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# Comparison of Plasmid- and Chromosome-Based Polymerase Chain Reaction Assays for Detecting *Chlamydia trachomatis* Nucleic Acids

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**Several laboratories have demonstrated that the polymerase chain reaction (PCR) is more sensitive than culture or enzyme immunoassay (EIA) for detecting *Chlamydia trachomatis* in genitourinary tract specimens when various DNA targets are used for amplification, including the cryptic plasmid, major outer membrane protein (MOMP), or rRNA genes. We compared the performances of five different PCR assays, including assays with two plasmid, two MOMP, and one rRNA targets, by amplifying serial dilutions of *C. trachomatis* DNA and testing genitourinary tract specimens. By using published procedures, two different plasmid primers had sensitivities of 0.1 fg for *C. trachomatis* plasmid DNA and 10 fg for total cellular DNA. The sensitivities of the assays with the two MOMP primers were 0.1 and 10 pg, and the sensitivity for the assay with the rRNA primers was 1 pg for cellular DNA. Both plasmid-based assays detected 38 of 38 confirmed Chlamydiazyme-positive specimens, whereas the assays with the MOMP and rRNA primers detected 36 of 38 and 29 of 38 confirmed Chlamydiazyme-positive specimens, respectively. Six of 18 Chlamydiazyme-negative specimens collected from individuals whose specimens were positive by culture or immunofluorescence were positive by both plasmid-based PCRs; 4 of these were positive by PCR with the MOMP primers and 3 were positive by PCR with the rRNA primers. The results obtained with both purified DNA and genitourinary tract specimens indicated that the plasmid-based PCRs are more sensitive than bacterial chromosome-based PCRs for detecting *C. trachomatis*.**

*Chlamydia trachomatis* infections have been diagnosed by cell culture, immunofluorescence (IF), enzyme immunoassay (EIA), direct DNA hybridization, and more recently, by polymerase chain reaction (PCR) amplification of specific target sequences. Comparisons of culture, IF, and EIA have indicated that IF and EIA are generally less sensitive than culture when culture is set as the "gold standard" (6, 16, 21, 28, 29). The true sensitivities of culture and EIA have recently been revealed in studies comparing culture with two or more antigen-based detection assays in which an expanded gold standard of positivity was used. Several laboratories that have used this approach have shown that culture and EIA have sensitivities of 80 to 90% when compared with the sensitivity of an assay with an expanded gold standard (5, 13, 16, 21). PCR has been shown to have equal or greater sensitivity than culture or EIA (2, 10, 18, 19, 22–27, 35–37). The improved sensitivity of PCR has been demonstrated for several types of specimens, including male urethral swabs, female urethral and endocervical swabs, and first-void urine specimens from symptomatic and asymptomatic men (18, 19). Targets for amplification have included both the cryptic plasmid (7, 8, 10, 18, 19, 22–24, 27) and chromosomal genes, including those for the major outer membrane protein (MOMP) (2, 11, 23, 25, 33), rRNA (7, 8, 36, 37), and cysteine-rich proteins (34). Since DNA targets and conditions for amplification have differed widely among several laboratories, it is not known how well these PCR assays perform relative to one another. In order to investigate possible differences between these PCRs, we compared five different PCR assays which have been described in the literature. In this report, we show that assays which amplify the plasmid are more sensitive than those that amplify chromosomal targets for detecting *C. trachomatis* in genitourinary tract specimens.

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## MATERIALS AND METHODS

**Specimens.** Urethral swabs were collected from asymptomatic or symptomatic men presenting either to a hospital-based sexually transmitted disease clinic (12% prevalence of *C. trachomatis* infection) or a family practice clinic (1% prevalence) as part of a *Chlamydia* prevalence study. Specimens were obtained from individuals who gave informed consent under a study protocol approved by a university ethics review committee. Urethral swabs and first-void urine specimens were collected from men for testing by EIA and PCR as described previously (5). Endocervical and urethral swabs were collected from women attending a birth control or planned parenthood clinic (combined prevalence of *C. trachomatis* infection of 8%) as described previously (6, 30).

**Culture.** *C. trachomatis* was isolated in McCoy cell cultures by using a 96-well microculture system, iodine staining, and one blind passage as described previously (6).

