DIAGNOSTICS

The superiority of polymerase chain reaction over an amplified enzyme immunoassay for the detection of genital chlamydial infections

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Background/objectives: The polymer conjugate enhanced enzyme immunoassay (IDEIA) and Cobas Amplicor polymerase chain reaction *Chlamydia trachomatis* (CT) (Amplicor PCR) are two commonly used assays for the diagnosis of CT infection. The performance of these assays was compared for the diagnosis of genital CT infection among 1000 consecutive patients attending a genitourinary medicine (GUM) clinic. Confirmation of positive results and the clinical significance of the absence of cryptic plasmid in chlamydia on the diagnosis of infection by Amplicor PCR were also investigated.
Methods: IDEIA, Amplicor PCR, and two nested in-house PCR assays targeting cryptic plasmid and *omp1* gene were performed on all samples. DNA from Amplicor PCR negative samples was pooled for in-house PCR assays. Each pool contained DNA from seven Amplicor PCR negative samples.
Results: Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and efficiency of IDEIA in the diagnosis of genital CT infection were 80%, 97%, 80%, 97%, and 95%, respectively. Sensitivity, specificity, PPV, NPV and efficiency of Amplicor PCR were 99%, 98%, 89%, 100%, and 98%, respectively. 16 (11%) of 144 Amplicor PCR positive results were identified as false positive by in-house PCR assays. No isolate of plasmid free CT was detected among the study population.
Conclusions: IDEIA should not be used for the diagnosis of CT infection because of its poor sensitivity. Although the analytic specificity of Amplicor PCR was 98%, because of the adverse medical, social, and psychological impact of false positive results for patients, confirmation of Amplicor PCR positive results by a different assay with comparable sensitivity is essential. Amplification assays targeting cryptic plasmid are appropriate for the diagnosis of genital CT infections.

The organism *Chlamydia trachomatis* (CT) is the most common sexually transmitted bacterium in the United Kingdom.[1] The enzyme immunoassay (EIA) is a commonly used front line assay for the diagnosis of CT infection. EIA reactive results are confirmed mainly by nucleic acid amplification tests (NAATs). Amplicor PCR (polymerase chain reaction) and the polymer conjugate enhanced enzyme immunoassay (IDEIA) are two commonly used assays for the diagnosis of genital CT infection. IDEIA has a distinct characteristic, dual amplification of signal, to increase the sensitivity of the assay. The increased sensitivity of IDEIA had been reported when compared to conventional EIA.[2] The sensitivity of IDEIA in comparison with ligase chain reaction (LCx Chlamydia, Abbott) was reported to be 92%.[3] Two small studies, performed mainly on female commercial sex workers reported comparable sensitivity for IDEIA and Amplicor PCR.[4 5] A recent review in this journal suggested further critical evaluation of IDEIA for the diagnosis of genital CT infection.[6] This is the first large study on the comparative performance of IDEIA and Amplicor PCR in the diagnosis of CT infection among patients attending a genitourinary medicine (GUM) clinic.

CT has 7–10 copies of cryptic plasmid that are highly conserved in sequence and size.[7] Many in-house and commercial assays including Amplicor PCR have chosen it as a target for amplification to enhance the sensitivity of detection. A number of studies have described clinical[8–11] and laboratory[12] isolates of CT that lack cryptic plasmid, suggesting that it is not essential for the growth of the organism. The impact of this phenomenon on the diagnosis of CT infection was investigated in this study. This issue of confirmatory testing of positive results, generated by Amplicor PCR, was also investigated.

METHODS

Patients

A total of 1000 consecutive patients attending the GUM clinic, from June to November 2003, at Addenbrooke's Hospital, Cambridge participated in this study. The study population comprised 437 males, median age 26 years (range 15–77) and 563 females, median age 23 years (range 15–58).

