Evaluation of the *Amplicor Chlamydia trachomatis* test versus culture in genital samples in various prevalence populations

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Abstract

Objective—To evaluate a newly developed polymerase chain reaction (PCR) assay, *Amplicor C trachomatis* for the detection of *C trachomatis* in genital samples using cell culture for comparison.

Subjects—501 patients (431 women and 70 men) attending an STD clinic in Hôpital Pellegrin (high-risk population) and gynaecological clinics (low-risk population) in Bordeaux, France.

Methods—The genital samples (cervical and urethral) were tested for the presence of *C trachomatis* using the *Amplicor* test and using standard cell culture identified by the immunofluorescence test using a monoclonal antibody to *C trachomatis*. Discrepancies between the results of culture and *Amplicor* were further analysed by major outer membrane protein gene (*omp1*)-PCR of the specimens taken in transport media and by direct fluorescent antibody (DFA) staining of elementary bodies in culture transport tubes.

Results—After analysis of discrepancies, the revised sensitivity and specificity of PCR were 95.3% and 100% and the positive and negative predictive values were 100% and 99.5%, respectively.

Conclusion—The present results indicate that the *Amplicor* assay is rapid, specific and more sensitive than the culture method. This test provides an excellent non-culture method for the detection of *C trachomatis* in various prevalence populations.

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Introduction

Chlamydia trachomatis, an obligate intracellular bacterium, is a common sexually transmitted disease-causing organism. The lack of evident symptoms contributes to the further spread of the disease and the most significant consequences of chlamydial infection for women include the increased risk of pelvic inflammatory disease, tubal infertility and ectopic pregnancy.¹

Therefore, it is important to have a reliable diagnostic test. Cell culture is considered the reference method for detection of *C trachomatis* in spite of less than 100% sensitivity. It is, however, labour intensive and requires two to

three days for results. Tests using direct immunofluorescence, enzyme immunoassays and DNA probe techniques² have been developed but some lack good sensitivity compared with cell culture.³ Nucleic acid amplification methods such as polymerase chain reaction (PCR) have the potential to provide specificities equal to that of culture and with higher sensitivity. Recently, there have been several reports of the use of PCR for the diagnosis of chlamydial urogenital infections.⁴⁻⁹ Two factors limiting the application of PCR techniques are the lack of standardisation and the lack of a simple method for amplification detection.

Roche Molecular Systems (RMS, Branchburg, NJ) has developed a standardised commercial chlamydia in vitro diagnosis assay, *Amplicor™ C trachomatis* test, using DNA amplification (PCR) of the cryptic plasmid and an enzyme oligoassay for the colorimetric detection.¹⁰ Described here is the evaluation of the new *Amplicor C trachomatis* test as compared with standard cell culture in high- and low-risk populations. Discrepant results were further analysed using direct immunofluorescence and a second PCR with primer set derived from a distinct genetic region.

Methods

Patient populations and specimen collection

Genital samples were collected at two sites in Bordeaux, France. At site A, specimens were collected from a low-risk population attending several gynaecological clinics. At site B, specimens were collected from a high-risk population attending the outpatient clinic for sexually transmitted diseases of the Hôpital Pellegrin.

A total of 501 samples were collected from 431 women and 70 men in sites A (349) and B (152) from September 1992 to March 1993. Three swabs were collected from each patient, one for *Neisseria gonorrhoeae* culture and the two others were for *C trachomatis* culture and *Amplicor*. The collection order of the three swabs was randomised.

The swabs for culture were placed in a transport medium containing sucrose-phosphate buffer (2SP) with 2% fetal bovine serum without antibiotics. The specimens from site A were transported to a private medical laboratory (Laboratoire d'Analyses Médicales, rue Mandron, Bordeaux) and tested within 5 hours after collection. The specimens from site B were transported to the

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hospital laboratory and stored at -70°C prior to processing (within three days).

The swabs for *Amplicor* from site A and site B were placed into 1 ml of specimen transport medium (*Amplicor*, RMS), transported to the hospital laboratory, stored at $+4^{\circ}\text{C}$ prior to processing using the *Amplicor* test (within six days). Medical histories were taken for patients with discrepant results.