**EIA.** Specimens for antigen detection were collected with manufacturer's collection kits and were tested by Chlamydiazyme (Abbott Diagnostics, North Chicago, Ill.) according

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to the manufacturer's instructions. Positive specimens were confirmed with the Chlamydiazyme blocking reagent (5).

**IF.** Those specimens that were collected for *C. trachomatis* detection by either culture or EIA and that gave discordant results were centrifuged at 3,000 × *g* for 20 min and sediments were stained with a fluorescein isothiocyanate-conjugated anti-MOMP monoclonal antibody (Syva Microtrak). The criterion for a positive result was five elementary bodies.

**DNA extraction.** *C. trachomatis* serovar L2, LGV 434/Bu, was propagated in McCoy cell culture, and elementary bodies were purified by differential centrifugation as described previously (20). Total bacterial or plasmid DNA was purified as described previously (1a) and was quantified by UV absorbance. The *C. trachomatis* serovar L2 plasmid cloned in pUC18 was obtained from Iain Clarke (University of Southampton, Southampton, United Kingdom).

**PCR.** Five different PCR assays targeting plasmid, MOMP, and rRNA DNAs were used in the study. The plasmid primers that were used included KL1-KL2 (KL1, 5′-TCCGGAGCGAGTTACGAAGA-3′; KL2, 5′-AATCAA TGCCCGGGATTGGT-3′), which have been described by Mahony et al. (18, 19), and T1-T2, which have been described by Claas et al. (7, 8). MOMP primers CT0005-CT06 have been described by Bobo et al. (2, 3). The second set of MOMP primers, JM15-JM16 (JM15, 5′-TTGCTTGGAGTG CTGGAGCT-3′; JM16, 5′-TCCTTAGTTCCTGTCACACC-3′) amplify a 208-bp fragment within the 1,182-bp MOMP sequence described by Stephens et al. (31). The rRNA primers were R1-R2 described by Claas et al. (7, 8). All PCR assays were initially run by using the conditions described in the original reports, including $MgCl_2$, primer, and *Taq* polymerase concentrations, annealing temperature, and number of cycles. CT0005-CT06 primers were used with 30 cycles of amplification consisting of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, since the number of cycles was not given by the authors (2, 3). The PCR assay with the JM15-JM16 primers was run with 2.5 mM $MgCl_2$, with amplification for 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C for 35 cycles. Since the conditions for the T1-T2 and R1-R2 PCR assays produced too many nonspecific products and a reduced sensitivity, $5 \times 10^{-4}$ M tetramethylammonium chloride (TMAC) was added to improve the fidelity of primer annealing (12). PCR products were analyzed by gel electrophoresis with 2% agarose gels and ethidium bromide staining. Serial dilutions of purified DNA and elementary bodies were tested by PCR. Genitourinary tract specimens collected in Chlamydiazyme collection tubes were diluted 1:10 with Chlamydiazyme specimen dilution buffer, and 10 μl was tested by PCR. Strict precautions including dedicated work areas, and aerosol-barrier pipette tips were used to prevent specimen contamination (14, 18).

## RESULTS

Evaluation of five different chlamydial PCR assays revealed striking differences in their performances. Plasmid primers KL1-KL2 amplified a 241-bp fragment, with no nonspecific products being visible on the gel (Fig. 1, lane 1). Plasmid primers T1-T2 yielded numerous nonspecific products of various sizes which obscured the predicted product of 517 bp (Fig. 1, lane 2). The addition of $5 \times 10^{-4}$ M TMAC minimized nonspecific priming events and produced the predicted 517-bp product (Fig. 1, lane 3). MOMP primers JM15-JM16 and CT0005-CT06 both gave specific products of 208 and 280 bp, respectively, with no nonspecific products being visible (Fig. 1, lanes 4 and 5). rRNA primers R1-R2 amplified a 208-bp product as well as larger and smaller products that were eliminated by the addition of TMAC (Fig. 1, lanes 6 and 7).

