the subjects in this study gave a history of the consumption of traditional, iron-laden beer, and two-fifths of them had increased serum ferritin.

In contrast to our studies, Wapnick et al. (7) reported that serum iron concentrations increased dramatically after oral administration of vitamin C to iron-loaded, scurvy subjects. The explanations for this difference probably lie in the timing of the vitamin C dose in relation to the serum iron measurement and the fact that the majority of the subjects in the present study did not have iron overload or ascorbic acid deficiency of the severity described by Wapnick et al. In addition, the vitamin C administered in this study was not sufficient to return leukocyte ascorbic acid to reference values in all of the iron-loaded subjects.

This study had limitations in that it was not possible to assess intracellular iron metabolism directly and to correlate these findings with serum concentrations of transferrin receptor, ferritin, and iron. In addition, we did not have a control group that did not receive ascorbic acid. Nevertheless, our results indicate that ascorbic acid status may influence serum transferrin receptor concentrations, probably through changes in intracellular iron metabolism.

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Reproducibility of the Roche Amplicor Polymerase Chain Reaction Assay for Detection of Infection by Chlamydia trachomatis in Endocervical Specimens, Gabriel M. Mulcahy, Ernest A. Albanese, and Bonita L. Bachl (UMDNJ-New Jersey Medical School, Newark, NJ 07103; * author for correspondence: Department of Pathology and Laboratory Medicine, UMDNJ-New Jersey Medical School, 185 South Orange Avenue, Newark, NJ 07103; fax 973-972-3199, e-mail mulcahy@umdnj.edu)

Several investigators have reported that the Roche Amplicor™ PCR assay for detection of infection by Chlamydia trachomatis in endocervical specimens (Roche Diagnostic Systems) has a sensitivity in the range of 89–97% (1–8), although a few studies have found lower sensitivity, in the range of 64–86% (8–10). All of the referenced studies have reported a specificity of 99–100%. Recently, however, the reproducibility of the assay has been questioned by investigators who found that 13 of 35 cervical specimens that were tested and then retested with the assay showed great variability in absorbance values (11). Because there is a paucity of information available concerning the reproducibility of the Amplicor assay, we report herein data informative on the issue that were obtained at UMDNJ-University Hospital. The protocol for this study was approved by the Institutional Review Board of UMDNJ-New Jersey Medical School.

The reproducibility studies reported here were derived from two data sets. The first set, 162 specimens, was drawn from a consecutive series of 5011 endocervical specimens examined by the Amplicor assay for detection of Chlamydia trachomatis in endocervical specimens.
of *C. trachomatis* (Table 1). This series was begun on January 18, 1994, and extended to May 27, 1994. The second data set, consisting of 717 specimens collected during various periods extending from May 31, 1994, to October 31, 1995, provided additional samples with differing quantitative assay absorbance values (A450) on initial examination. In all, 879 specimens (162 + 717) were used to assess reproducibility of the assay. Three types of reproducibility studies were performed: 571 specimens that were either positive or negative on initial testing were subjected to duplicate-repeat testing on a subsequent day; 79 specimens with initially equivocal results (A450, 0.2–0.5) were subjected to duplicate-repeat testing in triplicate (three sets of duplicate-repeat tests) to test the manufacturer’s algorithm for resolving the status of equivocal results; and a separate group of 229 specimens was subjected to within-run triplicate testing. Within each of the several absorbance categories that were established and that are defined in Table 1, the specimens tested for reproducibility were randomly chosen.

In accordance with the manufacturer’s directions, both initial and duplicate-repeat tests were performed within 10 days of specimen collection. One major exception to this protocol was that 47 of 132 specimens yielding initial absorbance values above 2.0 were retested between 11 and 17 days after specimen collection (all duplicate-repeat tests on these 47 specimens gave positive results). Approximately 70% of specimens were submitted by the hospital’s obstetrics and gynecology services, but specimens were also received from the emergency room (~20%) and from other clinical units. Specimens were stored before testing at 2–8 °C.

