Comparison of an in-house PCR assay, direct fluorescence assay and the Roche AMPLICOR Chlamydia trachomatis kit for detection of C. trachomatis

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To improve the control of Chlamydia trachomatis infection in India, a rapid, specific and cost-effective method is much needed. We developed an in-house PCR assay by targeting a unique genomic sequence encoding a protein from the C. trachomatis phospholipase D endonuclease superfamily that produces an amplified fragment of 368 bp. The specificity of the primers was confirmed using genomic DNA from other sexually transmitted disease-causing and related microorganisms and from humans. The assay was highly sensitive and could detect as low as 10 fg C. trachomatis DNA. Clinical evaluation of the in-house-developed PCR was carried out using 450 endocervical specimens that were divided in two groups. In group I (n=274), in-house PCR was evaluated against the direct fluorescence assay. The resolved sensitivity of the in-house PCR method was 97.22 % compared with 88 % for the direct fluorescent antibody assay. In group II (n=176), the in-house PCR was compared with the commercial Roche AMPLICOR MWP CT detection kit. The resolved sensitivity of the in-house PCR assay reported here was 93.1 % and the specificity was 97.46 %, making it a cost-effective alternative for routine diagnosis of genital infection by C. trachomatis. The method should facilitate early detection leading to better prevention and treatment of genital infection in India.

INTRODUCTION

Chlamydia trachomatis is one of the most common causative agents of sexually transmitted infections in developing countries including India. Infection with this agent is often asymptomatic (up to 80 % of women and 40 % of men) (Gaydos et al., 2004), making diagnosis and treatment difficult. Undetected genital infections may evolve into complications such as ectopic pregnancy, pelvic inflammatory disease, salpingitis with tubal scarring and infertility in female patients (Black, 1997; Semeniuk et al., 2002). In infected men, arthritis and epididymitis may result in urethral obstruction and decreased fertility. As asymptomatic and untreated patients can spread the disease to their partners, screening of all sexually active adolescents for C. trachomatis infection is recommended (CDC, 2002).

Detection methods for C. trachomatis infection include serology, culture method, ELISA, direct fluorescence assay (DFA) and nucleic acid amplification tests (NAATs). Although antigen-based diagnostic assays are as sensitive as the culture method, they show variability due to the methods of sample collection, transport and storage (Mårhd et al., 1981; Mahony & Chernesky, 1985). PCR and ligase chain reaction are more sensitive and specific compared to other diagnostic methods (Black, 1998; Wylie et al., 1998; Semeniuk et al., 2002) and have facilitated the use of less-invasive procedures for detection of asymptomatic C. trachomatis infection in female patients. Currently, several commercial NAAT-based assays such as Gen-Probe APTIMA Combo 2 (AC2), BD Probe Tec ET and Roche AMPLICOR (COBAS and manual) PCRs are available but their high cost prevents their routine use in developing countries. There is an urgent need for the development of a rapid, highly sensitive and cost-effective detection method considering the high prevalence of C. trachomatis infection in India (Divekar et al., 2000; Singh et al., 2002, 2003; George et al., 2003). In the present study, we have designed and established an in-house PCR assay for detection of C. trachomatis using primers against

Abbreviations: DFA, direct fluorescence assay; EBs, elementary bodies; FISH, fluorescence in situ hybridization; NAAT, nucleic acid amplification test; NPV, negative predictive value; PPV, positive predictive value; STD, sexually transmitted disease. Supplementary figures are available with the online version of this paper.

Received 11 December 2008
Accepted 9 March 2009

008698 © 2009 SGM Printed in Great Britain
genomic sequence. The performance of the in-house PCR method was evaluated against currently used diagnostic methods and was found to be cost-effective, highly specific and sensitive.

METHODS

Enrollment of patients. A total of 450 symptomatic female patients attending the Gynecology Outpatient Clinic of Safdarjung Hospital and Hindu Rao Hospital, New Delhi, India, were enrolled in this study following institutional ethical committee clearance and informed oral consent of patients. Women with clinical features of chlamydial cervicitis (mucopurulent cervical discharge or cervical ectopy with inflammation), urethritis or infertility were also enrolled.

Specimen collection. The vulva was examined for lesions and vaginal/cervical discharge and the cervix was examined for ulcers, wart and ectopy. After cleaning the exocervix with a cotton swab (Hi Media), two endocervical swabs were taken from each patient in order to avoid the swab sample variation that can occur when multiple swabs are taken. The clinical samples were divided into two groups (I and II). In the first group of 274 samples, a sterile cotton swab was used to collect each specimen in 1 ml transport medium (130 mM NaCl, 2.6 mM KCl, 10 mM Na2HPO4, 1.7 mM KH2PO4, 10 mM EDTA, pH 7.4). The specimen from the second swab was smeared on a clean glass slide, and air-dried for the DFA and fluorescence in situ hybridization (FISH) assay.

For the second group of 176 samples, two endocervical swab specimens were obtained. The first swab was placed into a vial containing AMPLICOR Specimen Transport Medium and the second swab was placed in 1 ml transport medium described above. All specimens were transported to the microbiology laboratory on ice within 1 h of collection and tested within 24 h or stored at −80 °C for subsequent use.

Primer design. More than 50 sequences of various genes of the C. trachomatis genome were obtained from GenBank. Short stretches of about 25–30 nucleotides were aligned using the BLAST program from NCBI to find matching sequence with other organisms if any. The sequence that was highly unique to C. trachomatis was selected for gene-specific primers. The selected sequence encoded a protein from the phospholipase D endonuclease superfamily (CT157; Entrez GeneID: 884104) producing an amplicon of size 368 bp. Primers were designed using Gene Runner 3.05 software. Their sequences are as follows: forward primer P1, TCTTTTTAAAACCTCGGAACCCCATTT, Tm 74.9; reverse primer P2, GGATGGCATCGCATAGCA-TTCTTTG, Tm 76.3 (US, UK, EU patent pending).

PCR amplification. For PCR assay, specimens were processed by a lysis method as described previously (Chaudhry & Saluja, 2002) and by an alternative method wherein 400 μl clinical specimen was centrifuged at 15 000 g at 4 °C for 30 min, and the pellet was resuspended in 40 μl 1× Tris-EDTA (pH 8.0) and boiled for 10 min at 100 °C (referred as crude lysate). Five microlitres of supernatant of processed sample or crude lysate was used for PCR in a reaction volume of 50 μl containing 1× Taq DNA polymerase buffer (50 mM KCl, 10 mM Tris/HCl pH 8.3, 1.5 mM MgCl2), 200 μM each of the four dNTPs (New England Biolabs), 25 pmol each forward and reverse primer and 1.0 U Taq DNA polymerase (Bangalore Genei India). Purified genomic DNA of C. trachomatis (kindly provided by Lynn Olinger, Francis I. Proctor Foundation, University of California, San Francisco, USA) was used as a positive control for each set of assays. Amplification was performed in a thermal cycler (1 Cycler; Bio-Rad) for 35 cycles: 95 °C for 5 min for initial denaturation, cycling of 95 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min, and final extension at 72 °C for 10 min. The amplicons were analysed on a 1.5% agarose gel by electrophoresis. The amplicons from positive samples (10%) were eluted using a DNA isolation kit (Biological Industries) according to the manufacturer’s instructions and sequenced using PCR primers (forward primer) with a Taq Dye Terminator Cycle Sequencing kit on a 377A autosequencer (Applied Biosystems) according to the manufacturer’s instructions. The DNA sequence of the amplified product was compared to known phospholipase D endonuclease superfamily nucleotide sequences (January, 2009) in the GenBank databases using the BLAST program to determine the percentage identity.

Roche AMPLICOR MWP CT detection assay. One hundred and seventy-six endocervical specimens of group II were tested by the Roche AMPLICOR CT detection kit (Roche Diagnostic Systems) according to the manufacturer’s instructions. For endocervical specimens, 1 ml specimen diluent was added to endocervical samples, mixed thoroughly by vortexing, and incubated for 10 min at room temperature. After overnight storage at 4 °C, 50 μl of the clinical sample was added to each PCR tube containing 50 μl of the Chlamydia PCR master mix. The PCR master mix contains primers for internal control as well. The assay was developed as per the instructions given by the manufacturer. To resolve the discrepant samples, genomic DNA was isolated from the aliquots of frozen specimens. Samples were centrifuged at 15 000 g for 30 min. Pellet was resuspended in 500 μl lysis buffer (50 mM Tris-EDTA pH 8.0, 400 μg proteinase K ml−1), incubated at 55 °C for 2 h and then boiled at 100 °C for 10 min with 1 mM DTT. Thereafter, it was extracted with phenol/chloroform and centrifuged at 12 000 g for 10 min. 2-Propanol with 1/10 volume of 3 M sodium acetate was added to the supernatant and incubated overnight at −20 °C. The pellet was collected by centrifugation at 12 000 g for 10 min, washed with 70% ethanol, air-dried and dissolved in water.

Evaluation of specificity and sensitivity. To evaluate the specificity of the primer pair, DNA extracted from C. trachomatis serovar L2 (kind gift from Dr Peter Braun, Department of Molecular Biology, Max Plank Institute for Infection Biology, Berlin, Germany), known positive clinical isolates of C. trachomatis (20 clinical isolates of serovar A and D obtained from Dr Sudha Salhan, Department of Obstetrics and Gynecology, Vardhman Mahavir Medical College and Safdarjung Hospital, New Delhi) and other sexually transmitted disease (STD)-causing and related micro-organisms, such as Mycoplasma sp. (6) (also a kind gift from Dr Sudha Salhan), Chlamydia pneumoniae (2), herpes simplex (1), Candida species (7), Ureaplasma (10), Trichomonas (6) (kind gift from the Department of Microbiology AIIMS, New Delhi, India), Neisseria gonorrhoeae (10), Neisseria meningitidis (genital isolates) (3), Neisseria lactamica 94D4 (1), Neisseria sicca 94C1 (1) and Neisseria subflava 86G7 (1) (kind gift from Professor J. W. Tapsall, WHO Collaborating Centre for STD and HIV, Department of Microbiology, The Prince of Wales Hospital, Randwick, New South Wales, Australia), was used as template for PCR. DNA was extracted from isolates of STD-causing and related micro-organisms as described above. Ten human genomic DNA samples were also used to evaluate the specificity of the primer pair.

To determine the sensitivity of the primer pair, serial dilutions from 100 pg to 1 fg of purified chlamydial genomic DNA and various dilutions of C. trachomatis positive clinical samples were used as template for PCR amplification. All assays were repeated at least five times.

DFA. For DFA (MicroTrak), specimens were centrifuged at 3000 g for 10 min and sediments were air-dried, fixed by incubation in methanol and stained with a FITC-conjugated anti-major outer membrane protein monoclonal antibody. The slides were examined
for typical apple-green fluorescent elementary bodies (EBs) at \( \times 1000 \) magnification. The presence of more than 10 fluorescent EBs was considered to be a positive result.