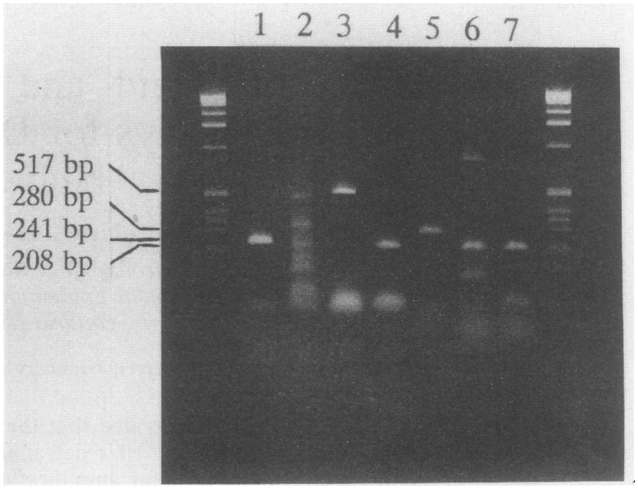


FIG. 1. PCR amplification of *C. trachomatis* DNA with various plasmid and chromosomal primers. DNA markers (1-kb ladder) are in the outside lanes. Lane 1, 241-bp product amplified with KL1-KL2 plasmid primers; lanes 2 and 3, 517-bp product amplified with the T1-T2 plasmid primers without (lane 2) or with (lane 3) TMAC added; lane 4, 208-bp product amplified with the JM15-JM16 MOMP primers; lane 5, 280-bp product amplified with the CT0005-CT06 MOMP primers; lanes 6 and 7, 208-bp product amplified with the R1-R2 rRNA primers without (lane 6) or with (lane 7) TMAC.

The sensitivities of the various PCR primer pairs were determined by testing serial dilutions of bacterial and plasmid DNAs (Table 1). Plasmid primers KL1-KL2 and T1-T2 had identical sensitivities, detecting 0.1 fg of plasmid DNA and 10 fg of total bacterial DNA. Both MOMP primer pairs had lower sensitivities than the plasmid primers; CT0005-CT06 detected 0.1 pg of bacterial DNA and JM15-JM16 detected 10 pg of bacterial DNA. The sensitivity of the rRNA primers R1-R2 was intermediate to those of the two MOMP primers, detecting 1 pg of bacterial DNA. The same pattern of relative sensitivities obtained with the plasmid- and chromosome-based PCR assays was seen with purified elementary bodies and by testing serial dilutions of two culture-positive specimens (data not shown). Both plasmid-based amplification assays were able to detect one inclusion-

TABLE 1. Sensitivities of five PCR primer pairs targeting plasmid or chromosomal DNA[a]

| Primer | Sensitivity | |
|---|---|---|
| | Plasmid DNA | Total DNA |
| Plasmid KL1-KL2 | 0.1 fg | 10 fg |
| Plasmid T1-T2 | 0.1 fg | 10 fg |
| MOMP CT0005-CT06 | ND | 0.1 pg |
| rRNA R1-R2 | ND | 1 pg |
| MOMP JM15-JM16 | ND | 10 pg |

[a] Each PCR was optimized by increasing the annealing temperature or adding TMAC, and then each reaction was run by using 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. MOMP primers JM15-JM16 were run for 40 cycles. Sensitivity was determined by testing serial 10-fold dilutions of purified plasmid and total bacterial DNA and is reported as the smallest amount of DNA that could be detected by PCR. ND, not done.