The PCR assay was performed in accordance with instructions in the package insert of the Amplicor *C. trachomatis* test kit (12), and the results of testing were interpreted in accordance with instructions in the insert: an A450 <0.2 was considered negative; values from 0.2 to 0.5 were considered equivocal; and values >0.5 were regarded as positive. Duplicate-repeat testing was performed on samples with equivocal values, which were then determined to be either positive or negative in accordance with the manufacturer’s criteria. Samples with two of three test results (the initial value and the pair of repeat values) showing an absorbance >0.25 were considered to be positive, and samples with two of the three results <0.25 were considered to be negative. The assay was performed in the clinical chemistry laboratory of UMDNJ-University Hospital, taking care to maintain separate working areas for reagent preparation, specimen preparation, and amplification and detection.

Systat 5.03 (Systat, Inc.) was used to calculate the confidence intervals shown in Table 1.

Table 1 shows the distribution of results obtained in the series of 5011 specimens and includes a breakdown of positive results based on a stratification of absorbance values. The overall rate of positive results for the series was 9.7%.

Table 1 also provides a summary of the results of repeat testing performed on 650 specimens: 332 initially negative, 239 initially positive, and 79 initially equivocal. The 332 negative specimens gave consistent (negative) results in 99.1% of duplicate-repeat tests. As shown in Table 1, the rate of reproducibility of positive results on duplicate-repeat testing varied greatly according to initial absorbance values, from 47.4% for specimens with initial absorbance values >0.5 to 1.0 to 98.8% for specimens with initial absorbance values >3.5. In calculating reproducibility rates for initially positive test results, a repeat test

<table>
<thead>
<tr>
<th>Test result</th>
<th>Absorbance</th>
<th>Number</th>
<th>%</th>
<th>95% confidence interval, %</th>
<th>Number of specimens tested for reproducibility</th>
<th>Agreement of duplicate-repeat tests with initial results</th>
<th>Agreement, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>&lt;0.2</td>
<td>4479</td>
<td>89.4</td>
<td>88.1–90.5</td>
<td>332</td>
<td>658 of 664</td>
<td>99.1</td>
</tr>
<tr>
<td>Equivocal</td>
<td>0.2–0.5</td>
<td>59b</td>
<td>1.2</td>
<td>0.8–1.7</td>
<td>79</td>
<td>63 of 79</td>
<td>79.7</td>
</tr>
<tr>
<td>Positive</td>
<td>&gt;0.5–1.0</td>
<td>26</td>
<td>0.5</td>
<td>0.3–0.9</td>
<td>57</td>
<td>54 of 114</td>
<td>47.4</td>
</tr>
<tr>
<td></td>
<td>&gt;1.0–1.5</td>
<td>7</td>
<td>0.1</td>
<td>0.0–0.4</td>
<td>15</td>
<td>23 of 30</td>
<td>76.7</td>
</tr>
<tr>
<td></td>
<td>&gt;1.5–2.0</td>
<td>15</td>
<td>0.3</td>
<td>0.1–0.6</td>
<td>35</td>
<td>54 of 70</td>
<td>77.1</td>
</tr>
<tr>
<td></td>
<td>&gt;2.0–2.5</td>
<td>10</td>
<td>0.2</td>
<td>0.1–0.4</td>
<td>14</td>
<td>26 of 28</td>
<td>92.9</td>
</tr>
<tr>
<td></td>
<td>&gt;2.5–3.0</td>
<td>13</td>
<td>0.3</td>
<td>0.1–0.5</td>
<td>24</td>
<td>44 of 48</td>
<td>91.7</td>
</tr>
<tr>
<td></td>
<td>&gt;3.0–3.5</td>
<td>40</td>
<td>0.8</td>
<td>0.5–1.2</td>
<td>10</td>
<td>19 of 20</td>
<td>95.0</td>
</tr>
<tr>
<td></td>
<td>&gt;3.5</td>
<td>362</td>
<td>7.2</td>
<td>6.2–8.3</td>
<td>84</td>
<td>166 of 168</td>
<td>98.8</td>
</tr>
</tbody>
</table>

Subtotal of positives on initial testing 473 9.4 8.5–10.4

Total 5011 650

* Includes specimens from both the consecutive series of 5011 specimens and from the supplemental series defined in the text.

* Of these cases, 14 were found to be positive, and 44 negative, according to the criteria of the test. One specimen was not tested further; its status was not resolved. Excluding this case, the positive rate was 487 of 5010, or 9.7% (95% confidence interval, 9.8–10.7%).

