JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1995, p. 251–253 Vol. 33, No. 1
0095-1137/95/$04.00+0

Influence of Volume of Sample Processed on Detection of *Chlamydia trachomatis* in Urogenital Samples by PCR

WIL H. F. GOESSENS,[1]* JAN A. J. W. KLUYTMANS,[1] NICOLE DEN TOOM,[1]
TINEKE H. VAN RIJSOORT-VOS,[1] BERT G. M. NIESTERS,[1] ERNST STOLZ,[2]
HENRI A. VERBRUGH,[1] AND WIM G. V. QUINT[3]

Departments of Clinical Microbiology and Antimicrobial Therapy[1] and Dermato-Venereology,[2] Erasmus University Rotterdam, 3000 DR Rotterdam, and Department of Molecular Biology, Diagnostic Centre SSDZ, 2600 GA Delft,[3] The Netherlands

Received 28 June 1994/Returned for modification 12 August 1994/Accepted 13 October 1994

In the present study, it was demonstrated that the sensitivity of the PCR for the detection of *Chlamydia trachomatis* is influenced by the volume of the clinical sample which is processed in the PCR. An adequate sensitivity for PCR was established by processing at least 4%, i.e., 80 μl, of the clinical sample volume per PCR. By using this preparation procedure, 1,110 clinical samples were evaluated by PCR and by cell culture, and results were compared. After discordant analysis, cell culture resulted in a sensitivity of 79.1% and PCR resulted in a sensitivity of 92.7%. Furthermore, it was shown that treatment with antibiotics immediately resulted in negative cell culture results but that PCR could give positive results up to 2 weeks posttreatment.

The PCR technique has become an important tool for the diagnosis of *Chlamydia trachomatis* urogenital infections (1, 2, 4, 6, 10, 11). Most laboratories use rather labor-intensive procedures for the isolation and concentration of target DNA in urogenital specimens (2). The phenol extraction method has a concentrating effect as well as a purifying effect. Sample volumes of 1 ml or more can easily be concentrated to 100 μl, and of this concentrated DNA, a predetermined volume is added to the PCR mixture. Claas et al. (2, 3) applied 100 μl of an initial sample volume to a final PCR analysis. By making use of this procedure, they established a PCR sensitivity of 100% and a specificity of 98.5% compared with *Chlamydia* cell culture (CC). In the investigative study of Kluytmans et al. (7), a modified DNA extraction procedure which was more suitable for processing large numbers of specimens was used. Although specificity values for both males and females were high (99.6 and 99.9%, respectively), sensitivities were low. Furthermore, the overall sensitivity correlated with the number of infectious, inclusion-forming elementary bodies (EBs), as determined by CC, indicating that this PCR assay was hampered by the presence of a limited number of DNA targets in the patient sample. In fact, only 2.2 μl of the initial sample volume was processed in the final PCR.

The present report describes the optimization of a sample preparation procedure suitable for processing large numbers of specimens and compares its clinical performance with that of CC with regard to the detection of *C. trachomatis* in urogenital specimens. Specimens were collected from 1,110 patients visiting the outpatient clinic for sexually transmitted diseases at the University Hospital of Rotterdam. Male urethral samples and female cervical samples were collected from patients who were clinically suspected of having *C. trachomatis* infections. Sample collection was performed by using a Dacron E.N.T. swab (Medical Wire and Equipment Co., Corsham, Willshire, United Kingdom), which was placed into 2 ml of 0.2 M sucrose phosphate buffer. Processing and CC procedures for the samples were performed as described previously (7). In order to determine the influence of sample preparation procedures on the sensitivity of the PCR, different DNA isolation procedures were performed on clinical samples categorized according to the number of infectious EBs present in the CC sample. The number of infectious EBs per patient sample was quantitated by microscopic determination of the number of inclusion bodies per 400-μl sample of specimen in a McCoy cell monolayer (7). Samples containing 1 to 5, 6 to 20, and more than 20 EBs per 400-μl sample volume were designated to contain small, intermediate, and large numbers of *C. trachomatis* organisms, respectively. Twenty samples from each category were tested. The following four procedures were evaluated.

Method a. For method a, a 200-μl sample of specimen was centrifuged for 10 min at 14,000 × *g* at room temperature. The pellet was resuspended in 75 μl of phosphate-buffered saline. Sixty microliters of this suspension was incubated with 10 μl of proteinase K (2.5 μg) and 10 μl of 6% Triton X-100 for 1 h at 37°C. After the solution was heated to 100°C for 10 min to inactivate the proteinase K, 100 μl of sterile water (distilled twice) was added to the sample. Ten microliters of this solution was subsequently added to 90 μl of PCR mixture.