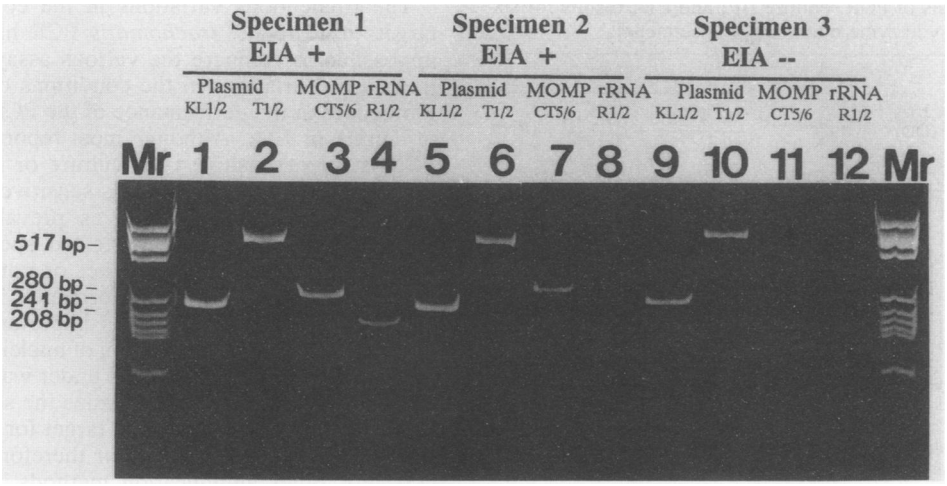


FIG. 2. PCR results for two positive and one negative Chlamydiazyme specimen by using four different primer pairs. Specimen 1 was an endocervical specimen with an absorbance of >2. Specimen 2 was an endocervical specimen with an absorbance of 0.3 (cutoff, 0.105). Specimen 3 was an urethral swab with an absorbance of 0.040 (cutoff, 0.105). The KL1-KL2 plasmid primers amplified a 241-bp product. The T1-T2 plasmid primers amplified a 517-bp product. The CT0005-CT06 MOMP primers amplified a 280-bp product. The R1-R2 rRNA primers amplified a 208-bp product. Outside lanes (Mr) contain 1-kb DNA ladder size markers.

forming unit of *C. trachomatis* serovar L2, while PCR with the MOMP and rRNA primers detected 10 to 100 inclusion-forming units (data not shown).

To further compare the sensitivities of these primers, three specimens containing different amounts of chlamydial antigen were selected by the Chlamydiazyme assay after confirmatory blocking. They were tested by PCR with plasmid, MOMP and rRNA primers (Fig. 2). One strongly positive specimen (absorbance, >2.0) was positive with all four primers, and both plasmid-based primers produced noticeably stronger bands than did either the MOMP and rRNA primers. The second specimen, with an absorbance of 0.3 (cutoff, 0.105), was positive by PCR with both plasmid primers, weakly positive by PCR with the MOMP CT0005-CT06 primers, and negative by PCR with the rRNA primers. The third specimen that was negative by Chlamydiazyme (absorbance, 0.052; cutoff, 0.105) was clearly positive by PCR with both plasmid primers but was negative by PCR with both MOMP and rRNA primers.

The sensitivities of the four PCR assays were further compared by testing 56 genitourinary tract specimens, including 38 confirmed positive specimens (35 endocervical and 3 vaginal swab specimens) and 18 negative specimens (11 endocervical, 5 female urethral, and 2 male urine specimens) by the Chlamydiazyme test. All 38 EIA-positive specimens were positive by PCR with both plasmid primers, whereas only 36 were positive by PCR with the MOMP primers and 29 were positive by PCR with the rRNA primers (Table 2). The two specimens that were missed by PCR with the MOMP primers were both endocervical specimens that had absorbances by EIA of 0.144 and 0.224 (cutoff, 0.113) and were blocked with the neutralizing antibody. The nine specimens (eight endocervical, one vaginal swab) missed by PCR with the rRNA primers had absorbances of 0.14 to 0.46 and all were blocked with the neutralizing antibody. For the 18 Chlamydiazyme-negative specimens, both plasmid-based PCRs detected six specimens as positive; four of these six specimens were also positive by PCR with the MOMP primers and three were positive by PCR with the rRNA primers. All six specimens were from women infected with *C. trachomatis*, as evidenced by at least one of the following: an accompanying culture-positive specimen or an IF-positive specimen. Table 3 shows the EIA, culture, IF, and PCR results for these six specimens.

## DISCUSSION

Our results obtained with both purified chlamydial DNA and clinical specimens demonstrated that plasmid-based PCRs are 10 to 1,000 times more sensitive than chromosome-based assays. This greater sensitivity of the plasmid-based amplified PCRs was also evident for genitourinary tract specimens, because PCR with MOMP and rRNA primers detected 7% (4 of 56) and 21% (12 of 56) fewer specimens, respectively (Table 2).