**FISH assay.** A FISH assay was performed following the method described by Kapur et al. (2006). Briefly, smears from endocervical swab specimens were prepared on clean glass slides treated with diethyl pyrocarbonate (DEPC) water. Smears were fixed in 4% paraformaldehyde in PBS for 3 h, immersed in 70% (v/v) ethanol for 15 min and air-dried. The slides were rinsed briefly in DEPC water, air-dried and hybridized with 5-carboxylfluorescein (FAM) fluorochrome labelled oligonucleotide probes complementary to the 16S rRNA gene sequence of *C. trachomatis*.

**Evaluation of assays.** All assays on clinical specimens were performed blinded to the results of one another. The original samples were taken for resolution of discrepancy. In group I, all 274 samples were tested by in-house PCR and DFA. In order to resolve 24 discrepant results in group I where samples were PCR-positive and DFA-negative, a FISH assay was performed. A specimen was considered true positive for *C. trachomatis* if it was positive by any two methods (DFA/FISH/in-house PCR). For group II of 176 samples, discrepant results (n=19) were resolved by PCR-based detection of two housekeeping genes, *ompA* (CT681; Entrez GeneID: 884473) and *gyrA* (CT189; Entrez GeneID: 884941). The treatment response of patients with discrepant results was also obtained. The treatment response was measured based on the resolution of clinical symptoms and doctors’ examination obtained from hospital records. A specimen was considered true positive when it was (i) in-house PCR and Roche PCR positive, (ii) Roche PCR positive and *ompA/gyrA* PCR positive or (iii) in-house PCR positive and *ompA/gyrA* PCR positive.

**Data analysis.** All statistical analysis was performed using SPSS v. 10.0 software. Sensitivities, specificities, positive predictive values (PPVs) and negative predictive values (NPVs) were calculated with 95% confidence intervals to test the significance of the estimates.

**RESULTS AND DISCUSSION**

**Specificity and sensitivity of primers**

Using the BLAST program from NCBI, sequences highly unique to *C. trachomatis* were selected for designing genus-specific primers. One of the sequences that was unique to *C. trachomatis* encoded a protein from the phospholipase D endonuclease superfamily (CT157, Entrez Gene ID: 884104) and was selected for primer designing. Amplicons of the desired size (~368 bp) were obtained when purified genomic DNA of different serovars of *C. trachomatis* (A, D and L2) was used as template for the in-house PCR (Supplementary Fig. S1 in JMM Online), while no amplicon was detected with the rest of the STD-causing and related micro-organisms as well as with human genomic DNA. The amplicons obtained from clinical samples were sequenced for at least 10% of positive samples, which further established the specificity of the in-house PCR. The DNA sequences of the amplicons were aligned with the known sequences of serovar A, D and L2 in the GenBank databases (Supplementary Fig. S2). The sensitivity of the primer pair was determined by testing serial dilutions of purified chlamydial genomic DNA for PCR. Using P1/P2 primers, amplified product could be detected with as low as 10 fg chlamydial DNA (equivalent to 9 inclusion-forming units) (Fig. 1). The sensitivity of the primer pair was also checked with *C. trachomatis* positive clinical samples. P1/P2 primers could amplify the specific product when crude lysate of clinical samples was used as template. PCR has been observed to be significantly more sensitive than conventional methods such as culture and antigen-based methods for diagnosis of *C. trachomatis* in clinical specimens (Wu et al., 1992; Mahony et al., 1993; Smith et al., 1993; Shattock et al., 1998; George et al., 2003). Many existing PCR assays, including the Roche MWP test, target the cryptic plasmid (Mahony et al., 1993; Smith et al., 1993; Shattock et al., 1998; George et al., 2003). The chromosomal targets most widely used are *ompA* (Mahony et al., 1993; Smith et al., 1993; Shattock et al., 1998; Wu et al., 1992) and *rRNA* encoding genes (Mahony et al., 1993). Plasmid-based PCR detection assays are considered to be more sensitive than chromosomal gene-based assays, as plasmid copy number is generally 7–10 per cell (Palmer & Falkow, 1986; Sriprakash & Macavoy, 1987; Ostergaard, 2002). NAAT-based diagnostic kits such as the Roche COBAS AMPLICOR CT assay and the Ligase Chain Reaction kit from Abbott (no longer available) have proven to be highly sensitive (92.9–100%) and specific (99–100%) for *C. trachomatis* detection (Loeffelholz et al., 1992; Bass et al., 1993; Jaschek et al., 1993; Smith et al., 1993; Kessler et al., 1994; Mahony et al., 1994; Catry et al., 1995; Chout et al., 1995; Semeniuk et al., 2002). However, a major obstacle in adopting these kits for routine diagnosis in clinical laboratories in India is the high cost (mean cost of a single assay is $50). The available diagnostic kits use plasmid-based amplification (Singh et al., 2002; George et al., 2003). Along with others (An et al., 1992; Schachter et al., 1996; Farencena et al., 1997), we have also observed that infection due to plasmid-free variants remains undetected (unpublished results) though it is still not a major clinical problem.

Comparison of the performance of different PCR assays including plasmid, *ompA* and rRNA gene targets has

![Fig. 1. Sensitivity of in-house primers using purified chlamydial genomic DNA. PCR amplification of purified genomic DNA (100 pg–1 fg) of *C. trachomatis* serovar D was carried out by using P1/P2 primers.](http://jmm.sgmjournals.org)
suggested that in general plasmid primers are 10–1000 times more sensitive (0.1 fg for plasmid DNA) than the genomic DNA primers for *ompA* (0.1–10 pg genomic DNA) and the rRNA gene (1 pg genomic DNA) (An et al., 1992; Joshi et al., 1994; Mahony et al., 1994; Crotchfelt et al., 1998; Niederhauser & Kaempf, 2003; Kapur et al., 2006). In contrast, our assay targeting the phospholipase D endonuclease superfamily gene showed higher sensitivity (10 fg–0.1 pg genomic DNA) than assays with other genomic targets.

**Clinical performance of the in-house PCR and comparison with DFA and the Roche AMPLICOR MWP kit**

Among 274 cases enrolled in group I, the median age of the patient population was 29 (range 18–57) years. Of 274 samples, 61 (22%) were positive and 189 (69%) were negative by PCR assay using the P1/P2 primer set and by DFA (Table 1). Of 24 discrepant samples, 11 samples were considered true positive, as 9 of these were PCR- and FISH-positive but DFA-negative while 2 were DFA- and FISH-positive but PCR-negative (Table 2). Thirteen samples were PCR-positive with the P1/P2 primer set but tested negative by DFA and FISH assay and were therefore considered true negatives. These 13 cases could not be reconfirmed because samples were insufficient. Follow-up specimens could not be collected because the patients had received antibiotic treatment by that time. Thus, in all, the P1/P2 primer pair detected a significant number of true-positive samples (70 out of 72) (*P* < 0.0001). In contrast, DFA detected 63 out of 72 true-positive samples. Based on our results, the prevalence of *C. trachomatis* infection was 27% among women visiting the gynaecology outpatient clinic when DFA was selected as the gold standard method.

Of 176 samples of group II, 42 were positive and 115 were negative by in-house PCR and commercial PCR assay (Table 3). Of 19 discrepant samples, 11 were positive by in-house PCR but negative by commercial PCR when processed as per the manufacturer’s instructions. However, all of them scored positive when purified DNA was used as a template and were considered true positives. Three samples were positive by in-house PCR but negative by Roche PCR. To resolve the discrepancy, purified genomic DNA of the samples was tested for two housekeeping genes: *ompA* and *gyrA*. The three samples tested negative for both *ompA* and *gyrA* and were treated as true negatives. One sample was positive by in-house PCR and negative by the Roche kit but found to be positive for *ompA* and *gyrA*, so it was taken as a true positive. Four samples were positive by the Roche AMPLICOR MWP kit and negative by in-house PCR but all of these tested positive for *ompA/*gyrA (Table 4). The response to therapy administered to the patients with discrepant results obtained from hospital records also supported the results obtained after resolving the discrepant samples. Thus, in all, the P1/P2 primer pair detected a significant number of samples (54 out of 58, 93%). The commercial PCR assay detected 98% of positive samples only when DNA was purified from 11 samples out of 58. The prevalence of *C. trachomatis* infection was 33% among women patients visiting the gynaecology clinic taking the Roche AMPLICOR MWP kit as the gold standard method.

**Sensitivity, specificity, PPV and NPV**

Compared with DFA, the in-house PCR with the P1/P2 primers demonstrated a sensitivity of 96.83%. After discrepant analysis by FISH, the sensitivity of the P1/P2 primers increased to 97.22% (Table 5). The specificity of PCR with the P1/P2 primers increased from 89.57% to 93.56%. The PPV of PCR with the P1/P2 primers increased from 73.5% to 84.33%. The NPV of PCR with the P1/P2 primers remained unchanged at 99%.

In the second part of the study, compared with the Roche MWP PCR assay, the sensitivity and specificity of the in-house PCR with P1/P2 was 91.3% and 88.46%. After discrepant analysis, the sensitivity of the PCR method increased to 93.1% and the specificity increased to 97.46%. The PPV of the in-house PCR increased from 73.7% to 94.73% while the NPV remained unchanged at 96.6% (Table 5).

The specificity of DFA is normally less than 90% in actual practice, thus to avoid the tendency to underestimate the specificity of the NAAT-based assay developed by us, the performance of discrepant samples was analysed with alternative methods of detection in order to determine whether they were true positives. Following discrepant analysis, the sensitivity and specificity of the in-house PCR improved considerably. All the discrepant samples (see Table 2) when retested by the in-house PCR method gave 100% reproducible results. The specificity of the in-house

**Table 1.** Comparison of in-house PCR with DFA: results for *C. trachomatis* before and after discrepant analysis

| Table 1. Comparison of in-house PCR with DFA: results for *C. trachomatis* before and after discrepant analysis |
|---|---|---|---|---|---|
| **PCR result (P1/P2)** | **DFA** | **After resolution of discordant results** |
| **Positive** | **Negative** | **Total** | **Positive** | **Negative** |
| Positive | 61 | 22 | 83 | 70 (61 + 9) | 13 (22 – 9) |
| Negative | 2 | 189 | 191 | 2 | 189 |
| Total | 63 | 211 | 274 | 72 | 202 |
PCR might have improved further if the follow-up samples from patients could be tested.

