Original Article

Performance of Strand Displacement Amplification Assay in the Detection of Chlamydia trachomatis and Neisseria gonorrhoeae

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SUMMARY: This study is a critical analysis of certain amplification assays for detecting Chlamydia trachomatis and Neisseria gonorrhoeae infections which have demonstrated that the plasmid-free variant of Chlamydia trachomatis is frequently responsible for infection in our patients. Specifically, we evaluated the performance of the strand displacement amplification (SDA) assay in detecting either C. trachomatis or N. gonorrhoeae in 1,190 clinical samples, both urogenital and ocular, from 1,005 consecutive patients. The results obtained with the BDProbeTec™ ET System were compared with three referenced amplification methods for C. trachomatis (detecting the 16S rRNA gene, the omp1 gene and the plasmid of C. trachomatis) and with both the culture method as well as an amplification assay followed by genetic identification performed using the MicroSeq 500 16S ribosomal DNA-based system for N. gonorrhoeae. The sensitivity of SDA (76%) in detecting C. trachomatis is significantly low when compared with that of other molecular techniques employing 16S rDNA or omp1 as a target. The specificity of the methods for detecting C. trachomatis was excellent, ranging from 99.4 to 100%. Furthermore, the results of SDA in detecting N. gonorrhoeae also provided excellent results (100% specificity and sensitivity).

INTRODUCTION

Chlamydia trachomatis and Neisseria gonorrhoeae are two of the most prevalent sexually transmitted pathogens, and often cause asymptomatic infections (1-6). Nucleic acid amplification tests (NAATs) have become widely used since they show greater sensitivity than other tests as well as good specificity (1,7-10). However, it is well known that the presence of certain inhibitors in clinical samples may be responsible for false negative results, while the concomitant presence of other bacteria could cause false positive results (5,11). Therefore, the diagnostic ability of any NAAT must be carefully evaluated. The performance of the BDProbeTec™ ET system (Becton Dickinson, Sparks, Md., USA) using strand displacement amplification (SDA) in detecting C. trachomatis has already been evaluated in several papers, although the majority of these utilized only certain clinical samples, such as urine, endocervical and urethral swabs (2,4,10,12). Less is known about vaginal swabs, or seminal or corneal samples, even though they are also quite frequently used. In general, studies on the evaluation of SDA in comparison with other amplification methods are based on the PCR technique using the plasmid of C. trachomatis (pCT) as a target, and are potentially biased due to the possible misdiagnosis of C. trachomatis plasmid-free variant (13,14). The purpose of the present paper was to evaluate the performance of SDA in detecting C. trachomatis and N. gonorrhoeae in urogenital samples and in ocular swabs, the latter being particularly useful in neonatal screening of children born of infected mothers. SDA in the detection of C. trachomatis was evaluated by a head-to-head comparison with PCR methods based on the amplification of 16S rDNA, omp1 and the plasmid for C. trachomatis, while the detection of N. gonorrhoeae was evaluated in comparison with reference standard culture methods and with the MicroSeq 500 16S ribosomal DNA-based system (Applera, Foster City, Calif., USA). Each sample showing positive results for either C. trachomatis or N. gonorrhoeae was confirmed by sequence analysis of the products (15).

METHODS

Samples: We examined a total of 1,190 samples from 1,005 different consecutive patients (320 men, median age 41.45 ± 10.33; 685 women, median age 36.62 ± 10.48) attending the outpatient department of the Policlinic of Tor Vergata (Rome, Italy) for a screening control. The samples were distributed as follows: 31 urine samples, 99 endocervical samples, 683 vaginal swabs (from 584 female patients since 99 women provided both cervical and vaginal samples), 86 seminal fluids, 260 urethral samples (from 174 male patients since 86 men provided both urethral and seminal samples) and 31 corneal swabs.

Urine sample collection, transport and processing for SDA assay: At least 10 ml of first-void urine were collected in sterile plastic preservative-free specimen cups. A urine-processing pouch (Becton Dickinson) was added to the sample, which was then maintained at room temperature for at least 2 h before being stored at 2-8°C until processed. Four milliliters of urine was centrifuged at 2,000 × g for 30 min. At the testing site, the pellet was then resuspended in 2 ml of the sample diluent (Becton Dickinson) and treated according to the manufacturer’s instructions.