Chlamydia culture

At the private medical laboratory, cultures were performed with McCoy cells in 24-well microtitre plates. A single well was inoculated per specimen, with a single passage. At the hospital laboratory, each specimen was inoculated in duplicate onto McCoy cell monolayers on glass coverslips in vials and examined after one and two passages. Following centrifugation, the cultures were incubated for 48 hours at 37°C in the culture medium containing glucose and cycloheximide. After incubation, the plates and one coverslip were fixed and stained with fluorescein-labelled monoclonal antibodies directed against the major outer membrane protein (MOMP) (Microtrak, Syva, Palo Alto, CA). Plates and coverslips were then examined for inclusions. The cells on the remaining coverslip were removed and inoculated onto two fresh McCoy cell monolayers in vials. A specimen was considered positive if one or more inclusions were identified in either the initial or blind pass cultures. Strains from positive specimens were subcultured and conserved in 2SP medium at -70°C .

Amplicor C trachomatis test

PCR amplification and detection of PCR-amplified DNA (amplicons) were carried out according to the manufacturer's instructions. The *Amplicor* kits were kindly provided by Roche Molecular Systems France. Each specimen was tested in duplicate. Briefly, 1 ml of specimen diluent (*Amplicor*, RMS) was added to the transport tube and mixed. An aliquot (50 μl) of the specimen was mixed with 50 μl of 2x PCR buffer (*Amplicor*, Master Mix, RMS) containing dATP, dCTP, dGTP, dUTP, biotinylated primers, AmpliTaq, using a micropipette with a plugged tip or positive displacement pipette. The Master Mix with AmpErase mixture was prepared in a dedicated area protected from the specimens and the amplicons aerosol. The remainder of the specimen was stored at -20°C . One amplification ready positive control and three amplification ready negative controls were included in each experiment. The sample tray was placed into a programmable model 9600 thermal cycler (Perkin-Elmer, Roche) for 30 cycles of denaturation at 95°C and hybridisation and annealing at 60°C . Biotinylated amplicons were denatured by the addition of 100 μl of NaOH-based denaturation solution (*Amplicor*, RMS) in a dedicated area. A 25- μl aliquot of the denatured amplicon and 100 μl of hybridization buffer were combined in a well of a 96-microwell plate that also contained an oligonucleotide "capture probe".

Hybridisation was carried out for one hour at 37°C . Then microwells were washed with washing buffer and 100 μl of avidin-horse-radish peroxidase conjugate was added. Following 15 minutes of incubation at 37°C and after five washes, 100 μl of substrate A/B (4 parts substrate A to 1 part substrate B; *Amplicor*, RMS) was added. After incubation for 10 minutes in the dark at room temperature, 100 μl of stop solution (*Amplicor*, RMS) was added and the reaction was read in an automated microwell plate reader at 450 nm. The cut-off between positive and negative result was an optical density (OD) of 0.25. A valid test consisted of negative controls with OD values less than 0.25 and a positive control with an OD greater than 2.

Analysis of discrepant results

All specimens producing an *Amplicor* result discrepant with culture were amplified by using PCR primers CT1/CT2 directed against the *C trachomatis omp1* gene¹¹ on the two transport medium.

Different amplification protocols were used for each transport medium, 2SP or *Amplicor* specimen transport medium. For the specimens in the 2SP transport medium, lysis, DNA amplification and detection were performed following published procedures with some modifications.^{11,12} Specimens were vortexed and 100 μl was mixed with 200 μl of lysis buffer [10 mM Tris-HCl, 1 mM EDTA (pH 7.5), 0.1% triton X100, 0.1 mg/ml proteinase K] and heated at 55°C for 90 minutes and then for 30 minutes at 95°C to inactivate the proteinase K. The amplification reaction was performed in a 50 μl -volume containing 10 μl of this prepared sample, 10 mM Tris-HCl (pH 9.5), 50 mM KCl, 0.1% triton X100, 1.5 mM MgCl_2 , 200 μM (each) dATP, dTTP, dGTP and dCTP, 1 μM of each primer and 2.5 U of *Taq* DNA polymerase (Promega, Madison, WI). Three drops of mineral oil were added to prevent evaporation. The amplification was performed in a PCR thermocycler (Perkin-Elmer, Cetus) and each cycle contained a denaturation step at 94°C for 1 minute, a primer annealing step at 55°C for 1 minute and an elongation step at 72°C for 1 minute, for 35 cycles. The amplified samples were electrophoresed on a 8% polyacrylamide gel. For confirmation of the specificity of the PCR products, a restriction enzyme digestion by *EcoRI* was used. The DNA fragments (77 and 52 bp) were separated by 8% polyacrylamide gel electrophoresis. DNA was visualised by ultraviolet fluorescence after staining with ethidium bromide.

