Original
articleDifferences in the sensitivity of the Amplicor
Chlamydia trachomatis PCR assay

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Purpose: To evaluate the use of the Amplicor PCR assay in two European centres (Amsterdam and Vienna) as a standard for amplification assays in comparative studies.

Study design: Both centres used the Amplicor PCR assay and their own standard diagnostic methods as the reference assay. Discrepant results were further analysed by an *Omp1*-PCR assay. In total 805 female patients and 614 male patients were included in the studies.

Results: The prevalence of *Chlamydia trachomatis* infections in female patients was 10.0% in Amsterdam and 2.5% in Vienna and in male patients 13.7% and 10.8%, respectively. The mean sensitivity of the Amplicor PCR assay with cervical specimens from female patients was 92.5% and the mean specificity was 99.2%. For the reference assays the mean values were 87.5% and 100.0% respectively. The mean sensitivity of the Amplicor PCR assay with urine specimens from male patients was 70.3% and the mean specificity was 97.6%. For the reference assays these values were 64.9% and 100.0% respectively. The mean sensitivity of the Amplicor PCR assay with urethral specimens from male patients was 67.6% and the mean specificity was 98.5%. For the reference assays these values were 62.2% and 100.0% respectively. Of all specimens together, 1.0% showed an OD value between 0.25 and 0.5 in the Amplicor PCR assay and had to be retested. The sensitivity of the Amplicor PCR assay was less than could be expected from previous studies comparing amplification assays under similar conditions. Also, the sensitivity of the Amplicor PCR assay varied considerably between study centres using urine specimens from male patients—45.9% and 94.6%, respectively.

Conclusion: The Amplicor PCR assay cannot be considered as a standard for amplification assays in comparative studies.

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Introduction

Chlamydia trachomatis is considered to be one of the major causes of sexually transmitted diseases and as such a major public health problem.¹ Direct and indirect costs of chlamydial infections are substantial and justify requests for more attention and a stronger multidisciplinary approach.^{2,3} In many countries there is a need for a better definition of the extent of the chlamydial epidemic in different populations.³ Since clinical symptoms and signs of *C trachomatis* infection are not specific, laboratory methods are required for a definitive diagnosis of *C trachomatis* infections for treatment and contact tracing. These methods include several direct and indirect techniques.⁴ None of the non-culture techniques, however, equals the sensitivity and specificity of optimal culture techniques, with the possible exception of the direct immunofluorescent assay. However, since this method requires highly skilled technicians, it is reserved for specialised research laboratories and has not been generally implemented as a routine diagnostic method.⁵ Reports claiming a high sensitivity and specificity of non-culture techniques have been disputed.⁶ When control programmes succeed and, as a result, prevalences decline there is a need for new detection techniques with higher sensitivities and, above all, higher specificities.⁷ The cell culture method has been considered the “gold standard” for detection of *C trachomatis* for a long time, although it is very laborious and its sensitivity is estimated to be

only in the range of 70–95%, even in experienced laboratories.⁸ Also, a substantial laboratory to laboratory variation in culture practices has been noted.⁹ The introduction of specific DNA amplification methods allowed the development of diagnostic methods for all kinds of micro-organisms, including fastidious and non-cultivable agents.^{10,11} Many reports have been published on homemade polymerase chain reaction (PCR) assays.¹² Since most laboratories have developed a different PCR protocol, comparison of results is difficult. To make valid comparisons standardisation of assays is necessary.¹³ Commercially available assays could provide a way to standardisation. The Amplicor PCR assay (Roche) is the first commercially available amplification assay.¹⁴ Here we report our experience with this assay in two different European centres.

Materials and methods

COLLABORATING CENTRES AND CLINICAL SPECIMENS

In two cities, Amsterdam and Vienna, the Amplicor PCR assay was evaluated in a routine diagnostic setting serving outpatient clinics for sexually transmitted diseases (STDs). The study population consisted of patients attending the STD clinics for a new consultation.

In Amsterdam 261 females and 270 males, who attended consecutively the STD outpa-

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tient clinic, participated in the study. From male patients, one urethral swab was taken for the Chlamydiazyme assay (Abbott) and a second for the Amplicor PCR assay. Male patients were also asked to urinate and 10 ml of the first void urine was sampled for the Amplicor PCR assay. Specimens were stored at 4°C and transported to the laboratory within 4 hours. From female patients, a cervical and a urethral swab were taken for the Chlamydiazyme assay and a second cervical and urethral swab for the Amplicor PCR assay. The order in which the swabs were taken was reversed halfway through the study.