Swab sample collection, transport and processing for SDA assay: Male and female urethral specimens were collected using rayon swabs (MiniTip Culturette™ Direct; Becton Dickinson). After having removed the mucus using a clean-
ing swab, a polyurethane-tipped swab (Culturette Direct; Becton Dickinson) was used to collect endocervical and vaginal specimens. The swabs were transported without preservative to the testing laboratory, where they were expressed into tubes supplied pre-filled with 2 ml of sample diluent. The swabs were discarded, and the sample fluid was heat lysed and cooled following the manufacturer’s instructions. To evaluate the possible use of vaginal swabs as an alternative to cervical swabs, 99 female patients provided both cervical and vaginal samples. The vaginal swabs were processed similarly to the cervical samples.

**Corneal swabs:** A polyurethane-tipped swab (Mini tip; Becton Dickinson) was used for the collection of cells during corneal scraping. At the testing laboratory, the swabs were transferred into tubes supplied pre-filled with 2 ml of sample diluent and treated in the same manner as the other samples.

**Seminal fluids:** One thousand microliters of each specimen were centrifuged at 2,000 × g for 30 min within 2 h of collection. After removing the supernatant, the pellet was washed twice with 200 μl of phosphate buffered saline (PBS), and transferred to a 4-ml tube containing 2 ml of sample diluent. The sample fluid was heat lysed and cooled as described for the other specimens. In order to evaluate the possible use of these samples in place of urethral specimens, 86 men provided both endourethral samples and seminal fluid specimens.

**Assay procedure:** For each tray of specimens assayed, one positive control and one negative control (Becton Dickinson) were included in a microwell tray and tested in the same way. The sample fluid was heat lysed and cooled following the manufacturer’s instructions; Bacterial DNA was extracted using PrepMan ultra (Applera) at 100°C for 10 min. The extraction mix was cooled at room temperature and centrifuged at 12,000 × g for 3 min. Two microliters of the supernatant of each bacterial extract were used for successive amplifications.

**Culture and amplification procedure for N. gonorrhoeae:** Endocervical, vaginal, urethral and corneal swabs were taken in duplicate: the first set of swabs was taken with a polyurethane-tipped swab (Culturette Direct) destined for the amplification technique; the second set was taken with the rayon swabs containing amies with charcoal (Oxoid Inc., Ogdensburg, N.Y., USA) as a transport medium. The latter were used for the culture method of N. gonorrhoeae. All specimens were cultured on modified Thayer-Martin and chocolate agar plates (Becton Dickinson) in an atmosphere enriched with 5% CO₂ at 37°C for 48 h. A Gram-negative, oxidase-positive colony was identified as N. gonorrhoeae using API NH (bioMérieux, Marcy l’Etoile, France).

**PCR amplification of a portion of 16S rDNA of N. gonorrhoeae:** Our PCR process utilized two couples of universal primers F8 and R531 (from the MicroSeq 500 16S ribosomal DNA-based system). All PCR products were confirmed by sequencing performed in an ABI Prism 310 Genetic Analyzer using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit, according to the manufacturer’s recommendations. Consensus sequences were compared with universal databases in the National Center for Biotechnology Information (NCBI) bank and in to the MicroSeq libraries (15, 16).

**Amplification assay for C. trachomatis:** To verify the reliability of SDA in identifying C. trachomatis, the samples were also subjected to PCR assay detecting both the 16S rDNA, the omp1 gene and pCT (14, 17). The results of these amplification assays are shown in Figure 1. For these PCR tests, the starting mix was that already used for the SDA assay (sample diluent preparation). Two volumes of 100% ethanol and 1/10 of volume of 3M sodium acetate were added.
to 2 ml of the sample diluent preparation, which was then centrifuged at 12,000 × g for 30 min. The pellet was purified using a QiAmp kit (Qiagen GmbH, Hilden, Germany) following the manufacturer’s instructions. Five microliters of the eluate were used in the PCR assays. The first 16S rDNA-PCR was performed following the method described by Pollard et al. (13). Employing our positive control, we determined the limit of detection of this PCR procedure to be 10 elementary bodies (EBs) of C. trachomatis.

The PCR detecting pCT was performed on 5 µl of the extraction mixture, according to the procedure described by ElNifro et al. (17). The PCR of the pCT reaction produced a 475-bp product. Using our positive control, we were able to determine the limit of detection of this PCR procedure to be 1 EB (that is, 10 pCT).

