. Cross-reactivity with seven different CsA metabolites ranged from 0.0% to 5.7% for the Dimension RxL assay compared with 0.4–15.9% for the TDx assay. Total imprecision (CV) averaged 6.2%, and within-run imprecision averaged 4.9%. Passing–Bablok linear regression analyses of all samples from two sites yielded the following: RxL = 0.81 × TDx – 16.8; and RxL = 1.12 × HPLC – 1.7.

Conclusions: The Dade Behring CsA assay for the random-access Dimension platform offers adequate performance characteristics for routine clinical use, does not require a manual pretreatment step, and demonstrates less cross-reactivity with CsA metabolites than another commonly used immunoassay.

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Cyclosporin A (CsA), a cyclic polypeptide of fungal origin, is an immunosuppressant commonly used in the management of solid organ and bone marrow transplant recipients. CsA specifically inhibits T-lymphocyte activation by interfering with the expression of the gene that encodes for the cytokine interleukin-2 via a signal transduction pathway that affects nuclear translocation and binding of the transcription factor nuclear factor-AT to the interleukin-2 promoter (1–4). CsA has the potential to cause substantial nephrotoxicity, hypertension, neurotoxicity, and seizures (5–7). It is administered intravenously or orally, and its absorption, distribution, and clearance are highly variable (8). In addition, CsA is subject to interactions with several drugs that may increase or decrease its bioavailability and metabolism (9, 10). The bioavailability of CsA may also change during the course of treatment (11, 12), presumably because of changes in gastrointestinal function and absorption of the drug. Elimination of the drug may also be saturable, leading to increased blood concentrations during the course of treatment (11). Taken together, the above facts indicate that there is clear consensus for the need to monitor CsA concentrations, with dosage adjustments guided by whole-blood CsA concentrations to minimize treatment failure and toxicity (13–15).

At room temperature and at therapeutic concentrations, CsA is partitioned between erythrocytes (40–50%),
leukocytes (10–20%), and plasma (30–40%) (16), where it is primarily bound by lipoproteins (16, 17). Because partitioning of the drug between these compartments is dependent on temperature (18, 19), hematocrit (20), and metabolite concentration (21), whole blood collected into EDTA-containing tubes is the recommended sample (22–25). The most common methods for routine CsA monitoring are HPLC and monoclonal antibody-based immunoassays, with HPLC currently considered as the gold standard (26). HPLC provides the most specific routine measurement of parent drug (27), whereas immunoassays using monoclonal antibodies have variable cross-reactivities with CsA metabolites (28–30). Recently, liquid chromatography–tandem mass spectrometry methods have been developed that will likely offer even more specificity (31). Nevertheless, all current methods require a labor-intensive pretreatment step to lyse erythrocytes before assay performance. Here we examine the performance of a new, no-pretreatment monoclonal antibody-based immunoassay for measurement of CsA in whole blood on the Dade Behring Dimension RxL™ random-access clinical chemistry system and compare results with those from another monoclonal antibody-based immunoassay and from a HPLC method.

Materials and Methods

CLINICAL SAMPLES
Three sites performed evaluations of the Dimension CsA assay: the Hospital of the University of Pennsylvania (site 1); The University of Wisconsin Hospital (site 2); and Washington University School of Medicine (site 3). Samples for the method comparison studies were obtained from patients receiving heart (35 patients), liver (44 patients), kidney (65 patients), pancreas (1 patient), kidney/pancreas (5 patients), bone marrow (12 patients), or lung transplants (12 patients) at sites 2 and 3. Excess, residual whole-blood samples from specimens submitted for routine CsA measurement were used. All sites analyzed samples on the Dade Behring Dimension RxL and the Abbott TDx. Samples from site 3 were sent to site 2 for HPLC analysis. Samples were analyzed within 24–48 h of being drawn or were stored at −20 °C until analysis and were subjected to no more than one freeze-thaw cycle. The Human Studies Committees of all three institutions approved this study.