In Vienna, 240 female patients participated in the first group comparing the Amplicor PCR assay with the PACE 2 (Gen-Probe) assay and 304 female patients participated in the second group comparing the Amplicor PCR assay with the MicroTrak enzyme immunoassay (Syva). In both groups cervical swabs were taken and the order in which the swabs were taken was reversed one or more times during the study. From 344 male patients urethral swabs were taken for the Amplicor PCR assay and for the PACE 2 assay. Also, first void urine was obtained for testing with the Amplicor PCR assay and with the MicroTrak enzyme immunoassay. The order in which the swabs were taken was changed several times during the study.

Detection of *Chlamydia trachomatis*

The swabs taken for the Amplicor PCR assay were placed in the transport medium and agitated vigorously. The swab was removed from the medium after expressing the liquid from the swab against the side of the tube. The assays were carried out according to the instructions of the manufacturer. When the optical density (OD) value of a specimen was less than 0.250 the specimen was considered negative and when the OD value was greater than 0.500 the specimen was considered positive. When the OD value was between 0.250 and 0.500 the assay was repeated. When the OD value of the repeated test was greater than 0.250, the specimen was considered positive, otherwise it was considered negative.

The swabs taken for the Chlamydiazyme assay were stored at 4°C and processed according to the instructions of the manufacturer. A blocking test was performed on all positive specimens. A reduction in the observed OD of 50% or more was considered a positive result. The result of the blocking test

was reported as the result of the Chlamydiazyme assay. The swabs taken for the PACE 2 and the MicroTrak assay were stored and processed, and results were reported according to the instructions of the manufacturer. Positive results were confirmed by a blocking confirmation test. The results of the blocking tests were reported as the final results.

Analysis of discrepancies

In case of any discrepancy the tests were repeated using the same algorithm as in the first run. When discrepancies were still present, the Amplicor specimens were frozen at -20°C, shipped to the manufacturer, and analysed by the manufacturer for the presence of chlamydial DNA by a PCR assay using primers amplifying a sequence from the gene encoding the outer membrane protein 1 (*Omp1*-PCR). The results of this test were considered decisive in determining the presence of *C. trachomatis* in a specimen.

Statistical analysis

In each population the "gold standard" was defined as confirmed positive cases. A case was considered confirmed positive when at least two independent assays (for example, the Amplicor PCR assay and the MicroTrak assay) reacted positively with one or more specimens from that patient after resolution of discrepant results. The assay statistics—sensitivity, specificity, and positive and negative predictive values—were calculated as described by Griner *et al.*¹⁵ from 2 × 2 tables. These statistics were calculated for each centre individually and for the combined results. The differences in results of the Amplicor PCR assays and the reference assays were analysed by McNemar's test. A *p* value of less than 0.05 was considered statistically significant. The results of the Amplicor assay with female cervical specimens and those of confirmed positive cases from both study centres were analysed in one model by logistic regression. Sensitivity, specificity, predictive values, and their respective 95% confidence limits were calculated as described by Coughlin *et al.*¹⁶ The same analysis was carried out for male urine specimens.

Results

DETECTION OF *C. TRACHOMATIS* IN CLINICAL SPECIMENS

The definition of a confirmed case mentioned in the methods section was used to estimate the prevalence of *C. trachomatis* infections in both study populations. The prevalence in females ranged from 2.5% in Vienna to 10.0% in Amsterdam and the prevalence in males from 10.8% in Vienna to 13.7% in Amsterdam (table 1). Overall, the prevalence of confirmed cases was higher in males than in females. The definition of a confirmed case was used as a reference to calculate the sensitivity, specificity, and predictive values of each assay employed in the separate studies. These values were calculated from the respective 2 × 2 tables. The sensitivity of the Amplicor assay

Table 1 Prevalence of confirmed infections (that is, at least two independent assays positive) with *Chlamydia trachomatis* in females and in males

Sex and study centre	Number of patients	Prevalence (%)
Females:		
Amsterdam	261	10.0
Vienna 1	240	2.5
Vienna 2	304	2.6
All females	805	5.0
Males:		
Amsterdam	270	13.7
Vienna	344	10.8
All males	614	12.1
All patients	1419	8.0