The estimated sensitivity and specificity range of DFA is 61–92% and 99–100%, respectively, in different laboratory settings when compared to those of the culture or non-culture method (Thejls et al., 1994; Schachter et al., 1996). The overall sensitivity of DFA in the present study was 88%. Since the cut-off for DFA is established to get the best combination of sensitivity and specificity, most assays will miss some positive samples by compromising sensitivity to achieve specificity. Although DFA is widely used as a reference method for evaluating diagnostic kits and for checking inter-laboratory variation, Thomas et al. (1993) reported that the DFA kit has its limitations. According to the report, about 30% of the clinical samples contain <10 EBs as judged by examining the smears stained in the assay. Therefore, the diagnostic performance of the DFA test is highly dependent on the number of chlamydial EBs that should be seen in order to obtain a positive sample. Similar

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>Test results</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P1/P2</td>
<td>PCR</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>+</td>
<td>-</td>
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Table 2. Resolution of discrepant results by FISH assay for C. trachomatis (n=24)

<table>
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<th>PCR result (P1/P2)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
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<tr>
<td>Positive</td>
<td>42</td>
<td>15</td>
</tr>
<tr>
<td>Negative</td>
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<tr>
<td>Total</td>
<td>46</td>
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Table 3. Comparison of in-house PCR with the Roche AMPLICOR MWP kit: results for C. trachomatis before and after discrepant analysis

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>P1/P2</th>
<th>Processed and developed with Roche</th>
<th>Purified DNA, developed with Roche*</th>
<th>ompA</th>
<th>gyrA</th>
<th>Symptoms</th>
<th>Treatment response</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
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<td>NR</td>
<td>NR</td>
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<td>11†</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
<td>-</td>
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<td>-</td>
<td>+</td>
<td>+</td>
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<td>True positive</td>
</tr>
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<td>+</td>
<td>NR</td>
<td>+</td>
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<td>True positive</td>
</tr>
<tr>
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<td>-</td>
<td>+</td>
<td>NR</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>True positive</td>
</tr>
</tbody>
</table>

NR, Not required.

*Total DNA from the patient sample was purified, followed by NAAT-based detection of C. trachomatis using the COBAS AMPLICOR MWP kit.
†The patient samples when processed and tested with the COBAS AMPLICOR MWP did not give positive results, even for the internal control, suggesting that the PCR was inhibited due to the presence of inhibitors in the processed patient samples.
‡Patients were asymptomatic, hence they were not given treatment for C. trachomatis infection.

Table 4. Resolution of group II discrepant results by PCR-based detection of ompA and gyrA of C. trachomatis (n=19)

<table>
<thead>
<tr>
<th>In-house PCR assay</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% 95% CI</td>
<td>% 95% CI</td>
<td>% 95% CI</td>
<td>% 95% CI</td>
</tr>
<tr>
<td>Group I</td>
<td>97.22 93.4–100</td>
<td>93.56 90.2–96.9</td>
<td>84.3 76.5–92.2</td>
<td>99 96.8–100</td>
</tr>
<tr>
<td>Group II</td>
<td>93.1 86.6–96.6</td>
<td>97.46 94.6–100</td>
<td>94.7 88.9–100</td>
<td>96.6 92–100</td>
</tr>
</tbody>
</table>

CI, Confidence interval.
results were also observed by Shattock et al. (1998), wherein they compared various detection methods for C. trachomatis. This can present a problem in a sample that is scored DFA-negative but gives an amplicon by PCR method. In our study, we observed that 13 samples out of 274 were positive with the P1/P2 primers but negative by DFA and FISH assay. These samples, which repeatedly were positive by our PCR, may have contained chlamydial genomic DNA, but unfortunately no follow-up samples were available to confirm this as patients either did not return or had taken the therapy.

We also evaluated our in-house PCR method against the commercially available and widely used Roche MWP kit. It is pertinent to mention that to avoid the swab sample variation that may occur when multiple swabs are taken (especially when infection load is low), only two swabs were taken for each patient. After discrepant analysis, the number of true-positive samples increased from 42 to 58, resulting in increased sensitivity and specificity of the in-house PCR. More importantly, the PPV improved significantly. The Roche AMPLICOR MWP kit gave positive results for four samples that tested negative by the in-house PCR. Twelve samples were positive by in-house PCR but negative by the Roche AMPLICOR MWP kit.

One major problem that we encountered during this study was occurrence of inhibition of the amplification reaction when the Roche MWP kit was used, as has also been observed by others (Shattock et al., 1998; Niederhauser & Kämpf, 2003). When DNA was purified for all discrepant samples (19 samples) and assayed again by the Roche MWP kit, the 11 samples which initially tested negative turned out to be positive (Table 4). These practical limitations prevented us from confirming the four samples that tested positive by in-house PCR but were negative by the Roche MWP kit. One of these patients was symptomatic and also gave an amplicon when PCR was performed for ompA and gyrA, while the other three were asymptomatic and were negative for ompA and gyrA. It is also possible that a plasmid-free variant of C. trachomatis may be present in the population.

The major objective of this study was to develop an in-house PCR method that is at least as sensitive as and specific as the commercial method. An additional advantage of the in-house PCR method would be of great consequence in disease management. We thank Dr Sudha Salhan (Safdurjung Hospital, Delhi) and Dr Nisha Jain (Hindu Rao Hospital, Delhi) for providing samples, and Mrs Madhu Badhwar and Mrs Asha Rani, IOP, for the excellent technical assistance. This study was supported by a grant of the Department of Biotechnology (BT/PRO 910/MED/09/168/98).

ACKNOWLEDGEMENTS

We thank Dr Sudha Salhan (Safdurjung Hospital, Delhi) and Dr Nisha Jain (Hindu Rao Hospital, Delhi) for providing samples, and

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Prevalence of Chlamydia infection among women visiting a gynaecology outpatient department: evaluation of an in-house PCR assay for detection of *Chlamydia trachomatis*

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Abstract

**Background:** Screening women for *Chlamydia trachomatis* infection in developing countries is highly desirable because of asymptomatic infection. The existing diagnostic methods in developing countries are not effective and their sensitivity fall below 45.0% which leads to further spread of infection. There is an urgent need for improved and cost effective diagnostic tests that will reduce the burden of sexually transmitted infections in the developing world.

**Methods:** Prevalence of *C. trachomatis* infection among women visiting gynaecology department of Hindu Rao hospital in Delhi, India was determined using Roche Amplicor Multi Well Plate kit (MWP) as well as using in-house PCR assay. We used 593 endocervical swabs for clinical evaluation of the in-house developed assay against Direct Fluorescence Assay (DFA; Group I n = 274) and Roche Amplicor MWP kit (Group II, n = 319 samples) and determined the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) of the in-house developed assay.

**Results:** We detected 23.0% positive cases and there was a higher representation of women aged 18-33 in this group. An in-house PCR assay was developed and evaluated by targeting unique sequence within the *gyrA* gene of *C. trachomatis*. Specificity of the reaction was confirmed by using genomic DNA of human and other STI related microorganisms as template. Assay is highly sensitive and can detect as low as 10 fg of *C. trachomatis* DNA. The resolved sensitivity of in-house PCR was 94.5% compared with 88.0% of DFA assay. The high specificity (98.4%) and sensitivity (97.1%) of the in-house assay against Roche kit and availability of test results within 3 hours allowed for immediate treatment and reduced the risk of potential onward transmission.

**Conclusions:** The in-house PCR method is cost effective (~ 20.0% of Roche assay) and hence could be a better alternative for routine diagnosis of genital infection by *C. trachomatis* to facilitate improved screening and treatment management.

Background

Genital infection due to *Chlamydia trachomatis* is one of the most common sexually transmitted infections. Worldwide, an estimated 92 million new cases of *C. trachomatis* infection occur each year. More than two-thirds of these cases occur in the developing world, where diagnostic and treatment services are almost absent [1]. Asymptomatic (nearly 80.0% of women and 40.0% of men) [1] and untreated genital infections have serious ramifications for the reproductive health of women as it may evolve into complications such as ectopic pregnancy, pelvic inflammatory disease, salpingitis with tubal scarring and infertility in female patients [1-3]. In infected men, arthritis and epididymitis may result in urethral
obstruction and decreased fertility. Chlamydia genital tract infection is an important risk factor for human papillomavirus induced cervical neoplasia as well as human immunodeficiency virus (HIV) transmission [4-6]. Undiagnosed and untreated chlamydial infections are thus not only creating major health problems and consequences for individuals but also result in major epidemiological, social and economical problems. The developing countries have a high incidence of new chlamydial infection, however, with the exception of sporadic testing, screening for Chlamydia is rare. Using various diagnostic tests with different performance characteristics, the prevalence of chlamydial infection among women in developing countries specifically sex workers varies from 8.5% to 37.0% [7-10]. The prevalence among female sex workers ranged from 27.0-36.0% in Philip-ppines [11,12] while it is 24.0% in Indonesia [7].

The prevalence of sexually transmitted diseases could be as high as 17.6% among females from tribal population [13] to varying degree in metropolitan cities in India [14]. The incidence of chlamydial infection in female sex workers in Surat was estimated to be 8.5% using PACE2 test (non-amplified DNA probe assays for Chlamydia; Gen-probe San Diego, USA) while in Ahmedabad it was almost double [8]. Although national screening programmes are in place in developed countries, such programmes are non-existent in most of the developing countries even among high risk population such as sex workers. Consequently for symptomatic patients WHO recommends a syndromic approach to case management [15] but unnecessary treatment is the major disadvantage of syndromic management. The major limitation for screening programmes is the lack of simple and cost effective diagnostic tests. Intracellular localization of the pathogen creates an additional challenge for routine diagnosis. Diagnosis of chlamydial infection is even more difficult in asymptomatic and in chronic or persistent infections where the pathogen load would be low. The large pools of asymptomatic infected people are not only at the risk of developing serious long-term sequelae but would also transmit the infection. Urdea et al., speculated that approximately 3 million vic-tims suffering from Disability Adjusted Life Years (DALYs) can be saved, more than 12 million incidence of gonorrhea and Chlamydia infections can be averted, about 161,000 HIV infections can be prevented among female commercial sex workers in sub-Saharan Africa, China and Southeast Asia with a diagnostic method that requires minimal laboratory infrastructure but has 85.0% sensitivity and 90.0% specificity for both gonorrhoea and Chlamydia [16]. A test that requires no laboratory infra-structure could save ~4 million DALYs, avert >16.5 million incidence of gonorrhoea and Chlamydia infections and prevent >212,000 HIV infections [16].

Nucleic acid amplification test (NAATs) are the tests of choice for the diagnosis of C. trachomatis genital infections because of their high sensitivity, specificity and suitability for various types of sample, including vulvovaginal swabs and first void urine (FVU), [3,14,17]. NAAT has also facilitated the use of less invasive proce-dures for detection of asymptomatic C. trachomatis infection in female patients. Several commercial NAATs are available and they make use of different technolo-gies: conventional PCR; quantitative PCR (Roche Diag-nostics, Abbott IL, USA); strand displacement amplification (Becton Dickinson, NJ, USA); transcription-mediated amplification (Gen Probe) and nucleic acid sequence-based amplification (Bio Merieux, Nancy L’etoile, France). The high cost of these kits and lack of appropriate infrastructure are the major deterrents for using these kits for large screening programmes to be conducted in developing countries including India. The prin-cipal detection methods of C. trachomatis infections in India still remain to be the culture method and Direct Fluorescent Assay (DFA). In addition, most of the resource limited clinics continue to practice syndromic management. Therefore there is a paucity of information regarding epidemiology of STD including C. trachomatis infections in India. However, considering the high pre-valence of infection in India there is an urgent need to design tests that are simple, inexpensive and can be used to improve diagnosis as well as specificity of the syndromic management [18-21].