The improved sensitivity of PCR with plasmid targets may be due in part to the presence of multiple copies of the *C. trachomatis* plasmid. Estimates of the number of copies of the plasmid have varied, but recent estimates are between 7 and 10 copies per bacterial cell (32). The presence of a single MOMP gene and a limited number of two or three tandem

TABLE 2. Performances of four PCR assays for the detection of *C. trachomatis* in Chlamydiazyme-reactive and -nonreactive specimens

| Chlamydiazyme | PCR results (no. positive/no. tested) (% sensitivity)[a] | | | |
|---|---|---|---|---|
| | Plasmid | | MOMP primer CT0005-CT06 | rRNA primer R1-R2 |
| | KL1-KL2 | T1-T2 | | |
| Reactive | 38/38 (100) | 38/38 (100) | 36/38 (94.7) | 29/38 (76.3) |
| Nonreactive[b] | 6/18 (100) | 6/18 (100) | 4/18 (66.7) | 3/18 (50) |
| Total | 44/44 (100) | 44/44 (100) | 40/44 (90.9) | 32/44 (72.7) |

[a] The sensitivity of each primer was calculated by dividing the number positive by each set of primers by the total number positive by the plasmid primers multiplied by 100. For the Chlamydiazyme-reactive specimens, 38 was used as the denominator, and for the nonreactive specimens, 6 was used as the denominator, for a total of 44.

[b] Nonreactive specimens were below the Chlamydiazyme cutoff of 0.113.

TABLE 3. Analysis of PCR, culture, IF, and EIA results for six chlamydiazyme-nonreactive specimens[a]

| Specimen | Type[b] | EIA (OD)[c] | Culture | IF | PCR | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Plasmid | | MOMP CT0005-CT06 | rRNA R1-R2 |
| | | | | | KL1-KL2 | T1-T2 | | |
| BBC 472 | Cx | − (0.034) | + | + | + | + | − | − |
| BBC 552 | Ure | − (0.080) | + | + | + | + | + | + |
| SHS 5153 | Cx | − (0.103) | − | + | + | + | + | + |
| SHS 597 | Cx | − (0.098) | + | + | + | + | + | + |
| CUSS 117 | FVU | − (0.056) | ND[d] | + | + | + | − | − |
| CUSS 425 | FVU | − (0.052) | ND | + | + | + | + | − |

[a] PCR and IF were performed on the Chlamydiazyme specimens. Culture was done on a separate specimen collected before the specimen used for Chlamydiazyme was collected.

[b] Cx, endocervix; Ure, urethra; FVU, first-void urine specimen.

[c] Chlamydiazyme absorbance is indicated in parentheses (OD, optical density); the cutoff was 0.106.

[d] ND, not done.

repeats of the 16S rRNA genes (30a) are consistent with a more sensitive plasmid-based PCR assay. The fact that the PCR with the MOMP primers gave different levels of sensitivity with purified DNA (0.1 pg for the CT0005-CT06 primers and 10 pg for the JM15-JM16 primers) suggests that other factors may play a role in determining the level of sensitivity of various primers. Since sensitivity is linked to specificity, several factors that affect specificity, including primer length and concentration, GC content, 3′-terminal base, annealing temperature, $MgCl_2$, deoxynucleoside triphosphate, and *Taq* polymerase concentrations, affect the sensitivity of an individual PCR (35). Great care is therefore required in selecting primers and optimizing all parameters in order to obtain conditions that will result in a high fidelity of primer annealing with minimal primer mismatching. In our study, we first analyzed each PCR by running the reaction under the conditions described in the original report of the PCR. The T1-T2 primers gave nonspecific products, necessitating the addition of TMAC and an annealing temperature increase from 42 to 55°C. These changes dramatically improved the performance of the assay (Fig. 1). Similarly, the conditions used with the R1-R2 rRNA primers were changed by increasing the annealing temperature to 55°C and adding TMAC, which again resulted in improved specificity. In order to compare the sensitivity of each PCR, we standardized the cycles using 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C and used 35 cycles for each PCR (except the PCR with the MOMP primers JM15-JM16, which gave visibly less product, so we used 40 cycles). We have shown that the use of TMAC improved the specificity and sensitivity of PCR for detecting *C. trachomatis* when certain primers are used, but even under the same conditions, different primers gave various levels of sensitivity. TMAC was not required with all sets of primers and could be omitted, provided that other aspects of the reaction mentioned above were optimized. Although we did not use it in our study, the incorporation of a "hot start" to minimize primer mismatching has been reported to improve the sensitivity and specificity of PCR (9). During the present study, a third set of plasmid primers, set II of Ostergaard et al., was evaluated, but these primers failed to amplify the 473-bp product, as reported (24). We subsequently were informed (1) that the downstream primer was written in the reverse direction in the original report (24).

