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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 *C. 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 *omp*1 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 *omp*1 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 dispacement 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 wass 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, *omp*1 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).

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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 $\times g$ 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 as previously treated samples. The two phases of priming and amplification were performed according to the manufacturer's instructions. In each molecular assay, a suspension of reference standard strains of *N. gonorrhoeae* ATCC 49981 and *C. trachomatis* D serovar (from our institute collection) was also used as an artificial amplification control. 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 $\times g$ 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% CO_2 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 *omp*1 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

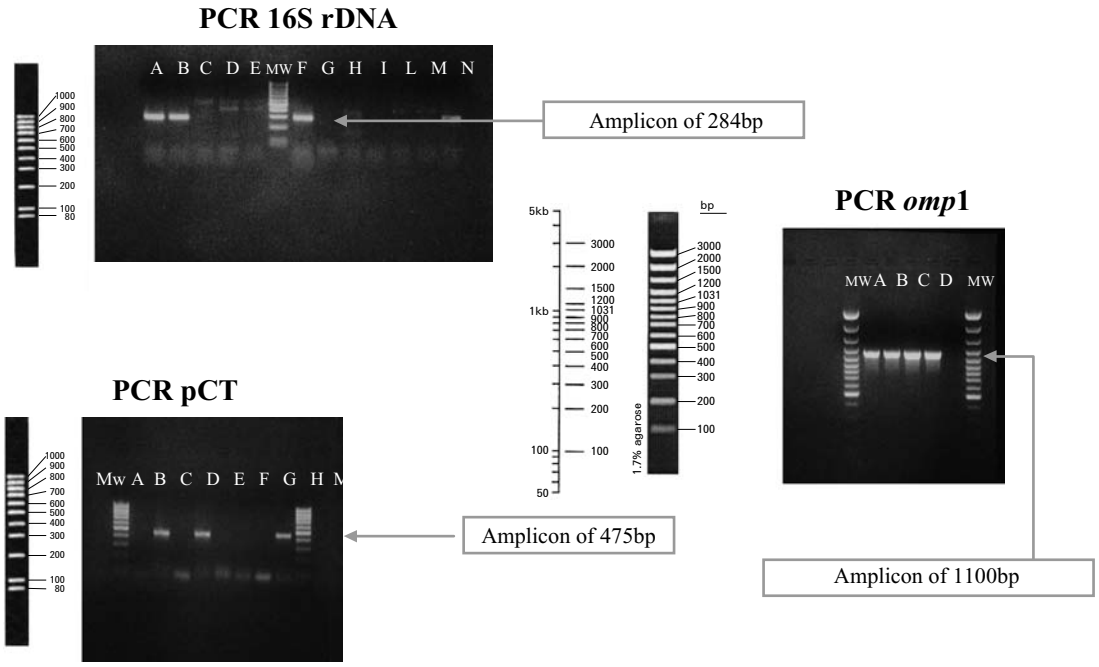

Fig. 1. 16S rDNA amplifcation method. Lanes A, B and F are some positive samples; N positive control from *C. trachomatis*; Lanes C-E, G-M are some negative samples. Mw, molecular weight marker.
PCR pCT method. Lanes A,C, E, F and G are some negative samples; Lanes B and D are two positive samples, H positive control.
PCR of *omp*1. Lanes A-D are positive samples.

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 *omp*1 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 *omp*1 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-*omp*1, PCR-pCT and SDA were considered to show true positive results for *C. trachomatis* when at least two of the referenced PCR methods produced an amplicon, and when sequence analysis confirmed the amplification products as belonging to the *C. trachomatis* genome. *N. gonorrhoeae* spp. were considered true positive when the culture was positive and the amplification method and sequence analysis of the PCR product, performed using the MicroSeq 500 system, confirmed the identification (15, 16).