Table 2 Sensitivity, specificity, and predictive values calculated from 2 × 2 tables of the results of the detection assays using cervical specimens from female patients relative to confirmed cases (that is, at least two independent assays positive)

Study centre	Assay	Sensitivity (%)	Specificity (%)	Predictive values (%)	
				Positive	Negative
Amsterdam	Amplicor	92.3	99.1	92.3	99.1
	Chlamydiazyme	88.5	100.0	100.0	98.7
Vienna 1	Amplicor	100.0	98.3	60.0	100.0
	PACE 2	100.0	100.0	100.0	100.0
Vienna 2	Amplicor	87.5	100.0	100.0	99.7
	MicroTrak	75.0	100.0	100.0	99.3
All centres	Amplicor	92.5	99.2	86.0	99.6
	Combined reference assays	87.5	100.0	100.0	99.4

Table 3 Sensitivity, specificity, and predictive values calculated from 2 × 2 tables of the results of the detection assays using specimens from male patients and confirmed cases (that is, at least two independent assays positive)

Study centre	Assay	Specimen	Sensitivity (%)	Specificity (%)	Predictive values (%)	
					Positive	Negative
Amsterdam	Amplicor	urine	94.6	98.3	89.7	99.1
	Amplicor	urethra	64.9	97.9	82.8	94.6
Vienna	Chlamydiazyme	urethra	59.5	100.0	100.0	94.0
	Amplicor	urethra	70.3	99.0	89.7	96.5
Vienna	PACE 2	urethra	64.9	100.0	100.0	95.9
	Amplicor	urine	45.9	97.1	65.4	93.7
All centres	MicroTrak	urine	70.3	100.0	100.0	96.5
	Amplicor	urine	70.3	97.6	80.0	96.0
All centres	Combined reference assays	urine	64.9	100.0	100.0	95.4
	Amplicor	urethra	67.6	98.5	86.2	95.7
All centres	Combined reference assays	urethra	62.2	100.0	100.0	95.1

Table 4 Sensitivity, specificity, and predictive values of the Amplicor PCR assay using cervical specimens calculated by logistic modelling of the results relative to confirmed cases (that is, at least two independent assays positive)

Study centre	Sensitivity (%) (95% CI*)	Specificity (%) (95% CI)	Predictive values (%)	
			Positive (95% CI)	Negative (95% CI)
Amsterdam	92.6	96.4	92.6	99.2
	73.6–98.3	96.4–99.8	81.8–97.2	97.7–99.7
Vienna 1	98.2	98.2	58.9	100.0
	92.5–99.6	92.8–99.6	81.7–90.0	99.7–100.0
Vienna 2	79.8	99.8	91.2	99.5
	37.6–96.3	98.7–100.0	72.5–97.6	97.6–99.9
All centres	92.5	99.2	86.0	99.6
	79.2–97.6	97.5–99.8	72.2–93.6	99.1–99.8

*CI = Confidence interval.

Table 5 Sensitivity, specificity, and predictive values of the Amplicor PCR assay using urine specimens from males calculated by logistic modelling of the results relative to confirmed cases (that is, at least two independent assays positive)

Study centre	Sensitivity (%) (95% CI*)	Specificity (%) (95% CI)	Predictive values (%)	
			Positive (95% CI)	Negative (95% CI)
Amsterdam	81.2	96.2	77.1	97.0
	71.2–88.4	93.5–97.8	60.3–88.1	93.6–98.6
Vienna	59.3	98.7	84.4	95.3
	41.1–75.2	97.3–99.4	73.8–91.2	91.3–97.5
Both centres	70.3	97.6	80.0	96.0
	58.9–79.6	96.1–98.5	68.5–88.0	92.9–97.8

*CI = Confidence interval.

ranged from 87.5% to 100% using cervical specimens, while the specificity was always higher than 98% (table 2). Using urethral specimens from males the sensitivity of the Amplicor assay ranged from 64.9% to 70.3% and using urine specimens from 45.9% to 94.6% (table 3). The specificity was always higher than 97%. A logistic regression model was calculated combining all data from female cervical specimens and from male urine specimens separately. This analysis provides a more reliable estimate of a 95% confidence interval of each statistic. Thus, the confidence interval of the sensitivity and the specificity ranged

from 79.2% to 97.6% and from 97.5% to 99.8% respectively for female cervical specimens (table 4), and from 58.9% to 79.6% and from 96.1% to 98.5% respectively for male urine specimens (table 5).