* The number of cases in which application of the manufacturer’s criteria to each of three sets of duplicate-repeat tests led to the same conclusion as to whether an initially equivocal specimen was positive or negative.
result was considered to be in agreement with the initial result if the manufacturer’s criteria for a positive test result were met; quantitative agreement of absorbance values was not required.

Initially equivocal results represented a special case in this evaluation. For only 63 (80%) of the 79 specimens that gave initially equivocal results and on which duplicate-repeat testing in triplicate was performed did the manufacturer’s algorithm produce consistent results as to whether specimens were positive or negative (49 negative and 14 positive). Discrepant results were obtained for the other 16 specimens (20%). In each of these 16 cases, two sets of duplicate-repeat test results led to one conclusion as to whether the specimen was positive or negative, but the third set of results led to the opposite conclusion.

The overall rate of reproducibility of the Amplicor assay was calculated to be 98.4%. This rate was determined by summing the products obtained when the rate of reproducibility for each absorbance subset of initial results (Table 1) was multiplied by the frequency of the respective subset in the consecutive series of 5011 specimens (also shown in Table 1). Specimens yielding absorbance values <0.2 and >=3.5 contributed most to the high rate of overall reproducibility for the assay because (a) absorbance values <0.2 and >=3.5 together accounted for 96.6% of all assay results in the consecutive series of 5011 specimens, and (b) both of these absorbance categories produced consistent results on replicate testing in ~99% of cases. Specimens with absorbance values ranging from 0.2 to 3.5, which had lower rates of reproducibility, accounted for only 3.4% of total accessions, and thus had relatively little effect on the rate for overall reproducibility of the assay. The overall rate of reproducibility for initially positive results was calculated to be 94.5%.

The high reproducibility on duplicate-repeat testing was confirmed in the series in which triplicate analyses of specimens were performed on the day of initial testing. Of 229 specimens in this series, 227 (99.1%) gave results that were either consistently positive (18) or consistently negative (209).

Only limited data on the reproducibility of the Roche Amplicor chlamydial assay in testing endocervical specimens have been published. In a series of 501 cervical and urethral specimens for which duplicate assays were performed (13), 10 (2%) gave discrepant results.

The causes of such discrepant results as we observed have not been established. Contamination of specimens by chlamydial DNA is always difficult to rule out completely as a possible cause of false-positive results, and specimen mix-up and technical error are also possible explanations for the discrepancies encountered. In this case, however, other explanations seem more probable. Negative and positive controls have consistently produced expected results in our runs of the assay, and we have found evidence for a “nearest neighbor” cross-contamination effect causing false-positive results.

For those specimens yielding negative test results initially but positive results on repeat testing, two likely causes of false-negative reactions especially must be considered. One is that the specimens could have contained polymerase inhibitors that were active at the time of initial testing but then became inactivated in the interval between initial and repeat testing. A second possibility is that the specimens could have had low copy numbers of the plasmid target for amplification, and therefore, particular aliquots of the specimens could have had numbers of target plasmids either above or below the threshold of the assay detection limit.

In respect to polymerase inhibition of the Amplicor assay, investigators have reported instances in which specimens yielded negative results, but after storage for a variable number of days (4, 5, 8, 9, 14), after phenol-chloroform or phenol extraction (1, 2, 8), or after specimen dilution (4, 9), gave positive results on repeat testing. Verkooyen et al. (15) found that, in specimens to which inocula of C. trachomatis were added, a combination of 10-fold dilution of specimens with heating to 95 °C for 10 min before testing produced an inhibition rate of only 41%, compared with a rate of 19% for untreated samples.

Support for the importance of copy number in detection of chlamydial DNA with the Roche assay was provided by the work of Toye et al. (7). Roche’s package insert for the Amplicor chlamydial assay states that the detection limit of the assay is one inclusion-forming unit, or ~10 plasmid copies (12), but Miyashita et al. (16) estimated that the detection limit of the assay is ~20–40 plasmid copies. Another possible cause of false-negative results, loss of chlamydial target DNA from specimens, is improbable, given DNA’s well-known resistance to degradation.