RESULTS

Of our 1,190 specimens from 1,005 different patients contemporaneously tested by SDA, PCR-16S rDNA, PCR-*omp*1 and PCR-pCT, a total of 34 samples (3.4%) showed 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-*omp*1 (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

Table 1. Distribution of *C. trachomatis* (CT) and *N. gonorrhoeae* (NG)-positive samples in different specimens

Sample (No)	SDA CT-positive	PCR-16S DNA/*omp*1 CT-positive	PCR pCT positive	SDA NG-positive	Culture and PCR-16S DNA (by MicroSeq 500) NG-positive
Urine (31)	7	8	8[2)]	0	0
Cervical swabs[3)] (99)	3	4	4[2)]	2	2
Vaginal swabs (683)	5+1	7	5	5	5
Seminal fluid (86)	3+1	8	6	2	2
Urethral swabs[4)] (260)	6+1	4	3	2	1[5)]+1
Corneal swabs (31)	2	3	2	0	0
Total (1,190)	26+3[1)]	34	28	11	11

[1)]: 3 false positive results which were not confirmed by sequencing.
[2)]: 2 samples among the urine and cervical specimens which resulted positive to PCR of pCT and indeterminate (grey-zone) with the SDA.
[3)]: cervical swabs from patients who also provided vaginal swabs. The results of vaginal swabs from women who also provided cervical swabs were concordant.
[4)]: including 86 urethral swabs from patients who also provided seminal fluid specimens. The results of urethral swabs and seminal fluids, provided from the same patients, were concordant.
[5)]: a sample for which the NG-culture method furnished a false negative result.

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

Tests for 1,190 specimens from 1,005 patients	Sensitivity	Specificity	PPV	NPV
SDA-CT	76% (26/34)	99.7% (968/971)	89.6% (26/29)	99.1% (968/976)
PCR-CT 16S rRNA/*omp*1	100% (34/34)	100% (971/971)	100% (971/971)	100% (971/971)
PCR pCT	82.3% (28/34)	99.4% (971/977)	99.4% (971/977)	99.4% (971/977)
SDA-NG	100% (11/11)	100% (994/994)	100% (994/994)	100% (994/994)
Culture for NG	90.9% (10/11)	99% (993/994)	99% (993/994)	99% (993/994)
PCR- NG/MicroSeq 500	100% (11/11)	100% (994/994)	100% (994/994)	100% (994/994)

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

female patients to evaluate the presence of *C. trachomatis* and *N. gonorrhoeae* demonstrated that the results for vaginal swabs were consistent with those obtained for cervical samples in the same patient (positive results in vaginal samples were concordantly positive in cervical swabs from the same patients). Furthermore, results from 86 male specimens showed that all *C. trachomatis* and *N. gonorrhoeae*-positive urethral specimens but one were contemporaneously positive in the seminal samples. The only exception was a sample from a patient who tested positive for *N. gonorrhoeae* in the seminal specimen but negative in the urethral sample.

DISCUSSION

SDA is undoubtedly a good system, successfully performing combined *C. trachomatis* and *N. gonorrhoeae* determinations either on the basis of its performance or because the method on the BDProbeTec™ ET System is efficiently automatized (2,4,9,10,12,20). Therefore, the aim of the present study, in addition to a simple comparison among different systems, also included a critical analysis of target choice in the amplification assay (particularly that of pCT) as well as an evaluation of the possible extension of this technique to other types of clinical samples.

We first evaluated the reliability of this system in detecting *C. trachomatis* and *N. gonorrhoeae* in urogenital and ocular specimens, comparing data with those obtained from three other referenced methods of detecting *C. trachomatis* (based on 16S rDNA PCR, on *omp*1 and on pCT PCRs) and two techniques for *N. gonorrhoeae* (the culture method and a PCR method based on the amplification of 16S rDNA by MicroSeq 500). The results provided by the PCR-pCT assay and the SDA method are substantially similar, however a significant lack of sensitivity was noted (76 and 82%, respectively) when compared with the 100% sensitivity shown by other *C. trachomatis* PCR assays. This demonstrates that the use of pCT as a target for amplification methods is not always the most appropriate choice (14,21). No significant differences were identified when the specificity of all methods were compared (99.4 to 100%). Even though it has been demonstrated that not all strains of *C. trachomatis* possess this element of extra-chromosomal DNA, this does not mean that those strains are less pathogenic or would be unable to infect the host cells (21). Therefore, a possible disadvantage of chlamydial-plasmid based amplification is the potential occurrence of a plasmid-free variant of *C. trachomatis*, which could give a false negative result. Currently, the opinion that *C. trachomatis* 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 alterative could also be to amplify the region codifying for the major outer membrane protein (*omp*1 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

good results.

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