In the present study we have designed and established a simple PCR based assay for diagnosis of C. trachoma-tis using primers against genomic sequences. The eva-luation of the test for its specificity, sensitivity, positive and negative predictive values against currently used diagnostic methods suggest that in-house PCR assay is highly comparable in its performance to that of commer-cial kits and is considerably less expensive. Availability of the test result within few hours could allow treatment at the initial visits and helps in preventing further transmission of the disease.

Methods
Enrolment of Patients
Total 593 out of the 2800 patients (median age 29 years and aged between 18-60 years), visiting gynaecology department, were enrolled for the study. Enrolment of patient was determined on the basis of vaginal discharge and other STD related symptoms. All participants were informed and oral consent of patients was taken.

Specimen collection
A thorough vulval examination was done for lesions and vaginal/cervical discharge. Perspeculum examination was carried out for wart, erosions and abnormal growth by
Amplification was performed using thermal cycler Taq DNA polymerase (Bangalore Genei India Pvt. Ltd., Bangalore, India). Purified genomic DNA of *C. trachomatis* was used as a positive control for each set of assays. 10 pmole each of forward and reverse primers, 0.7 U of Taq DNA polymerase (Bangalore Genei India Pvt. Ltd., Bangalore, India). Purified genomic DNA of *C. trachomatis* (kindly provided by Lynn Olinger, Francis L. Proctor Foundation, University of California, San Francisco) was used as a positive control for each set of assays. Amplification was performed using thermal cycler (I cycler, Bio-Rad, Richmond, USA) for 35 cycles; 95°C for 5 min for initial denaturation, followed by 35 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, final extension at 72°C for 5 min. The amplicons were analyzed on 1.5% agarose gel by electrophoresis. The amplicons from ten percent of positive samples were eluted using a DNA isolation kit (Biological Industries Ltd., Kibbutz Be’er Haemek, Israel) according to the manufacturer's instructions and sequenced by Roche. For endocervical specimens, 1 ml specimen lysis buffer was added to endocervical samples, mixed thoroughly by vortexing and were incubated for 10 min at room temperature. After overnight storage at 4°C, 50 μl of clinical sample was added to each PCR tube containing 50 μl of PCR master mix. The PCR master mix contained primers for internal control as well. The assay was developed as per instructions given by manufacturers. Samples for which the two methods described above showed discrepancy, genomic DNA was isolated from the aliquots of frozen specimens. Samples were centrifuged at 15,000 x g for 30 min. Pellet was resuspended in 500 μl of lysis buffer (Tris EDTA 50 mM pH 8.0, Proteinase K 400 μg/ml) and incubated at 55°C for two hours and then boiled at 100°C for 10 min with 1 mM DTT. Thereafter DNA was extracted with phenol:chloroform and centrifuged at 12,000 x g for 10 min, DNA was precipitated with isopropanol in the presence of 0.3 M sodium acetate and incubated overnight at -20°C. Pellet was collected by centrifugation at 12,000 x g for 10 min, washed with 70% ethanol, air-dried and dissolved in PCR grade water.

**Evaluation of specificity and sensitivity**

To evaluate the specificity of the primer pair, DNA extracted from positive controls, pathogens causing STI and those representing general microflora of cervix were used as templates in PCR reaction (Table 1). DNA was extracted from these microorganisms as described above. Ten human genomic DNA samples were also used to evaluate the specificity of primer pair. To

**Roche AMPLICOR MWP Chlamydia trachomatis Detection Assay**

Three hundred and nineteen endocervical specimens of group II were tested by Roche AMPLICOR CT detection kit (Roche Diagnostic Systems) according to the manufacturer's instructions. For endocervical specimens, 1 ml of specimen lysis buffer was added to endocervical samples, mixed thoroughly by vortexing and were incubated for 10 min at room temperature. After overnight storage at 4°C, 50 μl of the clinical sample was added to each PCR tube containing 50 μl of PCR master mix. The PCR master mix contained primers for internal control as well. The assay was developed as per instructions given by manufacturers. Samples for which the two methods described above showed discrepancy, genomic DNA was isolated from the aliquots of frozen specimens. Samples were centrifuged at 15,000 x g for 30 min. Pellet was resuspended in 500 μl of lysis buffer (Tris EDTA 50 mM pH 8.0, Proteinase K 400 μg/ml) and incubated at 55°C for two hours and then boiled at 100°C for 10 min with 1 mM DTT. Thereafter DNA was extracted with phenol:chloroform and centrifuged at 12,000 x g for 10 min, DNA was precipitated with isopropanol in the presence of 0.3 M sodium acetate and incubated overnight at -20°C. Pellet was collected by centrifugation at 12,000 x g for 10 min, washed with 70% ethanol, air-dried and dissolved in PCR grade water.

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determine the sensitivity of primer pair, purified genomic DNA from *C. trachomatis* at 100 pg to 1 fg concentrations following serial dilutions and various dilutions of *C. trachomatis* positive clinical samples were used as the templates for PCR amplification. All assays were repeated at least five times. For clinical samples, repeat assays were performed for randomly selected samples.

**Direct Fluorescence Assay**
For DFA (MicroTrak, Co Wicklow, Ireland), specimens were centrifuged at 3000 × g for 10 min and pellets were air dried, fixed by incubation in methanol and stained with a fluorescein isothiocyanate-conjugated anti-MOMP monoclonal antibody. The slides were examined for typical apple-green fluorescent elementary bodies (EBs) at 1000× magnification. The presence of more than ten fluorescent EBs was scored as a positive case.

**Fluorescence In-situ Hybridization**
FISH assay was performed as described previously [23].

**Definition of a positive sample**
All 274 clinical specimens in group I were tested by DFA and in-house PCR. FISH assay was carried out on discrepant results as a confirmatory test. Samples were considered positive if they tested positive by any of the two methods. All 319 clinical specimens of Group II were tested by Roche Amplicor MWP kit and by the in-house PCR assay. Amplification of known *C. trachomatis* genes ompA (CT681; Entrez GeneID: 884473) and pilE (CT157, Entrez Gene ID: 884104) were carried out on discrepant samples. Samples were considered positive if they tested positive by at least two PCR methods: Roche Amplicor MWP kit/in-house PCR/PCR amplification of ompA or pilE gene. The prevalence of *C. trachomatis* infection was determined based on the total number of positive samples after discrepant analysis.

**DATA analysis**
All statistical analysis was performed using GraphPad Prism version 5.03 software. Sensitivity, specificity, PPVs and NPVs were calculated with 95% confidence intervals to test the significance.

**Results and Discussion**
The cardinal sign for chlamydial infection according to syndromic case management guidelines is discharge from the cervix. Out of 2800 female patients who visited the gynaecology department for reproductive health problems and contraception, 593 patients were enrolled for this study. Almost all participants were symptomatic, including vaginal discharge (58.0%), lower abdominal

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**Table 1** List of various organisms and their source; used for evaluation of specificity of the C2/C5 primers

<table>
<thead>
<tr>
<th>Organism/Strain/Isolate</th>
<th>Number of Specimens tested</th>
<th>Source(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. trachomatis</em> serovar L2</td>
<td>1</td>
<td>Dr. Peter Braun, Max Plank Institute for Infection Biology, Berlin, Germany</td>
</tr>
<tr>
<td><em>C. trachomatis</em> Serovar A &amp; D</td>
<td>12 isolates from STD cases</td>
<td>Dr. Sudha Salhan, Department of Obstetrics and Gynaecology, Vardhman Mahavir Medical College and Safdarjang Hospital, New Delhi, India.</td>
</tr>
<tr>
<td>Mycoplasma spp.</td>
<td>8</td>
<td>Department of Microbiology AIIMS, New Delhi, India.</td>
</tr>
<tr>
<td>Chlamydia pneumoniae</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td><em>Candida</em> spp.</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Ureaplasma</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas</em> aeruginosa</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>Acinetobacter baumannii</em></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Herpes simplex virus 1 (HSV-1) and Herpes simplex virus 2 (HSV-2)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Cytomegalovirus</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>BK virus</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Trichomonas spp.</td>
<td>8</td>
<td>Department of Microbiology AIIMS, New Delhi, India &amp; Department of Poultry Science, University of Georgia, Athens, USA.</td>
</tr>
<tr>
<td>Neisseria gonorrhoeae</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Neisseria meningitidis (genital isolates)</td>
<td>3</td>
<td>Prof. J. W. Tapsall, WHO Collaborating Centre for STD and HIV, Department of Microbiology, The Prince of Wales Hospital, Randwick, New South Wales, Australia.</td>
</tr>
<tr>
<td>Neisseria lactamica 94D4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Neisseria sicca 94C1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Neisseria subflava 86G7</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>
pain (32.0%), infertility (4.3%) and other STD related symptoms. In our study overall prevalence of STD among females with STI related symptoms was 21.2% (593/2800). STI was more common among patients in the age group of 18-33 years (391/567). In an earlier study, STI prevalence was found to be 16.2% among patients attending STD clinics at a regional STD centre at New Delhi [24]. A relatively high prevalence (36.5%) of STI is reported in tribal population of central India [25]. In a study from Surat, 47.5% of female sex workers were reported to have STI [8], while in Calcutta 59.0% sex workers had STD [26]. In the present study, genital chlamydial infection, as detected by Roche Amplicor test, DFA and in-house PCR assay, was 25.2% among the symptomatic females enrolled. Although \textit{C. trachomatis} prevalence in patients of different age groups was not significantly different (Figure 1), more than 92.0% (118/128) of the total patients positive for \textit{C. trachomatis} were from reproductively active age group of 18-41 years. During the course of the study (2003-2009), the prevalence of patients infected with \textit{C. trachomatis} ranged from 24.0% to 30.0% (Figure 2). Among female sex workers in Surat, India, the prevalence of genital chlamydia by PACE2 test was found to be 8.5%, while in Ahmedabad, India it was reported to be twice as much [8]. Among the tribal populations (patients/general population) from central India although STI was high (36.5%), only 4.0% chlamydial infection is reported [24] which is similar to that observed among patients in Azerbaijan 3.1% [27] and Bangladesh 3.4%, [28]. High chlamydial infection has also been reported in Manila 23.3%, Cebu, Philippines 37.0% and 14.0% in Nicaragua [21,29].