The samples in *Amplicor* specimen transport medium, were transported to Roche Molecular Systems in Basel (Switzerland). The procedure was essentially identical to that used for amplification of the cryptic plasmid, except for the use of biotinylated *omp1*-derived primers, and the use of a *omp1* DNA-specific capture probe fixed to plate wells. For culture-positive and *Amplicor*-negative specimens, the *Amplicor* test was repeated on

diluted specimens before concluding. A negative *omp1*-PCR result on specimens collected in the *Amplicor* specimen transport medium indicated the presence of a PCR inhibitor or a sampling error. A positive-*omp1*-PCR result on specimens collected in the 2SP transport medium indicated a culture true positive result. The isolated strains were tested with *Amplicor* test to verify the presence of the plasmid. The samples consisted of 10 µl of the 2SP medium containing the strain, mixed with 1 ml of *Amplicor* specimen transport medium.

For culture-negative and *Amplicor*-positive specimens, a positive-*omp1*-PCR result whatever the transport medium, confirmed the *Amplicor*-positive result and indicated a culture false-negative result. A negative-*omp1*-PCR result indicated that the initial *Amplicor* result was a false positive result. In addition, a DFA test was performed on the 2SP transport medium. After centrifugation of the 2SP transport medium, 1 µl of the resulting pellet was placed on a slide, fixed with methanol for five minutes and stained with *C trachomatis* monoclonal antibodies (Syva, Microtrak) for the direct detection of elementary bodies (EBs). A specimen was considered positive if at least five EBs were identified. A summary of the protocol is given in table 1.

Statistical analysis

A sample was considered true positive if either the cell culture was positive or if the cell culture being negative, both PCR tests, plasmid and *omp1*, were positive. A true negative sample was a sample for which the cell culture and the PCR tests were negative. However, a negative cell culture result in a treated patient was considered as true negative culture in spite of a positive *Amplicor* result. Sensitivity was calculated as the percentage of positive cell cultures or PCR tests among the true positive samples. Specificity was calculated as the percentage of negative cell cultures or PCR tests among the true negative samples.

Table 1 Flow chart of *Chlamydia trachomatis* detection

1—Specimen collection
1-1 two sites—site A, gynecological clinics
—site B, STD clinic
1-2 two swabs from 501 patients (349 site A, 152 site B), one in 2SP transport medium, one in specimen <i>Amplicor</i> transport medium
2—Specimen analysis
2-1 <i>C trachomatis</i> culture in a private laboratory for specimens from site A and in hospital laboratory for specimens from site B
2-2 <i>C trachomatis Amplicor</i> in hospital laboratory for all specimens
3—Analysis of discrepant results
3-1 <i>Amplicor</i> + /culture –
—On <i>Amplicor</i> specimen transport medium repeat <i>Amplicor omp1</i> -PCR
—On 2SP transport medium <i>omp1</i> -PCR
DFA test
3-2 <i>Amplicor</i> – /culture +
—On specimen <i>Amplicor</i> transport medium repeat <i>Amplicor</i> on diluted specimen <i>omp1</i> -PCR
—On 2SP transport medium <i>omp1</i> -PCR

Positive and negative predictive values were calculated according to Bayes' rules¹⁴ by using the true prevalences calculated according to the criteria mentioned above.

Results

A total of 501 patients were evaluated for *C trachomatis* using the *Amplicor* assay. Forty-one specimens were *Amplicor* positive and 460 were *Amplicor* negative. In three out of 27 experiments, the OD values of the positive control were lower than 2 and the tests had to be considered invalid. After a second test, the OD values of the positive control became greater than 2 and the outcome of the samples remained unchanged. When analysing the results of the specimens tested in duplicate, 491 pairs given concordant results, only 10 pairs yielded two borderline positive-negative PCR results. These samples were tested again and were considered negative. Concerning the culture method, 30 specimens were positive and all positive specimens were always detected in the initial culture.

The comparison of cell culture and *Amplicor* test results is shown in table 2. Twenty eight specimens were *Amplicor* and culture positive, 458 specimens were negative in both tests, two were culture positive and *Amplicor* negative, and 13 were culture negative and *Amplicor* positive. Details of results of the different populations are shown in table 2. The *Amplicor* results of the 28 positive concordant specimens ranged from 0.3 to above 3 OD units. Of these, 27 yielded OD values above 3. In one case, the *Amplicor* assay was initially negative with an OD value below the cut off (repeated three times) and became positive with an OD value >3 after dilution of the sample. Therefore, the specimen was considered positive by both techniques. The specimens which were negative using culture and *Amplicor* yielded OD values of less than 0.25 (0.06 to 0.239).