The tremendous variations in the conditions and DNA targets used for *C. trachomatis* PCR have made it nearly impossible to compare the various assays described in the literature. Variations in the conditions of PCR can explain differences in the performance of the PCR in relation to that of culture or EIA. Although most reports have shown that PCR is more sensitive than culture or EIA, some reports have indicated that PCR is less sensitive than culture or IF (24, 25). Other factors such as prevalence of infection, number and type of specimens tested, spectrum bias, and the method of analysis, viz. whether or not an expanded gold standard is used will affect performance. The knowledge that all PCRs are not equivalent is important for a number of reasons. The commercialization of nucleic acid amplification tests, and PCR in particular, is under way, and manufacturers will naturally strive to optimize the sensitivities of these tests. The choice of a plasmid target for *C. trachomatis* for the Roche Chlamydia Amplicor therefore seems warranted (15). For other amplification methods, such as the ligase chain reaction or sequence self-sustained replication, careful analysis of *C. trachomatis* DNA will also be required to select appropriate targets. The reduced sensitivity of a chromosome-based assay, i.e., the MOMP target versus a plasmid target, may present an obstacle to laboratories wanting to use a second PCR to confirm a PCR-positive result when culture or EIAs were negative. Ossewaarde et al. (23) have recently shown that not all positive results by plasmid-based PCRs are positive when tested with MOMP primers. Loeffelholz et al. (15) have also reported the detection of culture- or EIA-positive specimens that are positive by Amplicor but that cannot be confirmed by PCR with MOMP primers. These discordant results would have to be confirmed as positive by another method such as IF. For this reason, we have used a second plasmid-based PCR instead of a MOMP-based PCR for confirmation (18, 19). Despite this limitation, future tests for *C. trachomatis* could involve coamplification of both a plasmid sequence for initial detection and a MOMP sequence for confirmation and/or serovar typing.

Despite an apparent advantage of PCR of increased sensitivity over conventional tests such as culture, EIA, and IF, there remain some areas of concern. EIAs may miss some specimens containing small number of organisms, as shown by our results. We found that 6 of 18 Chlamydiazyme-nonreactive specimens with absorbances of between 0.05 and the cutoff were positive by three different PCR tests and positive by IF or culture. Williams et al. (36) have recently shown that 7 of 44 (16%) specimens tested by the Chlamydiazyme assay with readings of between 0.05 and 0.09 were positive by PCR; 12 of these were tested by IF and 8 had detectable elementary bodies. Since cutoffs for EIAs and IFs are established to provide the best combination of sensitivity and specificity, sensitivity is sometimes sacrificed for improved specificity, and therefore, most assays will miss some positive specimens. This can present a problem when a given patient has a culture-negative or EIA-negative specimen together with a PCR-positive result. These situations are generally rare, but as more laboratories begin evaluating PCR and other amplification tests, this situation will occur more frequently. Judicious application of IF staining for elementary bodies, antibody neutralization tests for EIA, and confirmatory PCR with a second primer pair should help to resolve most of the discordant results. Some situations will arise, however, in which there is a positive PCR result and all other tests are negative. In this case, collection of a follow-up specimen from the patient may be helpful. Recent

studies with cynomolgus monkeys have indicated that ocular specimens from experimentally infected animals remained positive for weeks longer when tested by PCR than by culture (11). PCR-positive but culture-negative specimens may indicate a low level of replication with a small number of organisms below the level of detectibility by culture, or alternatively, microorganisms may have been neutralized by secretory immunoglobulin A antibody (4) or the specimen lost its viability because of transport and storage (17). Detection of *C. trachomatis* by sensitive nonculture tests such as PCR should reduce false-negative results, leading to the better prevention and treatment of ectopic pregnancies, pelvic inflammatory disease, and tubal infertility.

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