**Specificity and sensitivity of primers**

Using the BLAST program from NCBI, \textit{gyrA} gene of \textit{C. trachomatis} (Entrez GeneID: 884941) was selected for primer designing. Amplicons of desired size (463-bp) were obtained when purified genomic DNA of different serovars of \textit{C. trachomatis} (A, D and L2) were used as templates for the in-house PCR, while no amplicon was detected when genomic DNA from humans as well as other STD causing and related microorganisms were tested as templates. The specificity was further confirmed by sequencing the amplicons obtained from 10.0% of positive clinical samples. The DNA sequence of the amplicons were aligned to the known sequences of serovars A, B, D, E, G, L2 and Sweden2 of \textit{C. trachomatis} in the GenBank databases (Additional file 1). The primer pair was highly sensitive as an amplicon could be seen when as low as 10 fg of purified chlamydial genomic DNA (equivalent to 9 IFUs) was used as template (Figure 3). C2/C5 primers amplified the target sequence even when crude lysates of clinical samples were used in the PCR reaction. NAAT based diagnosis has been observed to be significantly more sensitive than conventional methods such as culture and antigen based methods for diagnosis of \textit{C. trachomatis} in clinical specimens. Several existing PCR assays including the Roche MWP test target the cryptic plasmid while the chromosomal targets most widely used are ompA and rRNA coding genes [20-35]. Plasmid based PCR detection assays are considered to be more sensitive compared to chromosomal gene based assay, as plasmid copy number is generally 7 to 10 per
cell [36-38]. NAAT based diagnostic kits like COBAS AMPLICOR CT (Roche Diagnostic system) and Ligase chain reaction kit from Abbott (presently out of market) were shown to be highly sensitive (92.9 to 100%) and specific (99.0 to 100%) for *C. trachomatis* detection [3,39-42]. However, major obstacle in adopting these kits for routine diagnosis in clinical laboratories in India is their high cost (average cost of single assay is $50). It may also be emphasized that the commercially available diagnostic kits use plasmid-based amplification. A new variant of *C. trachomatis* (Sweden2), carrying a 377-bp deletion within the plasmid, was reported in Sweden [43]. This deletion includes the target sequences used in the commercial diagnostic assays of Roche and Abbott. We have also observed infection due to plasmid free variants as reported in other populations [44,45], which remains a challenge for detection (unpublished results).

Comparison of the performance of different PCR assays including plasmids, *ompA* and *rDNA* targets has suggested that in general plasmid primers are 10 to 1000 times more sensitive (0.1 fg for plasmid DNA) than the genomic DNA primers for *ompA* (0.1 pg to 10 pg genomic DNA) and *rDNA* (1 pg genomic DNA) [34,46-48]. Our assay targeting *gyrA* gene showed high sensitivity (10 fg to 0.1 pg genomic DNA) compared to other genomic targets.

**Clinical performance of in-house PCR and its comparison with DFA and Roche Amplicor MWP kit**

To check the performance of in-house PCR assay, the samples were divided into groups as mentioned under methods. The median age of patients for group I (274 patients) was 29 years (range: 18 to 57 years). Out of 274 patient samples, 59 (21.5%) were positive and 191 samples (69.7%) were negative both by PCR assay using C2/C5 primer set as well as by DFA (Table 2). Out of the 28 discrepant samples, 15 samples were considered true positive, as 11 samples were PCR and FISH positive but DFA negative while 4 were DFA and FISH positive but PCR negative (Table 3). The remaining thirteen discrepant samples were considered true negative as they tested negative both by DFA and FISH assay. These 13 cases, which were PCR positive, could not be reconfirmed as follow-up specimens could not be collected because the patients had received treatment. Based on our results, it is evident that C2/C5 primer pair detected significant number of true positive samples (70 out of 74, p < 0.0001) as compared to the DFA method that detected 63 out of 74 as true positive samples.

Among the 319 samples enrolled in group II, 244 were negative while 60 samples were positive by both in-house PCR and commercial PCR assay (Table 2). To resolve the discrepancy, the genomic DNA of discrepant samples (as described under methods) was purified to remove PCR inhibitors if any, and tested for amplification of two housekeeping genes: *ompA* and *plde* as well as by in-house PCR and commercial PCR method. Out of 15 discrepant samples, 13 samples that were positive by in-house PCR but negative by commercial PCR, 9 samples tested positive for *ompA/plde* and hence were considered true positive. The remaining four in-house PCR positive samples, tested negative for *ompA* and *plde* and Roche PCR and were thus scored as true negatives. Two discrepant samples that were positive by Roche Amplicor MWP kit and negative by in-house PCR, tested positive for *ompA/plde* and were therefore considered true positive (Table 3). The response to therapy administered to the patients with discrepant results obtained from hospital records also supported our final results shown in Table 3.

Once again as for group I, C2/C5 primers detected a significant number of samples (69 out of 71, 97.0%) and its efficiency is comparable to that of commercial PCR assay which detected 98.0% of positive samples. However, the commercial PCR kit detected many samples only when DNA was purified from these samples. Based on our results, the prevalence of *C. trachomatis* infection was 25.5% among women visiting gynaecology outpatient clinic when DFA was selected as gold standard.

**Table 2 Comparison of in-house PCR with DFA and Roche amplicor MWP kit, results for *C. trachomatis* before and after discrepant analysis.**

<table>
<thead>
<tr>
<th>PCR results C2/C5</th>
<th>After resolution of discordant results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group I</strong> DFA</td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>59</td>
</tr>
<tr>
<td>Negative</td>
<td>4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>63</td>
</tr>
<tr>
<td><strong>Group II</strong> Roche</td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>60</td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>62</td>
</tr>
</tbody>
</table>
while it was found to be 21.6% when Roche Amplicor MWP kit was taken as gold standard. The overall prevalence of *C. trachomatis* was found to be 23.0% among symptomatic patients enrolled in the study.

**Sensitivity, specificity, PPV and NPV**

The estimated sensitivity and specificity range of DFA is 61.0% to 92.0% and 99.0% to 100% respectively in different laboratory settings when compared to that of culture or non-culture methods [44]. This could be due to sample preparation and handling conditions in the clinical set up as well as the laboratory. To avoid such factors, the discrepant samples were analyzed with alternative methods of detection in order to determine their true status. The in-house PCR (C2/C5) primers demonstrated sensitivity of 93.6% which increased to 94.5% after resolution of the discrepant cases by a second method for group I (Table 4). Similarly, the specificity of PCR by C2/C5 primers increased from 88.6% to 93.6% after discrepant analysis by FISH. The positive predictive value (PPV) of in-house PCR increased from 71.0% to 84.3% while the negative predictive value (NPV) remained unchanged at 98.0% even after confirming the status of discrepant samples. The estimates of specificity of in-house PCR might have improved further if the follow up samples from patients could be obtained.

The overall sensitivity of DFA in the present study was 88.0% which is close to suggested value of 92.0% [44]. In general, the cut-off for DFA is established to get the best combination of sensitivity and specificity, as a result, sometimes one may miss out a positive sample by compromising sensitivity to achieve specificity. Thomas *et al.* [33] have reported that DFA kit has its limitation as a reference method for evaluating a new diagnostic kit or to check inter-laboratory variation. According to their report the diagnostic performance of DFA test is highly dependent on the number of chlamydial EBs that should be seen in order to score a sample as positive sample. Since about 30.0% of the clinical samples contain ≤ 10 EBs, they are scored as negative. Shattock *et al.* [32] observed similar limitation when they compared various detection methods for *C. trachomatis*. Thus a sample may be DFA negative as it contains ≤ 10 EBs but can score positive by PCR method. In our study we observed 28 samples out of 274 that were positive by C2/C5 primers but negative by DFA or FISH assay. These samples, which repeatedly were positive by our PCR, may have contained chlamydial genomic DNA, but unfortunately no follow up samples were available to confirm this as patients responded to treatment and in some cases they did not return to the clinic.

We also evaluated our in-house PCR method against commercially available and widely used Roche MWP kit. It is pertinent to mention that to avoid the swab sample variation that may occur when multiple swabs are taken (especially when infection load is low); two swabs were taken for each patient. We observed inhibition of amplification reaction when internal control provided by Roche MWP kit also did not amplify. Similar observation is reported previously [32,48]. However, when DNA from these samples was purified and retested by Roche MWP kit, they were found to be positive. Compared with Roche MWP PCR assay our in-house PCR by C2/C5 shows 96.7% sensitivity and 95.0% specificity. Subsequent to resolving discrepancy the sensitivity of PCR method increased to 97.1% and specificity increased to 98.4% and the PPV of in-house PCR increased from 82.1% to 94.5% while the NPV remained unchanged at 99.0% (Table 4).

**Conclusions**

The major objective of this work was to develop an in-house PCR assay that is cost effective and has high

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**Table 3 Resolution of discrepant results for *C. trachomatis* infection.**

<table>
<thead>
<tr>
<th>No. of discrepant samples</th>
<th>Conclusion</th>
<th>C2/C5 DFA</th>
<th>FISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (n = 28)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 + - +</td>
<td>True positive</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>4 - + +</td>
<td>True positive</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13 + - -</td>
<td>True negative</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Group II (n = 15)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 + - +</td>
<td>True positive</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>4 + - -</td>
<td>True negative</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2 - + +</td>
<td>True positive</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

A second test was applied for each sample. FISH was taken as the gold standard for group I and *ompA/plde* for group II.

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**Table 4 Performance of in-house PCR assays based on expanded spectrum of positivity after confirmatory FISH assay and *ompA/plde* PCR.**

<table>
<thead>
<tr>
<th>In-house PCR Assay</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>95% CI</td>
<td>%</td>
<td>95% CI</td>
</tr>
<tr>
<td>Group I</td>
<td>94.5</td>
<td>86.7-98.5</td>
<td>93.6</td>
<td>89.1-96.5</td>
</tr>
<tr>
<td>Group II</td>
<td>97.1</td>
<td>90.2-99.7</td>
<td>98.4</td>
<td>95.9-99.6</td>
</tr>
</tbody>
</table>

* CI, Confidence interval.
sensitivity and specificity so that it can be used for diagnosis of *C. trachomatis* infection. Despite the high prevalence of STI in developing countries, the laboratory confirmation of infection is not carried out because of poor resource settings and due to lack of simple, cost effective diagnostic tools. The existing diagnostic methods in developing countries are not effective and their sensitivity fall below 45.0% which leads to further spread of infection [49]. The annual incidence of STIs in India is about 5.0% with 40 million new cases every year [50]. This prompted WHO to emphasise on syndromic approach for case management in developing countries including India. A number of authors suggest that syndromic management based on vaginal discharge syndrome either miss out a significant proportion of cases of genital chlamydia or lead to treatment even in the absence of infection of *C. trachomatis* [8,51,52]. In the present study we enrolled patients having symptoms and expecting treatment. In spite of this selection bias, the chlamydial infection was observed to be high among females in Delhi as was evident by laboratory investigations. Quick and inexpensive diagnostic test developed in the present study can help in regular clinical and laboratory screening for *C. trachomatis*. Consistent high prevalence of chlamydial infection as well as the potential synergistic role of STI in HIV and HPV transmission [4-6] suggest that syndromic management along with periodic screening may prove to be more effective approach to achieve long term goals of STI and HIV control through sustained access to effective preventive and treatment services.