The prevalence of *C trachomatis* as measured by positive cell culture was 5.98% overall, 4% in site A and 10.5% in site B (table 3). On the basis of these data, the overall sensitivity of the *Amplicor* assay was 93.3%, its specificity was 97.2%, its PPV was 68.2% and its NPV was 99.5%.

There were 15 discrepant results: 13 were

Table 2 Comparison of *Amplicor* test and cell culture results for *C trachomatis* detection

	No of samples using <i>Amplicor</i> test	
	Positive	Negative
All patients (n = 501)		
Culture positive	28	2
Culture negative	13 (8)*	458
Site A (n = 349)		
Culture positive	14	0
Culture negative	6 (3)*	329
Site B (n = 152)		
Culture positive	14	2
Culture negative	7 (5)*	129

(*) number of antibiotic-treated patients.

positive by *Amplicor* and negative by culture with OD values between 1.6 and >3, and two were positive by culture and negative by *Amplicor*. Of the 13 specimens which were *Amplicor*-positive and culture-negative, 13/13 were PCR positive in *Amplicor* specimen transport medium by the alternative *omp1*-PCR and 9/11 in the 2SP transport medium, 3/10 had five or more EBs in DFA of spun culture 2SP transport medium vials. In eight cases, the patients were treated for a suspected chlamydial infection. The details of these results are presented in table 4. These 13 discrepant results must be considered as *Amplicor* true positive results. The prevalence of *C trachomatis* as measured by the positive *Amplicor* assay was 8.1% overall, 5.7% in site A and 13.8% in site B (table 3). The two *Amplicor* negative, culture-positive specimens, were reamplified from the *Amplicor* specimen transport medium using the *Amplicor* assay and the *omp1*-PCR and the results remained the same, even after dilution. In addition, the two specimens in the 2SP transport medium were positive by using *omp1*-PCR and the two strains isolated from these specimens were

positive by using *Amplicor* assay. These results indicated that the *Amplicor* test had failed and were considered to be false-negative *Amplicor* results. The comparison of cell culture and *Amplicor* test results after analysis of discrepant results is shown in table 5. The adjusted sensitivity, specificity, PPV and NPV of the *Amplicor* assay increased to 95.3%, 100%, 100% and 99.5%, respectively (table 3). When analysing the results by sites, the sensitivity, specificity, PPV and NPV were 100%, 100%, 100%, 100% in site A and 91.3%, 100%, 100%, 98.4% in site B, respectively. The sensitivity of cell culture for all the samples in this study compared with true positive samples was 85.7%.

Discussion

DNA amplification techniques are rapidly gaining importance in diagnostic microbiology, especially in detection of *C trachomatis* because the sensitivity of the PCR technique is estimated to be higher than that of culture. However, several steps such as sample pretreatment, amplification parameters, analysis and control of specificity of PCR products are required to optimise PCR test results. Comparison of the performance of published PCR tests is difficult. An improvement has been achieved with the development of a standardised commercial PCR diagnosis-kit. The *Amplicor C trachomatis* test involves a simple sample processing method, a rapid DNA amplification of the common *C trachomatis* plasmid and a colorimetric microwell DNA hybridisation. We found the assay simple and quick to perform and in the vast majority of cases it gave very clear results, well above or below the cut-off level. Of the 41 *Amplicor*-positive specimens, only one yielded a borderline OD value of 0.3. This specimen contained only one inclusion in culture. Out of the 460 *Amplicor*-negative specimens, 450 yielded OD values of less than 0.25. Only 10 specimens tested in duplicate gave discrepant results. These results show that it is not necessary to test the specimens in duplicate systematically, but all results, yielding a borderline OD value, have to be tested again.

In the present study, the *Amplicor* test had a sensitivity superior to that of culture after resolution of discrepant results. In processing 501 specimens, there were no false-positive PCR results. Although the *Amplicor* test failed to detect three culture-positive specimens, it identified 13 true-positive specimens that culture failed to detect. In the culture-positive *Amplicor*-negative cases, the explanation for this result could be either a false-positive cell culture, because of contamination or a false-negative PCR test due to either PCR inhibitor or the presence of plasmidless strains or a sampling error. The fact that the alternative *omp1*-PCR tests were positive on culture transport medium, and on the two isolated strains, rules out the possibility that the cultures were false-positive and these isolates lacked the *C trachomatis* cryptic plasmid.