Analysis of specimens with OD values between 0.25 and 0.5 in the Amplicor PCR assay

In 2033 tests the number of specimens with OD values between 0.25 and 0.5 was 20 (1.0%; seven urine specimens and eight urethral specimens from male patients, and five cervical specimens from female patients). Of these specimens six were not available for retesting. Of the remaining 14 available specimens seven reacted negatively and seven reacted positively on retesting in the Amplicor assay.

Analysis of discrepancies

Specimens from females In the Amsterdam study, specimens from two patients reacted negatively in the Amplicor PCR assay and positively in the Chlamydiazyme assay. One of these reacted positively after retesting, and both reacted positively in the *Omp1*-PCR assay. Specimens from five patients reacted negatively in the Chlamydiazyme assay and positively in the Amplicor assay. Three of these reacted positively in the *Omp1*-PCR assay.

In the Vienna studies specimens from six patients reacted positively in the Amplicor PCR assay only. Three of these specimens had an OD value initially larger than 1.0 in the Amplicor PCR assay, but less than 0.1 after retesting. The OD value of another specimen was initially 0.25 and after retesting 3.0. The two other specimens reacted positively in the *Omp1*-PCR assay. From one patient the specimen reacted positively in the MicroTrak assay only. After retesting the corresponding specimen reacted positively in the Amplicor PCR assay (OD value 1.982).

Specimens from males In the Amsterdam study, urethral specimens from five patients reacted positively in the Amplicor PCR assay only. Upon retesting, four reacted negatively (all four had an OD value larger than 0.5 in the first test) and one remained positive. These specimens were not analysed further. Urethral specimens from 13 confirmed positive patients reacted initially negatively in the Amplicor PCR assay. Seven of these were positive on retesting in the Amplicor PCR assay. Urethral specimens from 15 confirmed patients reacted negatively in the Chlamydiazyme assay. Four urine specimens reacted positively in the Amplicor PCR assay only. Three of these specimens reacted negatively in the *Omp1*-PCR assay and one was not available for retesting. Urine specimens from two confirmed patients reacted negatively in the initial Amplicor PCR assay, after retesting, and in the *Omp1*-PCR assay. The urethral swabs of these patients reacted positively in the *Omp1*-PCR assay.

In the Vienna study, specimens from 12 patients reacted positively in the Amplicor

PCR assay only. Seven of these had an initial OD value larger than 0.5, but reacted negatively after retesting. Five reacted positively, when retested, but further confirmation data was not available. Eleven of the 20 urine specimens from confirmed positive patients that were negative in the Amplicor PCR assay were further analysed. Seven of these reacted positively when retested, two others reacted positively in the *Omp1*-PCR assay, while another two reacted negatively.

EVALUATION OF THE AMPLICOR PCR ASSAY

The studies were combined by type of specimen to calculate overall statistics. These are mentioned in tables 2–5. Altogether, 1419 specimens were analysed. All these specimens combined yielded an overall sensitivity of 78.1% (95% CI 69.6% to 84.7%), specificity of 98.5% (97.7% to 99.1%), and positive and negative predictive values of 82.4% (74.1% to 88.5%) and 98.1% (96.9% to 98.9%), respectively.

The results obtained with urethral specimens from males in the Amplicor PCR assay from each of the two studies separately and from both studies combined were not statistically significantly different from those obtained in the reference assays (McNemar's test; p values > 0.05).

The results obtained with urine specimens from males in the Amplicor PCR assay were statistically significantly different from those obtained in the reference assay in the Amsterdam study and in both studies combined (McNemar's test; $p < 0.001$ and $p < 0.05$, respectively), but not in the Vienna study.

The results obtained with cervical specimens from females in the Amplicor PCR assay and in the reference assays were not statistically significantly different in each study separately, or in all studies combined (McNemar's test; $p > 0.05$).

Discussion

Many studies have been published on the application of PCR techniques in the diagnosis of *C trachomatis* infections. Most papers deal with homemade assays. Since most of these assays have a different format, comparison of their results is difficult.¹² With the introduction of the commercially available Amplicor PCR assay¹⁴ standardisation of this type of detection technique could be possible.