DADE BEHRING DIMENSION ASSAY
The procedure for the Dade Behring Dimension assay is as follows: Whole blood (200 μL) from a potassium-EDTA collection tube, mixed either manually or on an inverter or rocker, is first added to a sample cup by the operator. The Dimension uses a sonication probe to mix the sample, lyses the blood cells by use of a saponin-based buffer and sonication, and then adds an anti-CsA antibody/β-galactosidase conjugate that binds CsA in the sample. Mixing and lysing should occur within 30 min of the sample being placed in the instrument. After CsA-coated magnetic beads are added to the reaction mixture, unbound conjugate is removed magnetically. The supernatant containing the CsA-antibody-enzyme complex is then transferred to a measuring cuvette, where it is mixed with chlorophenol red galactoside, which is hydrolyzed by the β-galactosidase to chlorophenol red. The change in absorbance at 577 nm (blanked at 700 nm) directly correlates to the amount of CsA in the sample. If CsA concentrations are expected to exceed 500 μg/L as might occur with a 2-h sample (C2) (32), a manual dilution protocol is performed using the Dimension CsA Level 1 Calibrator (0.0 μg/L).

ABBOTT TDx ASSAY
The TDx assay is a fluorescence polarization immunoassay performed according to manufacturer’s instructions, including the manual pretreatment step to lyse whole blood cells.

HPLC ASSAY
The HPLC assay was developed at site 2 for clinical use. The assay is performed as follows: One milliliter of whole blood is combined with 3.0 mL of precipitating reagent (water–acetonitrile–350 g/L zinc sulfate, 500:50:50 by volume) containing 300 μg/L cyclosporin G as an internal standard. The sample is incubated at room temperature for 10 min, and then centrifuged for 10 min at 3000g. The supernatant is extracted using a Gilson XL4 automated extraction instrument, and then 2.5 mL is applied to a 1.0-mL Empore C-8 cartridge that has been preconditioned with 0.5 mL of acetonitrile followed by 1.0 mL of acetonitrile–water (15:85 by volume). The cartridge is washed with 2.0 mL of acetonitrile–0.5 mol/L acetic acid (35:65 by volume), then with 0.5 mL of water. The drugs are eluted with 0.3 mL of acetonitrile followed by 0.25 mL of water. The combined eluate is placed in a sample vial, and 0.5 mL of isooctane is added. The vial is capped and placed on a rocking mixer for 10 min. The upper isooctane layer is aspirated and discarded; 50 μL of sample (lower layer) is injected onto a Zorbax SB-CN (cyanopropyl) HPLC column (4.6 × 75 mm; 3.5-μm particle size) maintained at 75 °C. The mobile phase is acetonitrile–water–acetic acid–triethylamine (620:280:0.2:0.1 by volume), and the flow rate is 0.3 mL/min. The drugs are detected at 214 nm. Relative retention times and relative peak areas are used for identification and quantification, respectively.

QUALITY-CONTROL MATERIALS
Six quality-control materials were used: (a) three levels of Elite QC, a nonmarketed product from Dade Behring prepared using preserved whole-blood hemolysate to which USP-grade CsA is added; and (b) three potassium-EDTA whole-blood pools containing 100, 200, and 300 μg/L CsA. The CsA metabolites AM9, AM19, AM1, AM1c, AM4N, AM1e9, and AM4n9 were purchased from Isotechnika. Each metabolite was purified by HPLC and quantified by HPLC using an internal standard.
PRECISION
Total and within-run imprecision were determined at each site by assaying the quality-control materials according to NCCLS protocol EP5-A. Total imprecision was calculated by measuring the materials in duplicate on 20 nonconsecutive days. Within-run imprecision was calculated by measuring each of the six control materials 20 times in a single analytical run.

LINEAR RANGE AND DETECTION LIMIT
The linear quantification range of the Dimension CsA assay was determined by measuring CsA in a series of parallel dilutions (n = 4 at each concentration), prepared using a 4000 μg/L stock solution of CsA diluted in CsA-free whole blood, and comparing observed vs expected values. The limit of detection for the assay was determined by measuring the Level 1 Calibrator (0 μg/L) 20 times. This was repeated three times at 2-week intervals at each of the three institutions. The limit of detection was defined as the mean plus 2 SD.