Sample collection and testing for *Chlamydia trachomatis*

This study was performed on routine diagnostic samples for genital CT infection. IDEIA chlamydia collection kit, S600730, was used for the collection of endocervical and urethral swabs from females and urethral swabs from males. Two swabs (endocervical and urethral) from every female were placed in a single tube and were treated as a single sample. A single urethral swab was analysed from each male patient. Both IDEIA and Amplicor PCR were performed on the same sample according to the manufacturers' protocols, DakoCytomation Ltd, Ely, UK and Roche Molecular Systems, Inc, CA, USA respectively. After completion of the diagnostic work, samples were anonymised, coded, and

Abbreviations: CT, *Chlamydia trachomatis*; EIA, enzyme immunoassay; GUM, genitourinary medicine; IDEIA, polymer conjugate enhanced enzyme immunoassay; NAATs, nucleic acid amplification tests; NPV, negative predictive value; PCR, polymerase chain reaction; PPV, positive predictive value

Table 1 Primers for plasmid and MOMP PCR

	Sequence	Position*	Amplicon size (bps)
First round MOMP PCR			
Outer forward primer	5′-TTGTTTTCGACCGTGTTTTG-3′	203	
Outer reverse primer	5′-AGCRTATTGGAAAGAAGCBCCTAA-3′	657	455
Second round MOMP PCR			
Inner forward primer	5′-AAACWGATGTGAATAAAGARTT-3′	223	
Inner reverse primer	5′-TCCCASARAGCTGCDCGAGC-3′	617	395
First round plasmid PCR			
Outer forward primer	5′-TTGGCYGCTAGAAAAGGCGATT-3′	7153	
Outer reverse primer	5′-TCCGGAACAYATGATGCGAAGT-3′	7365	212
Second round plasmid PCR			
Inner forward primer	5′-AACCAAGGTCGATGTGATAG-3′	7179	
Inner reverse primer	5′-TCAGATAATTGGCGATTCTT-3′	7328	150
DNA sequence of internal control			
5′-GTGCTCACACCAGTTGCCGCGGAAAGTATGTGGAATGTTAACACACCCACACCACACCCACACACGTGTTGGATCAATTTCGAGATGCGAGCTGCCAAGC-3′			
Forward primer for internal control	5′-GTGCTCACACCAGTTGCCGC-3′		
Reverse primer for internal control	5′-GCTTGGCAGCTCGCATCTCG-3′		

*Positions of primers for MOMP and plasmid PCR are according accession no. AF202457 and X06707, respectively.

stored at −20℃ for further quality assurance work. The Cambridge local research ethics committee approved this study.

DNA extraction

DNA for in-house PCR was extracted from 200 µl of each sample using the MagNA Pure LC total nucleic acid isolation kit and MagNA Pure LC Robot according to the manufacturer's protocol (Roche Molecular Systems, Inc, CA, USA). DNA was concentrated during extraction and was eluted in 60 µl of elution buffer.

In-house plasmid and MOMP nested PCR

Two in-house nested PCR assays targeting cryptic plasmid (plasmid PCR) and *omp1* gene (MOMP PCR) were performed on all samples. In all, 122 pools from 854 of 856 Amplicor PCR negative samples were prepared. The volume of a pool was 35 µl containing 5 µl of DNA from each of seven Amplicor PCR negative samples. The two remaining Amplicor PCR negative samples were processed separately. Individual in-house plasmid and MOMP PCR assays were performed on all samples in a pool which gave a positive result and 144 Amplicor PCR positive samples.