The third PCR procedure detecting the omp1 gene was performed on 5 µl of sample extraction mixture according to the procedure previously described (8,18). The PCR reaction provided a 1,100-bp product. The detection limit of this PCR procedure was found to be 10 EBs. To examine the amplicons obtained, 10 µl of the PCR products were analyzed by electrophoresis in a 2.0% agarose gel in a Tris-acetate-EDTA buffer and stained with ethidium bromide. The PCR products were studied in parallel with the following molecular weight markers: Gene Ruler 100 bp DNA Ladder and Gene Ruler DNA Ladder Plus (MBI Fermentas, Vilnius, Lithuania), only for omp1 PCR.

Amplification assay to exclude the presence of inhibitors: To exclude the possibility that some specimens could show negative results due to the presence of inhibitors, all negative samples were subjected to a second PCR assay for the purpose of amplifying the conserved gene of human β-actin (19).

Sequence analysis of PCR products: To exclude possible false positive results, all PCR products were subjected to nucleotide sequence analysis using an ABI Prism 310 Genetic Analyzer as described above.

Analysis of the results: Specimens tested in parallel by PCR-16S rDNA, PCR-omp1, PCR-pCT and SDA were considered to show true positive results for C. trachomatis; SDA produced a total of 26 positive results (2.5%). Table 1 shows the distribution of positive specimens in the different samples. PCR-16S rDNA showed 8 positive samples more than SDA and 6 more than the PCR-pCT method. The percentage of our sample which did not possess the plasmid was 17% of C. trachomatis-positive specimens (6 of a total of 34 positive specimens). In the present study, the possibility of false negative samples was excluded by the results of the PCR assay amplifying the conserved gene of human β-actin (19). The sensitivity of SDA and PCR-pCT are substantially similar and both are lower than those shown by PCR-16S-rDNA and PCR-omp1 (Table 2). Specifically, SDA showed three false positive samples, which were identified as false by sequencing analysis. The specificity rates of all systems do not show significant differences, and the present method is good. On the other hand, two samples which were positive when using the PCR-pCT technique, were “indeterminate” according to SDA.

Table 1 shows the results of detection of N. gonorrhoeae performed by SDA in comparison with results provided by the referenced methods. In this case, the number of SDA-positive specimens (1.1%) and their distribution in different samples are the same as those obtained by the other standard methods (culture and MicroSeq 500), except in one case in which the culture for N. gonorrhoeae resulted negative, while both the MicroSeq 500 16S ribosomal DNA-based system and SDA gave positive results. Consequently, the sensitivity of the culture method is less than that demonstrated by nucleic acid amplification techniques. In contrast, the specificity exhibited by all tested methods was found to be similar. The performance of the extraction procedure applied to some specimens (such as seminal fluids and vaginal and corneal swabs) never before tested using BDProbeTec™ (2,4,9,10, 12,20) was also good. Specifically, data obtained from 99

<p>| Table 1. Distribution of C. trachomatis (CT) and N. gonorrhoeae (NG)-positive samples in different specimens |</p>
<table>
<thead>
<tr>
<th>Sample (No)</th>
<th>SDA CT-positive</th>
<th>PCR-16S DNA/omp1 CT-positive</th>
<th>PCR pCT positive</th>
<th>SDA NG-positive</th>
<th>Culture and PCR-16S DNA (by MicroSeq 500) NG-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine (31)</td>
<td>7</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cervical swabs&lt;sup&gt;1&lt;/sup&gt; (99)</td>
<td>3</td>
<td>4</td>
<td>4&lt;sup&gt;2&lt;/sup&gt;</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Vaginal swabs (683)</td>
<td>5+1</td>
<td>7</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Seminal fluid (86)</td>
<td>3+1</td>
<td>8</td>
<td>6</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Urethral swabs&lt;sup&gt;3&lt;/sup&gt; (260)</td>
<td>6+1</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1&lt;sup&gt;1&lt;/sup&gt;+1</td>
</tr>
<tr>
<td>Corneal swabs (31)</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total (1,190)</td>
<td>26+3&lt;sup&gt;3&lt;/sup&gt;</td>
<td>34</td>
<td>28</td>
<td>11</td>
<td>11</td>
</tr>
</tbody>
</table>