RECOVERIES AND CROSS-REACTIVITY WITH CsA METABOLITES
Whole-blood hemolysates were prepared to assess recoveries and cross-reactivity with CsA metabolites. USP-grade CsA was added to the hemolysate at concentrations of 100, 200, 300, or 400 μg/L. The seven CsA metabolites were added separately at concentrations of 1000 μg/L to a hemolysate sample containing 200 μg/L CsA to measure cross-reactivity. The parent drug was also added to these samples to ensure that the measurements would fall within the linear range of the assay and to simulate clinical samples. Aliquots of these samples were analyzed in duplicate (Dimension and TDx) or once per day (HPLC) on 4 different days. Cross-reactivity with CsA metabolites was calculated as follows:

\[
\text{Cross-reactivity (\%)} = \frac{\text{test sample} - \text{control sample (200 μg/L CsA added)}}{1000 \text{ μg/L metabolite}} \times 100
\]

Mean results for the samples containing the metabolites from the TDx, Dimension, and HPLC assays were compared by a two-tailed t-test.

METHOD COMPARISON
At sites 2 and 3, TDx values were determined for therapeudic use, and excess sample was then assayed in duplicate on the Dimension RxL and then by HPLC. Samples from site 3 were frozen and sent to site 2 for HPLC analysis. Passing–Bablok linear regression analysis was performed on data for all categories of samples separately and on all samples combined.

ROLE OF THE SPONSOR
Dade Behring participated in the study design, data collection and analysis, and reporting of the results.

IMPRECISION
Total and within-run imprecision were calculated for the six different quality-control materials (Table 1). Total imprecision (CV) averaged 6.1%, and within-run imprecision averaged 4.9%. The highest average CV calculated for within-run imprecision was 8.1% at 100 μg/L, and the highest CV calculated for total imprecision was 11%, which was also at a concentration of 100 μg/L.

LINEARITY AND DETECTION LIMIT
In the series of parallel dilutions, the observed values differed from the expected values by <10% over a range of expected values from 30 μg/L (observed, 31.6 μg/L) to 500 μg/L (observed, 507.6 μg/L). Linearity was not evaluated at concentrations >500 μg/L, which is the concentration of the highest calibrator. At concentrations <30 μg/L, the observed and expected values differed by >10% (expected, 20 μg/L; observed, 26.2 μg/L). The limit of detection for the Dimension CsA assay averaged 9.4 μg/L (range, 5.3–16.9 μg/L at the three sites).

RECOVERIES AND CROSS-REACTIVITY WITH CsA METABOLITES
The recoveries obtained with the Dimension CsA assay were compared with those obtained with the TDx and HPLC by measuring CsA in samples containing 100, 200, 300, and 400 μg/L. USP-grade CsA by all three methods (Table 2). There were no statistically significant differences in recovery between the RxL and HPLC at sites 2 and 3, with the exception of the 400 μg/L sample, which showed a statistically significant difference (P <0.001) between RXL and HPLC at site 2. There were statistically significant differences for all concentrations of control materials between the TDx and HPLC methods at sites 2 and 3 (P ≤0.003). There were no clinically significant differences between parent drug alone and parent drug plus metabolite, as measured by HPLC (Table 2). Cross-

### Table 1. Total and within-run imprecision of CsA measurements in whole blood using the Dimension RxL.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean, μg/L</th>
<th>CV, %</th>
<th>Mean, μg/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC L1</td>
<td>96.3</td>
<td>5.3</td>
<td>104.3</td>
<td>8.8</td>
</tr>
<tr>
<td>QC L2</td>
<td>199.7</td>
<td>3.9</td>
<td>220.4</td>
<td>6.0</td>
</tr>
<tr>
<td>QC L3</td>
<td>283.7</td>
<td>3.8</td>
<td>311.6</td>
<td>5.8</td>
</tr>
<tr>
<td>WBP L1</td>
<td>128.8</td>
<td>6.6</td>
<td>134.1</td>
<td>5.8</td>
</tr>
<tr>
<td>WBP L2</td>
<td>221.1</td>
<td>4.6</td>
<td>222.6</td>
<td>5.9</td>
</tr>
<tr>
<td>WBP L3</td>
<td>342.5</td>
<td>4.8</td>
<td>360.2</td>
<td>4.8</td>
</tr>
</tbody>
</table>

*Total imprecision determined using 40 replicates for each quality-control material (three Elite QC and three whole-blood pools). Within-run imprecision determined using 20 replicates for each quality-control material.