Method b. For method b, a 30-μl sample of specimen was treated with 5 μl of proteinase K (1.25 μg) and 5 μl of 6% Triton X-100, incubated for 1 h at 37°C, boiled for 10 min, and added as a whole to 60 μl of PCR mixture.

Method c. For method c, a 200-μl sample of specimen was treated with 30 μl of proteinase K (7.5 μg) and 30 μl of 6% Triton X-100, incubated for 1 h at 37°C, and subsequently boiled for 10 min. From each lysate, 30 μl was added to 70 μl of PCR mixture.

Method d. For method d, a 400-μl sample of specimen was centrifuged for 30 min at 14,000 × *g*, and the pellet was treated with 40 μl of lysis buffer (50 mM Tris-HCl [pH 7.5], 1% Triton X-100, 1 mM EDTA, 250 μg of proteinase K per ml). After incubation at 37°C for 1 h, the lysates were boiled for 10 min and centrifuged briefly. From each lysate, 8 μl was added to 92 μl of PCR mixture. Method d is a modification of a method described initially by Ossewaarde et al. (9), and this method was applied in the clinical evaluation of 1,110 consecutive

* Corresponding author. Mailing address: Department of Clinical Microbiology and Antimicrobial Therapy, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. Phone: 011/3110/4633511. Fax: 011/3110/4633875.

TABLE 1. Sensitivities of different sample preparation procedures with clinical specimens containing small, intermediate, and large numbers of EBs in CC

Sample preparation procedure	Sensitivity (%) of PCR with samples containing the following no. of EBs in CC[a]:		
	Small	Intermediate[b]	Large[c]
a	40	30	70
b	65	40	55
c	ND[d]	55	100
d	75	100	100

[a] Twenty samples from each category were evaluated.
[b] Statistically significant differences (Fisher exact test) exist between results for methods a and d ($P < 0.001$), b and d ($P < 0.001$), and c and d ($P < 0.001$).
[c] Significant differences exist between results for methods a and c ($P = 0.020$), a and d ($P = 0.020$), b and c ($P = 0.001$), and b and d ($P = 0.001$).
[d] ND, Not Determined.

patient samples. Primers and PCR conditions were the same as those described in the paper of Kluytmans et al. (7). Discordant results were analyzed by a confirmatory PCR using primers recognizing the gene coding for major outer membrane proteins (MOMPs) (13).

The rationale for the four procedures selected is that they permit the addition of increasing initial sample volumes to the PCR without changing the method of sample processing significantly. If the dilution factors are taken into account, the actual clinical sample volumes processed in methods a, b, c, and d were 2.2, 30, 27, and 80 μl, representing 0.11, 1.5, 1.35, and 4% of the original 2-ml clinical sample volume, respectively. Another aspect of the four methods described that we studied was the time at which the lysis step took place, i.e., before (methods c and d) or after (methods a and b) the final subsample was taken for use in the PCR.

Table 1 shows the results obtained by application of the four different sample preparation procedures to clinical samples categorized according to the numbers of EBs they contained, as determined by CC. Assays of the 20 clinical samples containing large numbers of EBs showed that PCR using sample treatment procedure c or d resulted in a 100% concordance with CC results. Differences in sensitivity obtained, for instance, by methods b and c were statistically significant (Fisher exact test, $P = 0.001$). These differences are striking, as the same sample volume is applied in the final PCR in each method. This difference can be explained by the fact that in method c, 200 μl of the specimen is lysed as a whole before the sample for the final PCR is taken. In contrast, in method b the final PCR sample is taken before the lysis step. It is thus conceivable that lysis before sampling increases the chance of catching DNA available for amplification because of the release of multicopy plasmid DNA. In the case of clinical samples containing intermediate numbers of EBs, the difference in performance between methods b and c was still apparent; however, it was statistically not significant ($P = 0.527$). Analysis of the intermediate samples showed that method c missed 9 of the 20 samples. Method d, on the other hand, detected 20 of 20 samples, indicating that optimal sensitivity can only be reached by concentrating the original sample specimen. However, samples containing small numbers of EBs showed that even method d had a sensitivity limited to 75%. This limited sensitivity may have been due to sampling error, since we were dealing with samples containing less than five EBs per 400 μl of patient specimen. Statistically, only one EB or less was present in a volume of 80 μl, which was the final specimen volume applied in method d. On the basis of data obtained with samples containing large numbers of EBs and samples containing intermediate numbers of EBs, we concluded that method d constitutes the best sample treatment procedure. Method a, as applied in the study of Kluytmans et al. (7), proved to be the least sensitive procedure. In this protocol, the DNA was not purified and it was actually diluted instead of being concentrated. If the dilution factor is taken into account, only 2.2 μl of the original 200-μl sample of specimen (i.e., 0.11%) was used in the PCR assay.