<sup>1</sup>: 3 false positive results which were not confirmed by sequencing.
<sup>2</sup>: 2 samples among the urine and cervical specimens which resulted positive to PCR of pCT and indeterminate (grey-zone) with the SDA.
<sup>3</sup>: 3 false positive results which were not confirmed by sequencing.
false negative result. Currently, the opinion that plasmid-free variant of cells (21). Therefore, a possible disadvantage of chlamydial-extra-chromosomal DNA, this does not mean that those strains on the BDProbeTec™ ET System is efficiently automatized either on the basis of its performance or because the method combined is well known to be low; ii) An et al. have demonstrated the tion is weak because: i) the sensitivity of the culturing method does not exist (as it has never been isolated), or occurs with a low prevalence, is common (3). However, this argumentation is weak because: i) the sensitivity of the culturing method is well known to be low; ii) An et al. have demonstrated the possible role of a plasmid-free variant of C. trachomatis in human infection (14); iii) the present results clearly demonstrate that plasmid-free C. trachomatis strains are not infrequent; and iv) the present study, in its comparison of the performance of SDA with that of other methods discussed in previous studies (3,12,14,17,20-23), exposes a potential bias: the methods used in the other studies are all based on pCT detection. In our study the plasmid-free variant of C. trachomatis was found to show a prevalence of 17.6% (6 of 34 positive samples). Hence, an important issue in the future development of NAATs is how to improve the choice of the region to amplify to provide an assay with good sensitivity. In the present study, we again propose the use of the 16S rDNA gene, but a good alternative could also be to amplify the region codifying for the major outer membrane protein (omp1 gene) (8,18,24). The results of SDA in the present study in detecting N. gonorrhoeae were comparable to those reported in previous studies (4,9,20); additionally, the sensitivity and the specificity of the SDA system were excellent when compared with other molecular methods, and superior when evaluated with respect to the culture method. A useful aspect of our work is the possibility of performing SDA on samples such as seminal fluid, vaginal swabs and corneal swabs, which are annually processed in a diagnostic laboratory (10,11,23,25). The procedure of sample extraction does not require additional passages (with the exception of centrifugation for seminal fluids), and it is easy to perform. Regarding the possible use of vaginal swabs rather than cervical ones or seminal fluid as an alternative to urethral specimens, the results obtained in our study show that they are interchangeable in terms of rendering the SDA in the automation of the BDProbeTec™ ET, a good system for processing these samples. The type of biological sample does not influence the number of false negative results furnished by the SDA, as false negative results can occur both in traditional specimens (those certified by the producer) and in unconventional ones. Moreover, it is not unusual for SDA to provide some false positive results, which is quite normal for any screening method. In a recent publication, the Centers for Disease Control and Prevention (CDC) advised the confirmation of all positive results by other methods (11). The fact that SDA furnished two more false negative results than PCR of pCT (which amplifies the same target), combined with evidence from additional testing that these that these two were indeterminate, prompted us to further investigate a possible reconsideration of the positive cutoff of the method (23). In conclusion, considering the versatility and simple workflow of the ProbeTec™ ET system, after a more appropriate choice of the C. trachomatis target, we hope to extend this procedure for detecting either C. trachomatis or N. gonorrhoeae to all urogenital and ocular samples with

### Table 2. SDA compared with reference methods: sensitivity, specificity, PPV and NPV of C. trachomatis and N. gonorrhoeae assays

<table>
<thead>
<tr>
<th>Tests for 1,190 specimens from 1,005 patients</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDA-CT</td>
<td>76% (26/34)</td>
<td>99.7% (968/971)</td>
<td>89.6% (26/29)</td>
<td>99.1% (968/976)</td>
</tr>
<tr>
<td>PCR-CT 16S rRNA/omp1</td>
<td>100% (34/34)</td>
<td>100% (971/971)</td>
<td>100% (971/971)</td>
<td>100% (971/971)</td>
</tr>
<tr>
<td>PCR pCt</td>
<td>82.3% (28/34)</td>
<td>99.4% (971/977)</td>
<td>99.4% (971/977)</td>
<td>99.4% (971/977)</td>
</tr>
<tr>
<td>SDA-NG</td>
<td>100% (11/11)</td>
<td>100% (994/994)</td>
<td>100% (994/994)</td>
<td>100% (994/994)</td>
</tr>
<tr>
<td>Culture for NG</td>
<td>90.9% (10/11)</td>
<td>99% (993/994)</td>
<td>99% (993/994)</td>
<td>99% (993/994)</td>
</tr>
<tr>
<td>PCR-NG/MicroSeq 500</td>
<td>100% (11/11)</td>
<td>100% (994/994)</td>
<td>100% (994/994)</td>
<td>100% (994/994)</td>
</tr>
</tbody>
</table>

PPV, positive predictive value; NPV, negative predictive value.
good results.

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REFERENCES