Table 3 Performance of Amplicor test and cell culture

	Prevalence	Sensitivity	Specificity	Predictive value	
				Positive	Negative
All samples					
Amplicor	8.10%	95.3%	100%	100%	99.5%
Cell culture	5.98%	85.7%	100%	100%	98.9%
Site A					
Amplicor	5.7%	100%	100%	100%	100%
Cell culture	4%	82.3%	100%	100%	99.1%
Site B					
Amplicor	13.8%	91.3%	100%	100%	98.4%
Cell culture	10.5%	87.5%	100%	100%	98.5%

Table 4 Details of the 13 discrepant results, Amplicor + / Culture -

No of sample	omp1-PCR (Amplicor specimen transport medium)	omp1-PCR (2SP)	DFA (2SP)	Antibiotic treatment
1	+	+	-	-
2	+	ND*	ND	+
3	+	+	-	+
4	+	+	-	-
5	+	ND	ND	-
6	+	+	-	+
7	+	+	-	+
8	+	-	ND	-
9	+	-	-	+
10	+	+	+	+
11	+	+	-	+
12	+	+	+	+
13	+	+	+	-

ND*: not done.

Table 5 Comparison of Amplicor test and cell culture results after analysis of discrepant results

	Amplicor	Culture
True positive	41	30
True negative	458	466*
False positive	0	0
False negative	2	5

*Including eight treated patients Amplicor+/culture -.

The nature of the PCR inhibitors was not determined but in one case, after dilution of the specimen containing more than 20 inclusions per field, the *Amplicor* test became positive. It is possible that the presence of too much DNA matrix inhibits amplification. The question of the necessity to dilute all specimens before interpretation of results arises. Given that in this study, only one specimen in 501 required dilution, it is probably not necessary to dilute specimens routinely. Another possibility is that the two remaining false-negative *Amplicor* specimens resulted from swab to swab variability. The 13 culture-negative *Amplicor*-positive specimens were considered true-positive PCR results by using a second PCR assay with a different pair of primers. The use of two primer sets allows one to confirm these results.¹⁵ Ossewaarde⁶ suggested that the PCR with primers detecting at least two different specific chlamydial genes could be considered a new gold standard for laboratory use. A contamination is always possible but in our laboratory, we used separate areas for specimen processing, PCR set up, and product analysis to minimise carryover contamination. In addition, to avoid an aerosol contamination of a new clinical specimen with previously amplified DNA, the *Amplicor C trachomatis* test incorporate the use of AmpEraseTM containing uracil-N-glycosylase (UNG).

The results of the PCR required careful interpretation, however, with additional data such as medical history and previous use of antibiotics. Indeed, after treatment, the samples remained PCR test positive while cell culture samples became negative, and remained so for at least three weeks without further treatment.^{9,13} The PCR test and also DFA test can detect non viable organisms and some authors have suggested that when the PCR test is used for test of cure, an interval of three weeks after treatment course is required. In the present study, out of 13 false-negative culture cases, eight may be explained by a tetracycline treatment. In the other five cases, the culture was falsely negative and its sensitivity was 85.7%. This result is higher than those of some published studies. In these studies, sensitivity of the culture method ranged from 65.2% to 77.5%^{4,6,7} compared with PCR, but no clinical information was given. The advantage of PCR over culture for the eight patients who were suspected to be infected by *C trachomatis* and treated as such, is that the results of the PCR affirmed the diagnosis of chlamydia infection.

Two studies, reporting the evaluation of the *Amplicor C trachomatis* test in the USA, have been published. The first compared this test

with culture in 503 cervical specimens and with *Chlamydiazyme ELA* in 375.¹⁰ The second compared it with culture of urethral swabs in 530 male urine samples.¹⁶ In cervical specimens, our results are similar showing a high sensitivity, superior to 95% and a specificity near to 100%. In conclusion, these results show that the *Amplicor C trachomatis* test is more sensitive than the cell culture method, whatever the population studied. This test is simple, rapid, highly specific and sensitive and can be used for the rapid diagnosis of *C trachomatis* urogenital infections. It is certainly one of the best non-culture methods.

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