For specimens that gave positive results on initial testing and negative results on repeat testing, low copy number is again a plausible explanation. Furthermore, as suggested by Mahony et al. (4), polymerase inhibitors could also cause such a sequence of testing results if an initially inactive inhibitor became activated with aging of a specimen.

The results produced by the Amplicor assay for C. trachomatis are, overall, highly reproducible, but still show room for improvement. Clearly, if the information presented above on the resolution of equivocal results is confirmed by others, the algorithm constructed by the manufacturer for such resolution will require modification. In fact, there may be a small number of specimens for which results should be reported as indeterminate rather than as either positive or negative. In respect to the problem of false-negative results due to polymerase inhibitors, a number of investigators have recommended that an internal control (to flag possibly false-negative results) be incorporated into the assay’s protocol (4, 15, 17); pretreatment of specimens to reduce the activity of inhibitors, e.g., by dilution and heating, has also been advocated (4, 15). These measures appear quite reasonable, although in the case of dilution the potential benefit of a reduction in polymerase inhibitors must be weighed against the undesired effect of a reduction in...
plasmid copy number. It is notable that the Roche Cobas Amplicor automated PCR methodology includes an optional internal control (15, 18) and that the Cobas procedure for detection of C. trachomatis defines a category of “repeatedly equivocal” results that require additional testing on a new specimen for resolution (19).

References


Preparation and Validation of PCR-generated Positive Controls for Diagnostic Dot Blotting, Min-Hui Liang, Dennis R. Johnson, and Lee-Jun C. Wong. Molecular Diagnostic Laboratory, Institute for Molecular and Human Genetics, Georgetown University, Washington, DC 20007; 2 Dept. of Pathology and Pediatrics, Childrens Hospital, Los Angeles, CA 90027; 3 present address: Yale University, School of Medicine, New Haven, CT 06520-8066; 7 author for correspondence: Molecular Diagnostic Laboratory, Institute for Molecular and Human Genetics, 3800 Reservoir Rd., NW, Rm M4000, Georgetown University Medical Center, Washington, DC 20007; fax 202-784-1770, e-mail wonglj@gunet.georgetown.edu

Allele-specific oligonucleotides (ASOs) are small, single-stranded nucleotide polymers (~18–20 bases in length) of diagnostic utility given their ability to hybridize to single-stranded DNA target molecules in a sequence-specific, temperature-dependent manner. Accordingly, two target molecules differing in composition by a single base can be distinguished by hybridization with an ASO that is complementary to one of the target molecules but noncomplementary to the other by a single base. The latter target produces a single basepair-mismatched double strand that dissociates at a lower washing temperature than the fully complementary, double-stranded molecule.

Since first used to screen for beta-s globin mutations in sickle cell disease, ASOs have been widely used to detect known disease-causing mutations and disease-associated base substitutions (1–3). Diagnostic screening using ASOs for clinically relevant base substitutions (mutations/polymorphisms) frequently uses a dot blot format. PCR-amplified patient DNA is bound to a membrane, denatured to generate single-strand targets, and probed with both mutant and wild-type ASOs. Subsequent washing under optimized conditions denatures all but the entirely complementary double-stranded hybrids, which yield a positive signal. Dot blotting requires positive-control samples to monitor the sensitivity and specificity of each diagnostic blot. Given the rarity of some disease alleles, the acquisition of positive-control DNA is often rate-limiting in the establishment of a dot blot protocol for a given disease.

Mitochondrial disease phenotypes have been associated with an array of base substitutions in the mitochondrial genome, and diagnostic screening with ASOs is an effective approach to the identification of mitochondrial DNA mutations. For example, an adenine-to-guanine transversion at position 4136 (A4136G) of the human mitochondrial genome, which leads to the substitution of cysteine for tyrosine in the ND1 subunit of respiratory chain complex I, has been associated with Leber hereditary optic neuropathy (4–6). Using the A4136G substitution associated with Leber hereditary optic neuropathy as a prototype, we have validated a technical approach to the preparation of synthetic positive controls suitable for ASO dot blot analysis in any disease.

The approach involves generating PCR products containing the base substitution of interest by using a single-basepair-mismatched PCR primer to amplify normal target DNA. This produces abundant mutation-containing PCR