The CVs are the mean of values obtained at all three sites (range of CVs from all three sites in parentheses).

QC L1-L3, quality-control material levels 1, 2, and 3.

WBP L1-L3, whole-blood pools 1, 2, and 3.
reactivity with all metabolites except AM4N was lower on the RxL than on the TDx. The average cross-reactivity on the RxL ranged from 0.18% (AM1c9) to 5.2% (AM4N), whereas on the TDx, the average cross-reactivity ranged from 0.41% (AM1c9) to 15.3% (AM9). On the RxL, significant cross-reactivities occurred consistently (at two or three of the three evaluation sites) with metabolites AM9, AM1, and AM4N, whereas significant, consistent cross-reactivities occurred with metabolites AM9, AM19, AM1, AM1c, AM4N, and AM4n9 on the TDx.

**METHOD COMPARISON**

Shown in Fig. 1 are the combined data from sites 2 and 3, comparing CsA values from the Dimension RxL and HPLC (Fig. 1A) or TDx (Fig. 1B). Passing–Bablok linear regression analyses were performed on all individual data sets and are shown in Table 3, whereas regression data for all samples combined are provided in the legend for Fig. 1. The TDx method consistently produced higher CsA values than the Dimension method. The RxL does overestimate CsA concentrations, compared with HPLC, but not to the extent demonstrated by the TDx method.

**Discussion**

Monitoring of CsA concentrations is routine practice in the management of transplant patients. HPLC is considered the gold standard, but it is labor-intensive and time-consuming. Thus, >95% of clinical laboratories in the US use immunoassays (33). Most of these immunoassays are performed on batch analyzers, rather than random-access analyzers, and all require a time-consuming manual pretreatment step. Turnaround time can be important when assaying immunosuppressants, and results

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**Table 2. Recoveries and cross-reactivities with CsA metabolites.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Site 1</th>
<th>Site 2</th>
<th>Site 3</th>
<th>Site 1</th>
<th>Site 2</th>
<th>Site 3</th>
<th>Site 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 μg/L</td>
<td>96.7</td>
<td>98.1</td>
<td>86.7</td>
<td>111.0</td>
<td>121.8</td>
<td>123.3</td>
<td>97.5</td>
</tr>
<tr>
<td>200 μg/L</td>
<td>189.7</td>
<td>202.7</td>
<td>219.9</td>
<td>212.9</td>
<td>232.1</td>
<td>241.4</td>
<td>192.5</td>
</tr>
<tr>
<td>300 μg/L</td>
<td>295.4</td>
<td>313.0</td>
<td>271.8</td>
<td>314.2</td>
<td>344.0</td>
<td>343.9</td>
<td>287.8</td>
</tr>
<tr>
<td>400 μg/L</td>
<td>408.5</td>
<td>457.5</td>
<td>387.9</td>
<td>413.6</td>
<td>454.0</td>
<td>457.3</td>
<td>373.0</td>
</tr>
</tbody>
</table>

AM9: 201.9 (1.2) 218.3 (1.1) 187.4 (1.1)
AM19: 194.8 (0.5) 218.2 (1.8) 183.4 (0.6)
AM1: 203.6 (1.4) 209.7 (1.0) 177.8 (0.1)
AM1c: 199.6 (1.0) 210.7 (1.1) 180.2 (0.4)
AM4N: 246.8 (5.7) 256.9 (5.7) 218.8 (4.2)
AM1c9: 196.6 (0.7) 201.9 (0.2) 173.3 (0.0)
AM4n9: 209.2 (1.9) 200.0 (0.0) 182.9 (0.6)

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* Means are from duplicate determinations on 4 different days (n = 8). Cross-reactivity calculated as described in Materials and Methods.