TABLE 2. Results of CC and PCR for 1,110 clinical samples, obtained by using sample preparation method d

Result by PCR[a]	No. of samples with the following result by CC:		Total
	+	−	
+	79	33	112
−	8	990	998
Total	87	1,023	1,110

[a] The performance of PCR compared with that of CC was as follows: sensitivity, 90.8%; specificity, 96.8%; positive predictive value, 70.5%; and negative predictive value, 99.2%.

A prospective clinical evaluation was subsequently performed on 1,110 samples by using method d. Table 2 shows the results of this evaluation. If the CC results only are used as a "gold standard," the sensitivity and specificity of PCR were 90.8% and 96.8%, respectively. Of the 1,023 CC-negative samples, 33 were PCR positive. The results showed *C. trachomatis* prevalences to be 7.8% as determined by culture and 10.1% as determined by PCR. Eight culture-positive samples were missed by PCR.

To gain insight into the relation between the number of EBs in the culture-positive samples and PCR results, the samples were categorized into the predefined categories described above. In Table 3 it can be seen that six of the eight samples missed by PCR (i.e., 75%) contained fewer than five EBs per 400-μl sample of specimen. Of these six samples, four were available, and results obtained with them could be confirmed by PCR conducted on extracts of the corresponding CCs (data not shown). The remaining two CC-positive samples missed by PCR were in the intermediate- and large-number categories. Discrepancy analysis of these two samples performed by using a primer set aimed at the MOMP gene revealed that the intermediate-number category sample probably stayed negative because of inhibitory substances in the concentrated sample. The large-number category sample initially missed was positive with the MOMP primer set.

Furthermore, a discrepancy analysis was performed on the 33 PCR-positive, CC-negative samples by using the same

TABLE 3. Culture-positive samples categorized according to numbers of EBs

No. of EBs[a]	No. of samples	No. of PCR-positive samples	No. of PCR-negative samples[b]
Small	20	14	6 (70.0%)
Intermediate	17	16	1 (94%)
Large	50	49	1 (98.0%)
Total	87	79	8 (90.8%)

[a] Numbers of EBs determined to be present in 400-μl samples of specimen.
[b] The sensitivity of PCR is denoted in parentheses.

MOMP primer set. In this group, 23 of 33 samples were positive by the MOMP PCR. It should be realized that the MOMP gene is a single-copy gene and that this assay may, therefore, be less sensitive than a PCR procedure which targets the endogenous multicopy plasmid. In a paper of Ossewaarde et al. (9) it was demonstrated that a PCR performed with a MOMP primer set, on a twofold dilution of a serovar suspension, was fourfold less sensitive than a PCR performed with a plasmid primer set.

To help explain PCR-positive, CC-negative results, the medical records of the patients involved were analyzed for antibiotic treatment during the week preceding the time of sampling. Nine of the 33 (27.3%) PCR-positive, CC-negative patients had been treated. Seven of the nine samples from these patients were also positive in the PCR performed with the MOMP primer set. From four patients we received pretreatment samples and posttreatment follow-up samples, and it was observed that at 1 to 3 weeks after initiation of antibiotic treatment CCs were negative and PCR results were still positive. At 3 weeks posttreatment samples were PCR and CC negative for all patients. These results are in accordance with the findings of other authors (8, 12). The remaining 24 of the 33 PCR-positive, CC-negative patients had not received antibiotics, but each was screened for a *C. trachomatis* infection, since all patients were determined to be at risk on the basis of clinical and/or epidemiological criteria. After analysis of discordant results by MOMP PCR, the sensitivity and specificity of both CC and PCR were recalculated by defining another gold standard. The assumption that if CC was negative but PCR was positive with both primer sets a failure of CC had occurred was made. In addition to the 87 CC-positive samples, PCR detected 23 other true-positive samples. This adds up to 110 true-positive samples and 1,000 true-negative samples. CC detected 87 and PCR detected 102 of the 110 true-positive samples, resulting in sensitivity values of 79.1 and 92.7%, respectively. Because analysis of CC-positive, PCR-negative specimens was not performed, the specificity of CC was 100% by definition. The recalculated specificity of PCR was 99.0%.

On the basis of these data we conclude that even with PCR, a minimal volume of clinical sample (i.e., at least 4%) has to be processed to obtain an adequately sensitive assay. A few samples containing small numbers of EBs are missed. These latter findings are in accordance with the results of Roosendaal et al. (12) and Gilroy et al. (5), who also demonstrated that when cervical smears contain small numbers of EBs (<10 EBs), PCR can produce negative results.