Nested PCR

Two rounds of PCR were performed on each sample or pool of DNA in 50 µl volume. A volume of 5 µl of DNA from a single sample or 35 µl of DNA from each pool was used in the first round PCR. The reactions in the second round contained 2 µl of amplicons from the first round PCR. The amplification reactions also contained 200 µM of each of dNTP, 25 pmol of each primer, 4 mM $MgCl_2$, 5 µl of 10× PCR buffer and 1.5 units of *Taq* DNA polymerase recombinant (Invitrogen life technologies, Paisley, UK). DNA from CT LGV-II, strain 434 (ATCC VR-902B) was used as a positive control and nuclease free water was used as a negative control for amplification. Six molecules of 100 bases long synthetic DNA and 2.5 pmol of forward and reverse primers to amplify this DNA were also added in the reaction to identify inhibition of amplification. Tenfold serial dilutions of CT positive control DNA were used to investigate the sensitivity of MOMP and plasmid PCR assays. Samples and controls were denatured at 95℃ for 3 minutes, followed by 30 cycles of amplification in the first round and 25 cycles in the second round in a PTC-200 Peltier Thermal Cycler (Genetic Research Instrumentation Ltd, Essex, UK). Each cycle consisted of denaturation at 95℃ for 30 seconds, annealing of primers at 50℃ for 1 minute, and extension at 72℃ for 1 minute followed by final extension at 72℃ for 7 minutes. The sequence of primers and internal control are shown in table 1. The size of amplicons was ascertained in reference to PCR Markers (Promega, Madison, USA) by gel electrophoresis using 3% agarose (NuSieve 3:1 Agarose, Bio Whittaker Molecular Applications, Rockland, ME, USA).

Criteria for a true positive result

A positive or equivocal result by IDEIA was considered as a true positive if it was confirmed by two of the three PCR assays. A positive result by Amplicor PCR, plasmid PCR, or MOMP PCR was considered as a true positive if it was confirmed by two of the three PCR assays.

RESULTS

IDEIA and Amplicor PCR

According to the manufacturer's criteria, 113 samples were positive, 15 samples were equivocal, and 872 were negative for chlamydia antigen by IDEIA; 144 samples were positive and 856 samples were negative for cryptic plasmid DNA by Amplicor PCR. Internal control was detected in all samples identified as negative by Amplicor PCR. All equivocal IDEIA

Table 2 Results of IDEIA, Amplicor PCR, and in-house PCR assays

	Assays			
Results	IDEIA	Amplicor PCR	Plasmid PCR	MOMP PCR
Positive	128*	144	140	117
Negative	872	856	860	883
Total	1000	1000	1000	1000

*15 of 128 results were equivocal.

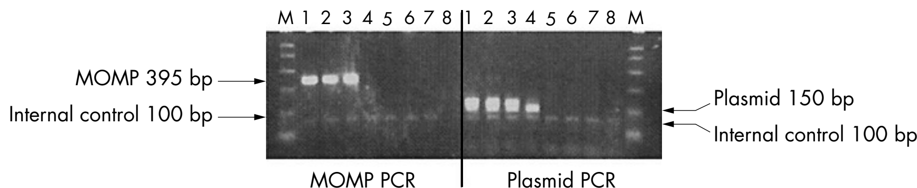

Figure 1 Sensitivity of in-house nested MOMP and plasmid PCR assays. M, PCR markers. Lanes 1–8, serial 10-fold dilutions of *C trachomatis* DNA. Arrows indicate the size of amplicons.

results were grouped with IDEIA positive results to investigate the performance of this assay. The results are shown in table 2.

In-house nested plasmid and MOMP PCR

MOMP PCR was able to detect up to a single genome copy of CT in the amplification reaction. The sensitivity of plasmid PCR was 10-fold higher than MOMP PCR (fig 1); 128 (89%) and 116 (81%) of 144 Amplicor PCR positive samples produced positive results with plasmid PCR and MOMP PCR, respectively. All 116 samples, which were positive by MOMP PCR, were also positive by plasmid PCR. One and seven of 122 pools from Amplicor PCR negative samples generated a signal with MOMP PCR and plasmid PCR respectively. Both MOMP and plasmid PCR detected one Amplicor PCR negative sample as positive. Plasmid PCR detected 13 Amplicor PCR and MOMP PCR negative samples as positive. The internal control was amplified in all pools. The results are shown in table 2.

According to the criteria described in the methods section, the results of IDEIA, Amplicor PCR, plasmid, and MOMP PCR are summarised in tables 3 and 4.