**Fig. 1.** Bland–Altman difference plots of whole-blood CsA measurements obtained by the Dimension RxL and HPLC (A) or TDx (B) at sites 2 and 3.

(A), Dimension vs HPLC linear regression results (n = 661): slope (95% confidence interval), 1.12 (1.10–1.14); intercept (95% confidence interval), –1.7 (–4.4 to 0.98) μg/L; Pearson correlation coefficient, 0.94; S_yx = 32.3 μg/L. (B), Dimension vs TDx linear regression results (n = 667): slope (95% confidence interval), 0.81 (0.80–0.82); intercept (95% confidence interval), –18.8 (–19.1 to –14.5) μg/L; Pearson correlation coefficient, 0.96; S_yx = 26.4 μg/L.
should be generated within a dosing interval so that changes in the next dose can be made if necessary (24). An assay for CsA that is available on a random-access analyzer that also eliminates the requirement for a manual pretreatment step could substantially decrease turnaround time.

The average detection limit for the CsA assay on the Dimension platform was 9.4 μg/L, and the linear quantification range was 30–500 μg/L, which is acceptable for routine clinical use because therapeutic ranges are generally 75–400 μg/L (25). The total imprecision of the new CsA immunoassay was 4.8–8.8% at concentrations of 100–360 μg/L, which is within the recommendation of 5% at a CsA concentration of 300 μg/L (24, 25). The Dimension CsA assay slightly exceeded these recommendations on one of the high-concentration quality-control materials, for which the average total imprecision was 5.8%. In comparing the Dimension assay with the TDx and to HPLC, we found that the precision of the Dimension CsA assay was comparable to that of the TDx, which is the routine assay at sites 1 and 3. At site 3, the CVs for the TDx assay ranged from 4.7% to 8.3% during the same time period as the CsA method evaluation. The imprecision of the Dimension assay was comparable to the imprecision of the HPLC assay at site 2, which had CVs ranging from 2.3% to 11% during the same time period, and is consistent with values in the literature (26). No recommendations have been issued regarding within-run imprecision, but the CsA assay evaluated here had an average within-run imprecision of 3.8–6.6%, which is similar to both published HPLC data [reported ranges, 4–7% (26, 28)] and to published TDx data [reported ranges, 2–6% (30)]. In addition, we found that the recoveries obtained with the CsA assay on the Dimension platform were superior to those obtained with the TDx assay, using HPLC as the comparison method.

Consensus documents recommend the following performance characteristics for CsA assays when compared with HPLC: a slope of 0.9–1.1; an intercept of ±15 μg/L or less; and an S_yx ≤15 μg/L (24, 25). Overall, the Dimension CsA assay performed within these recommendations with the exception of the S_yx. The CsA immunoassay for the Dimension chemistry analyzer showed a consistent positive bias when compared with HPLC, but less than that shown by the TDx method. These results are presumably attributable to variable cross-reactivities with CsA metabolites because the Dimension assay showed less cross-reactivity with all of the CsA metabolites tested except AM4N.

It has been shown that monoclonal antibody-based CsA immunoassays can demonstrate variable, and sometimes significant, cross-reactivity with various CsA metabolites. CsA is eliminated by the liver via cytochrome P450-mediated metabolism (34), and management of CsA therapy in liver recipients is therefore especially complex because liver dysfunction can alter its elimination and absorption. CsA concentrations may remain increased in blood until good bile flow is restored (35). Liver recipients frequently have cholestasis and are more likely to have high blood concentrations of CsA metabolites. For example, one study (36) showed the following average concentrations of metabolites in liver recipients at a parent drug