The principle goal of this study was to develop an in-house PCR method that is at least as sensitive and as specific as commercial method. An additional advantage of the in-house PCR method would be its low cost. Since our studies also suggest that there is a high prevalence of *C. trachomatis* (>23.0% in females visiting gynaecology department), there is a definite need to have a cost effective method for routine diagnosis in India. We consider that implementation of the specific and sensitive PCR assay, developed in the present study may allow clinical microbiology laboratories in developing countries to detect *C. trachomatis* rapidly, which would be of great consequences in disease management.

**Acknowledgements**

We thank Prof. Vani Brahmacari (Dr. B. R. Ambedkar Center for Biomedical Research) for reading the manuscript and providing valuable comments. PN and DS gratefully acknowledge the research fellowships from ICMR and CSR respectively. We are grateful to all the patients and clinicians for their cooperation in this study. Financial support from DRT (ET/PRO 910/MED/09/168/08 and 102/FD/SAN/PR1119/2007-2008) is gratefully acknowledged.

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**Authors’ contributions**

ALP participated in designing the experiments, executing them, performing data analysis and writing the manuscript. DS and PN contributed in designing and performing the experiments. UC helped in designing of experiments and writing of the manuscript. SC5 participated in performing the clinical evaluation of the in-house PCR. SM organized and supervised the collection of clinical specimens. DS (corresponding author) instigated the project, designed experimental settings, finalized data analysis and writing of the manuscript. All authors read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

**Received: 4 May 2010 Accepted: 8 September 2010**

**Published: 8 September 2010**

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Review Article

Indian J Med Res 134, October 2011, pp 419-431

An insight into the drug resistance profile & mechanism of drug resistance in Neisseria gonorrhoeae

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Received October 27, 2009

Among the aetiological agents of treatable sexually transmitted diseases (STDs), Neisseria gonorrhoeae is considered to be most important because of emerging antibiotic resistant strains that compromise the effectiveness of treatment of the disease – gonorrhoea. In most of the developing countries, treatment of gonorrhoea relies mainly on syndromic management rather than the aetiological based therapy. Gonococcal infections are usually treated with single-dose therapy with an agent found to cure > 95 per cent of cases. Unfortunately during the last few decades, N. gonorrhoeae has developed resistance not only to less expensive antimicrobials such as sulphonamides, penicillin and tetracyclines but also to fluoroquinolones. The resistance trend of N. gonorrhoeae towards these antimicrobials can be categorised into pre-quinolone, quinolone and post-quinolone era. Among the antimicrobials available so far, only the third-generation cephalosporins could be safely recommended as first-line therapy for gonorrhoea globally. However, resistance to oral third-generation cephalosporins has also started emerging in some countries. Therefore, it has become imperative to initiate sustained national and international efforts to reduce infection and misuse of antibiotics so as to prevent further emergence and spread of antimicrobial resistance. It is necessary not only to monitor drug resistance and optimise treatment regimens, but also to gain insight into how gonococcus develops drug resistance. Knowledge of mechanism of resistance would help us to devise methods to prevent the occurrence of drug resistance against existing and new drugs. Such studies could also help in finding out new drug targets in N. gonorrhoeae and also a possibility of identification of new drugs for treating gonorrhoea.

Key words Epidemiology - mechanism of drug resistance - Neisseria gonorrhoeae

Introduction

Despite the recent advances in diagnosis, surveillance and treatment, sexually transmitted diseases (STDs) remain one of the leading diseases throughout the world. Increased promiscuity and onset of sexual activity at an early age are two important contributing factors to the spread of sexually transmitted diseases. Neisseria gonorrhoeae (also known as the gonococcus) colonizes primarily in the human genitourinary tract, giving rise to the sexually transmitted infection gonorrhoea. It causes both symptomatic and asymptomatic genital and extragenital tract infections. Disease caused by this organism is a significant public health problem despite continual advances in treatment\textsuperscript{1,2}. Worldwide, there
are an estimated 62 million new cases a year, with an average of 22 million cases at any given time\textsuperscript{4-5}. *N. gonorrhoeae* inhabits mainly mucosal surfaces of the urethra in males and the cervix in females. As the signs and symptoms of infection are often absent or obscure, complications such as pelvic inflammatory disease (PID), infertility, ectopic pregnancy arise\textsuperscript{6}. Infection in pregnant women may lead to crucial perforation and blindness in the newborn. Gonococcal infections have also been documented to facilitate acquisition and transmission of HIV and HPV infections\textsuperscript{7,8}. Asymptomatic infections by *N. gonorrhoeae* largely contribute to the persistence and transmission of disease in a community\textsuperscript{9}. Therefore, to eliminate *N. gonorrhoeae* infections and in turn to control HIV and HPV infections it is important not only to screen high-risk population but also to treat them immediately with most effective drugs. Control of gonococcal infections has relied on effective single-dose antibiotic therapy given at the initial clinical visit, prior to any knowledge of the organism’s susceptibility pattern. In the recent past, there has been an alarming increase in the number of isolates of *N. gonorrhoeae* resistant to commonly used drugs\textsuperscript{10-14}. Surveillance is, therefore, necessary to understand ongoing resistance trends and to ensure the success of any therapy. The irrational and injudicious use of antibacterial agents, especially in the developing countries is encouraging this trend and the situation is expected to worsen unless appropriate steps are initiated. Thus, resistance of the gonococcus to antibiotics has been the cause of much concern in recent years and has been the subject of extensive investigation. The present review summarizes and trends of drug resistance in *N. gonorrhoeae*, mechanism of drug resistance and discusses the treatment regime. In addition, the need to look for new and alternative antibacterial agents is also emphasized.

**Characteristics of gonococcus**

*N. gonorrhoeae* is a Gram-negative diplococcus and is known to infect humans only. It is closely related to and probably derived from *Neisseria meningitidis*, but has become highly adapted to survival in the genital tract. It is transmitted by human-to-human contact and survives poorly outside the human body. *N. gonorrhoeae* is a very successful pathogen as it can evade or adapt to host defences, persist without severely damaging the host, and be transmitted to and infect other hosts, thereby maintaining itself. Particularly in women, gonococci may produce only mildly symptomatic or asymptomatic disease. This adaptation allows the organism to persist and disseminate over long periods\textsuperscript{15}.

The most important characteristics of *N. gonorrhoeae*, in the context of antimicrobial resistance pattern, are its phenotypic and genotypic variability which enables it to evade the host response. Phenotypic variability occurs through differential expression of existing parts of the genome. Genotypic variation is achieved by incorporation of new genetic material, which can be acquired either by conjugation or transformation. It is because of this feature that *N. gonorrhoeae* has acquired penicillinase producing plasmids. Another important feature of *N. gonorrhoeae* is its antigenic variability. This helps the bacterium to survive in its limited host, *i.e.*, humans. Antigenic variability of *N. gonorrhoeae* is partially due to its ability to acquire genetic material from related organisms\textsuperscript{16,17}.

**Epidemiology**

Single dose therapy for *N. gonorrhoeae* infection has become the norm in most of the countries throughout the world. The basic reason behind this is that single dose therapy is most effective and assures adequate treatment. World Health Organization (WHO) recommendations for selecting any treatment for gonorrhoea states that the antimicrobial prescribed should be such that the cure rate is about 95 per cent\textsuperscript{18,19}. Moreover, during the past few years *N. gonorrhoeae* has started developing resistance against most of the antimicrobials that are prescribed for its therapy. Therefore, surveillance of the antimicrobial resistance becomes very important in monitoring the emergence and spread of resistance and in planning appropriate treatment regimens. Gonorrhoea is a disease mainly found in resource-poor settings where laboratory facilities are limited or unavailable. Due to this reason, culture and antimicrobial susceptibility testing of *N. gonorrhoeae* is hardly done in the developing countries. Developed countries have collected the data in proper manner due to availability of adequate resources. Such planned studies are always of help in monitoring resistance pattern in the bacteria. However, resistance data obtained from developing countries are mainly from point prevalence studies, which cannot be used to follow the trend. Such epidemiological studies need to be done on a regular basis because the prevailing strains of the bacteria and their antimicrobial susceptibility profiles keep on changing. The data obtained on *N.
gonorrhoeae susceptibility, so far, are incomplete and there is an urgent need for proper surveillance throughout the world.

Pre-quinolone era

Sulphanilamide was introduced as an antimicrobial against *N. gonorrhoeae* as early as in 1937. However, the bacteria became resistant quickly against sulphanilamides within a span of two years. At the same time, when sulphanilamides were given as treatment for gonorrhoea, Alexander Fleming also documented the ability of penicillin to inhibit the growth of *N. gonorrhoeae* in his 1929 paper describing penicillin discovery. Thereafter, penicillin became the choice of antimicrobial for the treatment of gonorrhoea in 1943 and remained so for decades. Susceptibility profile of *N. gonorrhoeae* against penicillin (and other antimicrobials) was monitored throughout the world, and *in vitro* resistance to penicillin was expressed in a uniform manner in terms of minimum inhibitory concentrations (MIC). During the initial years of treatment of gonorrhoea with penicillin, all the isolates had an MIC of <0.0125 mg/l (0.02 IU/l) and were considered to be sensitive to the treatment. However, *N. gonorrhoeae* began developing low level resistance to penicillin. The MIC values of *N. gonorrhoeae* isolates gradually increased to >0.12 mg/l and gradually most of the strains became resistant to penicillin (MIC >0.5 mg/l). Due to this increase in the MIC values, it was necessary to increase the effective dose of penicillin from 50,000 units in 1945 to 4.8 million units by 1970. This increase in penicillin resistance was proved to be the additive effect of multiple chromosomal mutation resulting in altered penicillin binding proteins, increase in the antibiotic efflux system and probably decrease in the antibiotic uptake from the membrane. Chromosomal mediated penicillin resistance was found to be of low level as determined by the MIC values. Simultaneous to the development of chromosomal mediated resistance, high-level plasmid mediated resistant isolates of *N. gonorrhoeae* were also observed in various countries. These isolates were termed as penicillinase producing *N. gonorrhoeae* (PPNG) as these harboured a plasmid having a gene of β-lactamase, the product of which conferred resistance towards penicillin. Reports of such high level penicillin resistant isolates were documented in Africa, Asia, North America, Europe and Australia. Due to the emergence of penicillin resistant isolates (both chromosomal and plasmid mediated) of *N. gonorrhoeae*, penicillin was no longer considered an effective treatment for gonorrhoea by 1989 and, therefore, penicillin was also prohibited in most parts of the world.