DISCUSSION

The only two previously published reports to have compared the performance of IDEIA and Amplicor PCR for the diagnosis of genital CT infection reported comparable sensitivity.[4 5] However, this study demonstrated that IDEIA is 20% less sensitive than Amplicor PCR. The study population in the previous two studies mainly comprised high risk commercial female sex workers and separate samples were collected in two different transport media for IDEIA and Amplicor PCR. This study was performed on 1000 consecutive patients attending a GUM clinic, on a single sample from each patient in only one type of transport medium for both assays, hence removing a number of confounding factors that may have affected the previous studies.

A number of studies have reported reproducibility problems with Amplicor PCR for the diagnosis of CT infection.[13–15] Sixteen (11%) of 144 Amplicor PCR positive results were identified as false positive in this study. This finding is consistent with a previous study, which was performed on 733 endocervical swabs, reporting 13 (15%) of 87 Amplicor PCR positive results as false.[16] Because of the significant number of false positive results by Amplicor PCR, the need for confirmatory testing on Amplicor PCR positive samples is evident. Data presented in this study suggest that the combination of Amplicor PCR on all samples and plasmid PCR on Amplicor PCR reactive samples is adequate to identify all false positive results without affecting the sensitivity of diagnosis. MOMP PCR did not have a significant impact on either sensitivity or specificity.

A number of studies have used chromosome based PCR assays to confirm the reactive results generated by Amplicor PCR, which is essentially a plasmid based PCR. These studies have reported discrepant results between these two types of PCR assays in the range of 6%–12%.[17–19] MOMP PCR used in this study was 8% less sensitive than Amplicor PCR. With multiple copies of cryptic plasmid in CT, plasmid based PCR assays have shown up to 1000-fold higher sensitivity in comparison with chromosome based PCR assays.[20 21] Hence, chromosome based PCR assays should not be used to confirm the reactive results of plasmid based PCR assays. MOMP PCR, because of its nested format, detected up to a single genome copy/reaction, while sensitivity of plasmid PCR was 10-fold higher than MOMP PCR. Twelve samples did not appear to have even a single copy of CT genome copy/reaction but were positive with both plasmid PCR and Amplicor PCR. These were regarded as true positives. The clinical significance of positive results generated by plasmid based PCR

Table 3 Interpretation of results according to the criteria

	Assays			
Results	IDEIA	Amplicor PCR	Plasmid PCR	MOMP PCR
True positive	103	128	129	117
True negative	846	855	860	871
False positive	25	16	11	0
False negative	26	1	0	12
Total	1000	1000	1000	1000

Table 4 Performance of IDEIA, Amplicor PCR, and in-house plasmid and MOMP PCR

Assay	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Efficiency (%)
IDEIA	80	97	80	97	95
Amplicor PCR	99	98	89	100	98
Plasmid PCR	100	99	91	100	99
MOMP PCR	91	100	100	99	99

from samples, which do not have any chromosomal DNA of CT, needs further investigation.

IDEIA is not an appropriate test for the diagnosis of genital CT infection, because of its poor sensitivity in comparison to Amplicor PCR. Reactive results generated by Amplicor PCR should be confirmed by a different amplification assay. The sensitivity of the confirmatory assay should be comparable with the front line assay. Chromosomal based confirmatory assays for plasmid based PCR may lead to false negative confirmation. The absence of cryptic plasmid in CT was not identified in the population studied. The in-house assays reported in this study are nested PCR with gel electrophoresis. The assays in this format are not suitable for large scale routine diagnostic use owing to risk of contamination and their laborious nature. However, in-house real time PCR assays using closed systems will be useful to confirm positive results generated by commercial NAATs.

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CONTRIBUTORS
HJ, CS, and CC participated in study planning and design; HJ, HS, and AA performed practical work and data analysis; HJ was the lead writer; and all authors provided revision and commented on the paper.

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