The analytical sensitivity of the Amplicor PCR assay is a factor of 1000 higher than that of other non-culture, non-amplification assays. The detection limit is two to four elementary bodies.¹⁷ This high analytical sensitivity should facilitate the detection of low grade infections.

Previous reports describe three main applications—urethral swabs and urine from males, and cervical swabs from females. Using cervical specimens sensitivity and specificity are reported to be equal to or greater than 97% and 99.7% respectively,^{14 18–21} except in one study.²² In this study they were reported as 88% and 99.2% using a premarket kit and

60% and 99% using the commercially available kit. This study was carried out in Seattle, USA, in a research centre with an excellent implementation of the cell culture method. The reason for this discrepancy is not yet clear. Using urine or urethral swabs from males these values are reported to be equal to or greater than 95% and 99.8% respectively.^{21 23 24}

The prevalence values observed in our studies were similar to those reported before in the same centres.^{25 26} The prevalence of *C trachomatis* infections in females in Vienna ($< 5\%$) was lower than that in females in Amsterdam ($> 10\%$). Since the prevalence in males in both cities was comparable ($> 10\%$), this is probably related to differences in the populations attending the clinics. Previous data from the same female population in Amsterdam suggest that the Amplicor PCR assay detects only 50% of the additional positive specimens using the Chlamydiazyme assay as the baseline compared with an optimised homemade PCR assay in cervical specimens.²⁶ Using both urine and cervical specimens from female patients and a ligase chain reaction even more additional confirmed cases were found in this population.²⁷

The choice of a reference method when the true disease status is unknown is difficult. Since amplification methods are usually more sensitive than traditional methods, the use of these traditional methods as reference methods is not appropriate. Laboratory techniques are used to aid in the clinical diagnosis of patients. Therefore, we used a reference method that was considered to approximate the true disease status of the patient as much as possible. The sensitivity and specificity were designed to characterise straightforward 2×2 tables. Analysis by the logistic regression model yields strata specific estimates, that differ slightly from the results calculated from 2×2 tables for each centre, but these estimates include variation between the centres and probably provide a better estimate of the true values.¹⁶ Also, a confidence interval can be calculated based on this model.

The results of only one individual study were statistically significantly better than the reference method. Combining the results from all studies into one study the Amplicor assay was significantly better than the reference method only using urine specimens from males. In the Vienna study detection in urine specimens performed badly. All procedures were validated by Roche personnel. The reason for this observation remains unclear.

In these studies, not all specimens with discrepant results were available for further analysis. Confirmation of positive results in the Amplicor PCR assay was not always carried out. Therefore, the true values of sensitivity and specificity could be higher than those reported here.

Two types of problems were noted during these studies—false negative results and false positive results with an OD value less than 0.5. A number of specimens initially negative in the Amplicor PCR assay proved to be positive on

retesting. Several explanations exist for this phenomenon: (i) incomplete lysis of the sample; (ii) the presence of thermolabile inhibitors; or (iii) incomplete neutralisation of the first detergent in the transport buffer by the second detergent in the specimen dilution buffer. Specimens with an OD value between 0.25 and 0.5 should be interpreted carefully. The specificity of these results has been questioned before.²⁰

In the studies reported here, half of these specimens were actually negative. The reason they occurred might be contamination from highly positive specimens during the microtitre plate hybridisation procedure. Further studies are necessary to resolve both type of problems.

Other solutions for the low sensitivity of the Amplicor PCR assay have been suggested recently. Diluting specimens less than recommended by the manufacturer may result in additional positive specimens,²⁸ but eventually also in the presence of more inhibitors.¹² Since the occurrence of many false negative results seems to be related to the transport method an altered method of specimen transport seems to be a more suitable suggestion.²⁹

While selecting an assay for laboratory detection several items should be considered⁴ besides sensitivity and specificity. The prevalence and other characteristics of the patient population are also very important. Also, the number of repeated tests should be limited. In these studies 1.0% of the tests had to be repeated. This number as well as necessary controls per assay run have to be considered when calculating the test price per diagnosis.

The sensitivity and specificity values of the Amplicor PCR assay compare favourable with those of non-culture, non-amplification detection assays.⁴ Since the variability is too large and since there is still room for improvement, the current version of the Amplicor PCR assay can not be considered as an alternative for isolation in cell culture as the "gold standard" or as a new standard in the laboratory diagnosis of *C. trachomatis* infections.

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