A few documented reports of antimicrobial susceptibility data from India suggested a slow stepwise increase in penicillin resistance. Low level resistance of *N. gonorrhoeae* to penicillin was observed first in India as early as 1981 in Madras, where the first case of β-lactamase isolate of *N. gonorrhoeae* was documented. Thereafter, various other reports indicated the increase in the resistance profile of *N. gonorrhoeae* towards penicillin. Most of the isolates resistant to penicillin were found to be harbouring β-lactamase producing plasmid. Use of penicillin for the treatment of gonorrhoea was discontinued in India in 1990. As a result, penicillin resistance (both chromosomal and plasmid mediated) decreased subsequently. Thereafter resistance towards penicillin once again showed a steep rise to 42.4 and 66.7 per cent in 2000 and 2001 respectively, along with increase in isolation of penicillinase producing *N. gonorrhoeae* (PPNGs). Study conducted by Ray et al. showed high level of penicillin resistance from Hyderabad (79%) and Chennai (62.5%), while low level of resistance (20-33%) for penicillin was observed from isolates obtained from Kolkata, Nagpur and Pune. Coincident with the development of resistance to penicillin, gonococci also developed resistance to several other antibiotics, including tetracycline, chloramphenicol, erythromycin and streptomycin. Tetracycline was also considered as another important antimicrobial during the pre-quinolone era. Since tetracycline was not very expensive and thus was a widely used antimicrobial, and most importantly tetracycline was given as an adjunct therapy for *Chlamydia trachomatis*, it was not possible to evaluate the contribution of tetracycline in the management of gonorrhoea. Same was the case with azithromycin, even though it was considered to be a more expensive alternative. Gradually through constant use of tetracycline to treat such co-infections, *N. gonorrhoeae* acquired low-level resistance towards this antimicrobial. High-level chromosomally mediated tetracycline resistance emerged in the 1970s along with chromosomally mediated penicillin resistance. Plasmid mediated tetracycline resistant *N. gonorrhoeae* (TRNG) emerged in 1985 in Atlanta and The Netherlands and was probably a result of the acquisition on a plasmid, a *tet*-M determinant from streptococcal species.
Various reports from USA also indicated the presence of both chromosomal mediated tetracycline resistance and TRNG\textsuperscript{24,67}. In 1997, 25.6 per cent of isolates from USA were tetracycline-resistant, of which 17 per cent were chromosomally mediated and 8.6 per cent were TRNG\textsuperscript{68}. Regional data from USA showed an increase in TRNG from less than 5 per cent in 1990 to early 15 per cent in 1995. In the WHO Western Pacific study, TRNG were widely but unevenly distributed. In 1998, particularly high proportions of TRNG were seen in Singapore (84%), the Solomon Islands (74%) and Vietnam (35.9%), continuing a pattern observed in earlier years\textsuperscript{18}. In all other regions TRNG distribution was below 10 per cent. Reports of high level tetracycline resistance were also documented in Africa, Europe and Netherlands\textsuperscript{18}.

TRNG strains were also identified in the WHO South East Asia Region, and Thailand alone accounted for about 16 per cent of isolates in 1994-1997\textsuperscript{18}. An additional 55 per cent of strains had chromosomal-mediated resistance. Indonesia had particularly high rates of TRNG, and virtually all \textit{N. gonorrhoeae} isolates show one or the other forms of resistance\textsuperscript{49}. In India, decreased susceptibility towards tetracycline was reported as early as in 1971 in Mumbai with 28 per cent of the isolates with decreased susceptibility towards tetracycline\textsuperscript{50}. Bhalla et al\textsuperscript{54} found 28 per cent of 50 consecutive isolates in New Delhi to be TRNG. In 1997, 10 per cent of 94 isolates from Bangladesh were TRNG\textsuperscript{51}. In 2000/2001, a study conducted by Ray et al\textsuperscript{52} reported high percentage resistance in three centers of India (Hyderabad, Nagpur and Pune).

Another important antimicrobial, the aminocyclitol (spectinomycin) was introduced for the therapy against antibiotic resistant gonococci or in patients who were allergic to other drugs. Most gonococci have remained sensitive to spectinomycin, except for the report describing three strains in Europe, which are apparently single-step high-level resistant mutants\textsuperscript{62}. First case of spectinomycin resistant \textit{N. gonorrhoeae} isolate was reported in India in 2002\textsuperscript{53}.

**Quinolone era**

\textit{Quinolones in the treatment of N. gonorrhoeae:} In 1989, in response to the increasing frequency of isolation of penicillin, tetracycline, streptomycin and spectinomycin-resistant strains of \textit{N. gonorrhoeae} in the United States and also throughout the world, the Centers for Disease Control and Prevention (CDC) recommended the use of broad-spectrum cephalosporins or fluoroquinolones for the primary treatment of uncomplicated gonorrhoea\textsuperscript{54}. Similar policies were also adopted by various national organizations in other parts of the world. The quinolones most widely used for the treatment of gonorrhoea are second generation antimicrobials such as ciprofloxacin, norfloxacin and ofloxacin\textsuperscript{55}. The fourth-generation quinolones, such as trovafloxacin, have been tested for the treatment of gonorrhoea, but information on resistance to this antimicrobial is not available. It was noticed that among the quinolones, fluoroquinolones had excellent oral absorption and good tissue distribution, achieved excellent interstitial fluid levels and adequate penetration into macrophages, were free from any serious toxic side effects and induced low frequency of spontaneous single-step mutations. Due to their excellent safety and tolerability, these have become popular alternatives to penicillin and cephalosporin derivatives in the treatment of various infections including gonococcal infection. Soon fluoroquinolones were regarded to be as close as possible to the ‘ideal’ antimicrobial agent, since these possessed a broad spectrum of antimicrobial activity. By 1993, ciprofloxacin was recommended as the first line therapy to treat gonorrhoea throughout the world\textsuperscript{56}.

**Quinolone resistance profile in N. gonorrhoeae:** Initially most of the isolates of gonococci were found to be extremely susceptible to quinolones and more importantly, fluoroquinolones. Widespread use of fluoroquinolones in last 20 years and often misuse, coupled with emerging resistance, gradually compromised their utility\textsuperscript{44}. Since the importance of proper documentation of antimicrobial susceptibility studies was understood, data became available from all parts of the world\textsuperscript{57}. Knapp’s criteria for \textit{in vitro} resistance to quinolones have been followed in almost all the studies carried so far\textsuperscript{58}. In most of these studies, it was evident that the resistance towards fluoroquinolones, which is almost chromosomal mediated, develops in an incremental manner. Most of these studies indicate that the initial isolates which were less susceptible towards ciprofloxacin were found to have MIC values of 0.06 mg/l, which gradually increased to 1 mg/l (such strains being referred to as intermediate resistant) and later to as high as 16 mg/l (classified as resistant isolates). Such strains were referred to as quinolone resistant \textit{N. gonorrhoeae} (QRNG). Strains with MIC value >4 \textmu g/l were considered as high level resistance (HLR).
strains. Patients infected with these isolates showing decreased susceptibility or intermediate resistance towards ciprofloxacin, usually responded very well to 500 mg of ciprofloxacin. Later on, a few reports of treatment failure also appeared.

The ciprofloxacin resistant isolates were reported in mid 1980s from many parts of the world. By the end of 1992, more than 40 per cent ciprofloxacin resistant isolates were documented in Japan. Thereafter, ciprofloxacin resistant strains spread very quickly from Asia to Australia, Hawaii and North America. Studies from USA also indicated a rise in ciprofloxacin resistant isolates especially in California. Significant ciprofloxacin resistance emerged simultaneously from the WHO Western Pacific Region and SEAR. In these countries, it was thought that the emergence of ciprofloxacin resistance was accelerated mainly because of its use for the treatment of other diseases as well. There were two reports of increasing ciprofloxacin resistance from Bangladesh. In India, the use of ciprofloxacin, as the first-line therapy for gonorrhoea started in 1990. It was also included in the syndromic management in cases of suspected gonorrhoea. Resistance to norfloxacin soon appeared in 1996 from New Delhi, India. By the end of 2000, a burst in ciprofloxacin-resistant isolates was observed in India. Interestingly, with the emergence of fluoroquinolone resistant strains in India, a rapid decline of PPNGs was observed. Similar observation was also reported from other countries indicating a penicillinase producing plasmid curing effect on an ecological scale. In most of these studies, the molecular basis of antibiotic resistance was not investigated. In a study from India, decrease in ciprofloxacin resistant strains was observed which may be due to ciprofloxacin not being used for treatment of gonorrhoea in India. Studies from developed countries such as Australia, Canada and US suggest that quinolone resistant strains were introduced sporadically over many years. Once introduced into sexual networks, these subtypes spread and eventually achieved endemic transmission. In response to the increase in ciprofloxacin resistant isolates from throughout the world, the use of this antimicrobial to treat gonorrhoea was discontinued in early 2000s from most of the countries. In 2004, CDC discontinued the use of ciprofloxacin to treat gonococcal infections. The use of ciprofloxacin was continued in Europe till 2004 and was discontinued only in 2005. Simultaneously, the use of quinolone group of antimicrobials for the treatment of gonorrhoea was also discontinued in India.

**Post-quinolone era**

Consequent to the increase in the resistance profile of *N. gonorrhoeae* towards quinolones, third-generation cephalosporins, both injectable (ceftriaxone) and oral (cefixime and cefdinir), were the only available treatment recommended by the CDC and other national organizations for the gonococcal infections. In patients allergic to cephalosporins, spectinomycin was recommended as the drug of choice. Cephalosporins were discovered in 1945. These are known to work as other β-lactams, by inhibiting the cell wall synthesis through binding and inhibiting the action of enzymes responsible for inserting peptidoglycan cross-linkage structures into the cell wall.

Cephalosporins are known to be important antimicrobials for the last 10 years. Despite their historic reliability, resistance to cephalosporins also started developing in Asia and later on in other regions of the world as well. The resistance towards cephalosporins was documented as early as in 1996 and then later in 2000 in Japan. Several subsequent reports from Japan also indicated much higher MIC values for cephalosporins. Similar results were also documented from other countries like China, Hong Kong, Taiwan, Europe, US and Africa. A surveillance report from India, wherein isolates collected from different laboratories of India, Bangladesh, Nepal and Sri Lanka during 1999-2001, documented significant increase in the isolates with decreased susceptibility to ceftriaxone. In India, Bala et al. reported nine isolates with ceftriaxone MIC of 0.064 mg/l among the 382 isolates studied during 2002-2006. All cases were treated with ceftriaxone 400 mg and there were no treatment failures observed. Some *N. gonorrhoeae* isolates demonstrating reduced cephalosporin susceptibility also have reduced susceptibility to multiple drug classes, including quinolones, macrolides, penicillins, and tetracyclines. These ceftriaxone less sensitive strains almost always exhibited resistance to quinolones or quinolone and penicillin as reported from Australia, Japan and India suggesting increasing prevalence of these multi-resistant strains in these countries.