| Table 3. Comparison of Dimension, TDx, and HPLC for CsA measurement. |
|-----------------|-----------------|-----------------|--------|--------|
|                  | n | Slope | Intercept |  \( r \) |  \( S_yx \) |
| Heart            |   |       |           |        |        |
| Dimension vs HPLC | 111 | 1.10 (1.04–1.15) | 18.4 (7.6 to 29.2) | 0.89  | 40.5   |
| Dimension vs TDx | 111 | 0.76 (0.73–0.79) | −1.3 (−10.8 to 8.1) | 0.93  | 33.7   |
| TDx vs HPLC      | 111 | 1.32 (1.25–1.39) | 49.2 (35.4 to 63.0) | 0.88  | 51.7   |
| Kidney           |   |       |           |        |        |
| Dimension vs HPLC | 273 | 1.03 (1.0–1.1) | 5.5 (1.6 to 9.4) | 0.92  | 31.2   |
| Dimension vs TDx | 279 | 0.82 (0.81–0.84) | −16.9 (−19.7 to −14.1) | 0.97  | 20.8   |
| TDx vs HPLC      | 270 | 1.22 (1.19–1.25) | 30.5 (26.3 to 34.7) | 0.94  | 32.5   |
| Liver            |   |       |           |        |        |
| Dimension vs HPLC | 201 | 1.18 (1.16–1.21) | −10.0 (−13.5 to −6.4) | 0.96  | 24.7   |
| Dimension vs TDx | 201 | 0.81 (0.79–0.83) | −24.8 (−29.0 to −20.6) | 0.95  | 27.1   |
| TDx vs HPLC      | 201 | 1.33 (1.29–1.37) | 34.2 (28.4 to 40.0) | 0.92  | 40.4   |
| Miscellaneous    |   |       |           |        |        |
| Dimension vs HPLC | 77  | 1.07 (1.02–1.11) | 0.4 (−7.5 to 8.4) | 0.94  | 30.3   |
| Dimension vs TDx | 77  | 0.79 (0.76–0.82) | −11.1 (−18.5 to −3.8) | 0.95  | 26.9   |
| TDx vs HPLC      | 77  | 1.26 (1.20–1.32) | 28.0 (17.4 to 38.6) | 0.92  | 40.4   |

* Number of samples.
\(^{a}\) 95% confidence intervals for slope and intercept derived from Passing-Bablock regression.
\(^{c}\) Pearson correlation coefficient.
\(^{d}\) SD of the residuals.
\(^{e}\) Five samples had insufficient volumes for HPLC, and four were below linearity in one or more methods.
\(^{f}\) Includes samples from 12 lung, 12 bone marrow, 5 kidney/pancreas, and 1 pancreas transplant patient.
concentration of 187 ± 20 µg/L: AMn9, 29 ± 8 µg/L; AM19, 265 ± 32 µg/L; and AM1c, 14 ± 5 µg/L. Thus, there is a benefit to using assays that demonstrate the lowest cross-reactivity because the role of metabolites in immunosuppression continues to be debated (25). In fact, current consensus is that monitoring of metabolite concentrations is not warranted and that analytical methods should be specific for the parent drug (23–26). The Dimension and TDx methods use different monoclonal antibodies, which is the likely reason for the differences in observed cross-reactivities and the decreased bias compared with HPLC observed for Dimension CsA values.

Cross-reactivities and the decreased bias compared with HPLC observed for Dimension CsA values from patient samples. It should be noted that cross-reactivity was measured only at metabolite concentrations is not warranted and that analytical methods should be specific for the parent drug. Currently in use, and it appears to exhibit less cross-reactivity because the role of metabolites in pharmacokinetics. Immunol Today 1992;13:136–138.

In conclusion, the new, no-pretreatment CsA assay on the Dade Behring Dimension random-access clinical chemistry analyzer demonstrates adequate performance characteristics for routine clinical use and could allow more timely delivery of results. Values obtained with the Dimension assay compare well with other immunoassays currently in use, and it appears to exhibit less cross-reactivity for the metabolites examined here than most commonly used immunoassays.

This work was supported by Dade Behring, Inc. (Newark, DE). We thank Rhonda Sneeringer for technical assistance.

References
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