One of the problems with the current PCR techniques for detecting *C. trachomatis* is that with the sample processing methods available, the ratio of target DNA to aspecific DNA and inhibitory substances present in the processed sample is in favor of the latter two substances. Future diagnostic assays should be designed in such a way that in the sample processing procedure, only target DNA or RNA is isolated (captured) from large volumes of patient specimens and is added in total to the PCR assay. If this strategy is followed, the sensitivity of the assay will likely increase and the problems associated with false-negative results due to inhibitory substances will be circumvented. However, even without the use of sophisticated sample processing techniques we also demonstrated that the currently applied PCR detects additional positive patients, most of whom are truly positive for *C. trachomatis* infection. However, the clinical relevance of PCR-positive, CC-negative results, especially in relation to antibiotic treatment, has to be established in further studies.

(Part of this research has been presented at the 94th General Meeting of the American Society for Microbiology in Las Vegas, Nev. [22 to 27 May 1994]. The abstract number was C-452.)

REFERENCES

1. **Bobo, L., F. Coutlee, R. H. Yolken, T. Quinn, and R. P. Viscidi.** 1990. Diagnosis of *Chlamydia trachomatis* cervical infection by detection of amplified DNA with an enzyme immunoassay. J. Clin. Microbiol. **28:**1968–1973.
2. **Claas, H. C. J., W. J. Melchers, I. H. de Bruijn, M. de Graaf, W. C. van Dijk, J. Lindeman, and W. G. V. Quint.** 1990. Detection of *Chlamydia trachomatis* in clinical specimens by the polymerase chain reaction. Eur. J. Clin. Microbiol. **9:**864–868.
3. **Claas, H. C. J., J. H. T. Wagenvoort, H. G. M. Niesters, T. T. Tio, J. H. van Rijsoort-Vos, and W. G. V. Quint.** 1991. Diagnostic value of the polymerase chain reaction for chlamydia detection as determined in a follow-up study. J. Clin. Microbiol. **29:**42–45.
4. **Dutilh, B., C. Bébéar, P. Rodriguez, A. Vekris, J. Bonnet, and M. Garret.** 1989. Specific amplification of a DNA sequence common to all *Chlamydia trachomatis* serovars using the polymerase chain reaction. Res. Microbiol. **140:**7–16.
5. **Gilroy, C. B., B. J. Thomas, and D. Taylor-Robinson.** 1992. Small numbers of *Chlamydia trachomatis* elementary bodies on slides detected by the polymerase chain reaction. J. Clin. Pathol. **45:**531–532.
6. **Griffais, R., and M. Thibon.** 1989. Detection of *Chlamydia trachomatis* by the polymerase chain reaction. Res. Microbiol. **140:**139–141.
7. **Kluytmans, J. A. J. W., W. H. F. Goessens, J. W. Mouton, J. H. van Rijsoort-Vos, H. G. M. Niesters, W. G. V. Quint, L. Habbema, E. Stolz, and J. H. T. Wagenvoort.** 1993. Evaluation of Clearview and Magic Lite tests, polymerase chain reaction, and cell culture for detection of *Chlamydia trachomatis* in urogenital specimens. J. Clin. Microbiol. **31:**3204–3210.
8. **Ossewaarde, J. M., F. H. F. Plantema, M. Rieffe, R. P. Nawrocki, A. de Vries, and A. M. van Loon.** 1992. Efficacy of azithromycin and doxycycline in therapy of cervical infections caused by different serovars of *Chlamydia trachomatis*. Eur. J. Clin. Microbiol. Infect. Dis. **11:**693–697.
9. **Ossewaarde, J. M., M. Rieffe, M. Rozenberg-Arska, P. M. Ossenkoppele, R. P. Nawrocki, and A. M. van Loon.** 1992. Development and clinical evaluation of a polymerase chain reaction test for detection of *Chlamydia trachomatis*. J. Clin. Microbiol. **30:**2122–2128.
10. **Ostergaard, L., S. Birkelund, and G. Christiansen.** 1990. Use of polymerase chain reaction for detection of *Chlamydia trachomatis*. J. Clin. Microbiol. **28:**1254–1260.
11. **Palmer, H. M., C. B. Gilroy, B. J. Thomas, P. E. Hay, C. Gilchrist, and D. Taylor-Robinson.** 1991. Detection of *Chlamydia trachomatis* by the polymerase chain reaction in swabs and urine from men with non-gonococcal urethritis. J. Clin. Pathol. **44:**321–325.
12. **Roosendaal, R., J. M. M. Walboomers, O. R. Veltman, I. Melgers, C. Burger, O. P. Bleker, D. M. Maclaren, C. J. L. M. Meijer, and A. J. C. van den Brule.** 1993. Comparison of different primer sets for detection of *Chlamydia trachomatis* by the polymerase chain reaction. J. Med. Microbiol. **38:**426–433.
13. **Stephens, R. S., R. Sanchez-Pescador, E. A. Wagar, C. Inouye, and M. S. Urdea.** 1987. Diversity of *Chlamydia trachomatis* major outer membrane protein genes. J. Bacteriol. **169:**3879–3885.