**Potential alternatives in the treatment of N. gonorrhoeae infections:** Until 1980s, there was a parallel and consistent development of the new antibacterials, which were active against most of the resistant strains.
of bacteria. The increasing drug resistance in almost all bacteria in the recent past, has prompted scientists to look for possible alternatives such as immunotherapy, vaccination, identification of novel targets for drugs, probiotics, etc. Attacking virulence mechanisms rather than the whole bacterial structure offers a wide range of possibilities. An advantage of such a strategy is that it seems less likely to apply selection pressure. Although no work has been done for _N. gonorrhoeae_, in other bacteria, targets that have been investigated include receptor sites, sortases, quorum sensing signals, Shiga toxin, and staphylococcal enterotoxin B. In addition, the therapies derived from complementary and alternative medicine (CAM) used by the general public, need to be explored.

Spectinomycin can also be considered as a therapeutic option for persons with gonococcal urogenital infection who cannot tolerate cephalosporins. However, it would probably remain as an alternative treatment rather than a recommended one, because high levels of resistance developed when this antimicrobial was widely used in the mid-1980s. Azithromycin, 2 g, taken orally has been shown to be effective against uncomplicated gonococcal infection and could be thought as an option for persons who are allergic to cephalosporins. However, concerns about the development of antimicrobial resistance to macrolides with widespread use restrict current treatment recommendations to limited circumstances. Macrolides such as azithromycin and erythromycin, have also been associated with the multiple transferable resistance efflux system.

Several naturally occurring bacterial DNA gyrase inhibitors, such as the coumarins, which include novobiocin, chlorobiocin and coumermycin A1, have been shown to have antibacterial property. The coumarin derivatives inhibit ATPase activity of DNA gyrase by competing with ATP for binding to the B subunit of the enzyme. Recently activity of some medicinal plants has been evaluated against _N. gonorrhoeae_, which seems to have a promising future. Among the compounds that were evaluated, eugenol, a compound from _Ocimum sanctum_ was also found to be active against multi-resistant isolates of _N. gonorrhoeae_.

**Mechanism of drug resistance**

_N. gonorrhoeae_, originally highly susceptible to antibiotics can adapt to adverse conditions. A hostile environment in which antibiotics are present may select for the multiple changes which result in resistance and treatment failure. Mechanisms of antibiotic resistance in gonococci may be conveniently grouped as those that involve reduced access of the antibiotic to the target site and those that involve alteration of the target site itself. Access of antibiotics to the target site may be limited by reduced permeability of the cell envelope caused by changes in porin proteins; active export of antibiotics from the cell by means of efflux pumps; and destruction of the antibiotic before it can interact with the target. Alteration or deletion of the target site of the antibiotic results in a reduction of its affinity for the antibiotic. Genetically, these changes may be mediated by either chromosomal or extra-chromosomal elements (plasmids). Multiple resistance determinants may coexist in a single organism so that the level of resistance can increase incrementally and a single strain can be resistant to a number of different antibiotics.

In gonococci, chromosomally mediated resistance is generally slow to emerge and disseminate. In _N. gonorrhoea_, the process of genetic transformation is known to be responsible for acquiring drug resistance. But such a change is visible only if many such acquisitions of the determinant take place. Plasmid-mediated resistance, at present limited to penicillins and tetracyclines, is transmitted by means of conjugation. This process requires the presence of a conjugative plasmid to mobilize the plasmid carrying the resistance determinants. Since not all strains possess conjugative plasmids, the rate of spread of resistance may be limited to some extent. However, conjugative plasmids are also transferable during conjugation, so that some recipient strains then become donors themselves. Different rates of dissemination of extra-chromosomally mediated resistance have been observed. For example, the ‘Asian’ PPNG plasmid spread more rapidly than the ‘African’ PPNG plasmid because initially only strains carrying the former determinant also contained conjugative elements. In _N. gonorrhoeae_, plasmid-mediated resistance spreads more rapidly than chromosomally mediated resistance. Amongst non-quinolone drugs, several studies have been carried out to understand the mechanism of penicillin resistance, which has been summarized below.

(i) **Resistance to penicillins**: The penicillins were widely used for the treatment of gonorrhoea for many years (and still are in some regions). Originally, _N. gonorrhoeae_ was extremely sensitive to almost all the drugs, known so far, and treatment with 150,000 units of penicillin was effective in most instances. Later on
decreased *in vitro* susceptibility towards penicillin appeared and it was thought to be associated with treatment failure. Increasing the recommended dose of penicillin 'temporarily alleviated the clinical problems resulting from infection with these strains, but almost inexorably levels of resistance increased and large numbers of treatment failures again occurred, even with high-dose regimens'. This was an example of step-wise accrual of chromosomal changes over a period of many years. The targets of β-lactam agents are the penicillin binding proteins (PBPs), enzymes located in the cell envelope that participate in cell wall metabolism. Alterations in PBP-2 and PBP-1 decreased their affinity for the penicillins, and thus the susceptibility of the organism. PBP-2 is encoded by the *penA* locus. Changes in other loci such as *mtr* and *penB* produce additive effects. The *mtr* locus mediates resistance to a wide range of antibiotics, detergents and dyes through an active efflux system. Mutations in the *penB* locus, which affect a porin, result in reduced permeability of the cell envelope to hydrophilic antibiotics and other compounds. *N. gonorrhoeae* also has a *porA* 'pseudogene' which is not expressed. In contrast, *N. meningitidis* expresses two porins, PorA and PorB. The combined effect of *penA* mutations and increased expression of *mtr* is shown to increase the MIC of penicillin by 120-fold. Gonococci exhibiting these changes are termed chromosomally resistant *N. gonorrhoeae* (CMRNG). Reduced susceptibility to cephalosporins, tetracyclines and other agents is also mediated by chromosomal mechanisms in the concerned genes.

In addition to chromosome mediated resistance, resistance to penicillins is also mediated by a plasmid-borne, inducible TEM-1 type β-lactamase. β-lactamase is known to hydrolyze the β-lactam ring of penicillins, thus inactivating them. Chromosome mediated resistance is slow and incremental, on the contrary, resistance mediated by plasmid is a single step process. PPNG were detected at the same time in the United Kingdom and the USA. The first isolates were reported, respectively, from Africa and the Far East. Although the same TEM type of β-lactamase was present in both instances, the gene was carried on plasmids of different sizes, which became known as the ‘African’ and ‘Asian’ plasmids. Transmission of the resistance by conjugation required the presence of another mobilizing plasmid, which was already present in the Asian PPNG when it was first isolated, but was not found in the African strains until 1981. Thus, the Asian strain disseminated more widely and more quickly. Lactamase production (PPNG) and chromosomal changes (CMRNG) can co-exist in the same isolate. This is relevant because of the clinical use of penicillins in combination with β-lactamase inhibitors. These substances, such as clavulanic acid and sulbactam, prevent the β-lactamases from inactivating the penicillins. Combinations such as amoxycillin/clavulanic acid are widely used to treat other infections. In theory, and sometimes in practice these represent an effective oral therapy for PPNG infections, but more commonly single-dose regimens of penicillin/inhibitor combinations have failed. This appears to be due to PPNG strains having a high frequency of underlying intrinsic or chromosomally mediated penicillin resistance. Chromosomally mediated resistance can be measured reliably only after the organism is ‘cured’ of its plasmid and the MICs reassessed.

**(ii) Resistance to quinolones:** As already stated, the quinolone antibiotics most widely used for the treatment of gonorrhoea are ‘second generation’ agents such as ciprofloxacin and ofloxacin. As in the case of chromosome mediated penicillin resistance, resistance to these antibiotics has developed incrementally over a number of years and multiple chromosomal changes are involved. Access of quinolones to their targets is reduced by changes in cell permeability and possibly by efflux mechanisms. These events produce low-level quinolone resistance. The targets of the quinolones are topoisomerases, including DNA gyrase. High-level clinically relevant resistance is mediated by alteration of the target sites, initially via mutation in the *gyrA* gene. Multiple amino acid substitutions have been described which, when combined, result in high-level resistance. Multiple mutations also occur in the *parC* gene which codes for the production of topoisomerase IV, a secondary target for quinolones in gonococci, but again found in association with high-level resistance. Changes in ParC seem to arise in the presence of mutations affecting GyrA. The more recent (fourth generation) quinolones are more active against strains with altered ParC, but are less effective against GyrA mutants. Thus, these compounds will in theory, be active against some, but not all, ciprofloxacin-resistant gonococci. The newer quinolones have yet to be assessed for efficacy against gonorrhoea. One of these agents, trovafloxacin, has been withdrawn from use in many countries because of toxic side-effects.

**(iii) Resistance to cephalosporin antibiotics:** Altered gonococcal susceptibility to cephalosporin antibiotics is chromosomally mediated and is due to the same changes that account for decreased penicillin
susceptibility\textsuperscript{116,119}. There is cross-resistance between penicillins and early generation cephalosporins such as cefuroxime\textsuperscript{119,120}. However, this is not the case for the later generation cephalosporins such as ceftriaxone and cefixime. Not all cephalosporins are hydrolyzed by the TEM-1 type β-lactamase, and therefore, some of these compounds are active against PPNZ. Other β-lactamases (cephalosporinases), which are constitutively expressed by many other Gram-negative genera, have thus far not been detected in gonococci and there has been no transfer of genetic material encoding production of extended spectrum β-lactamases into pathogenic \textit{Neisseria}. If such an event occurs, it would be devastating for gonorrhoea treatment programmes that rely heavily on the third-generation cephalosporins. In the past five years, gonococci with decreased susceptibility to ceftriaxone has been reported though the mechanism of resistance has not been fully understood\textsuperscript{77}. Recent data also suggest that the emergence and spreading of cephalosporin-resistance gonococci is quite similar to the data showing the emergence of quinolone-resistance strains\textsuperscript{127}.

\textbf{Conclusion}

Despite a high prevalence of uncomplicated gonorrhoea and an increasing incidence of resistant isolates of \textit{N. gonorrhoeae}, throughout the world, standardized monitoring of the antimicrobial susceptibility profile has been restricted to Gonococcal Isolate Surveillance Project (GISP) in United States, Gonococcal Resistance to Antimicrobials Surveillance Program (GRASP) in England and Wales and Gonococcal Antimicrobial Susceptibility Program (GASP) in the America and the Caribbean. There is a need for better control of gonococcal disease including enhanced global surveillance of resistance and improved treatment. The cost associated with culture of gonococci for determination of antimicrobial resistance profile forbids routine determination of MIC before treatment. Hence, there is an undeniable need to simplify and standardize the \textit{in vitro} antibacterial susceptibility procedures. The E-test method though expensive, is an attractive alternative to the earlier, agar dilution technique, for gonococcal antibacterial susceptibility testing. Molecular technology can provide an alternate procedure to the culture method for the surveillance of the antimicrobial susceptibility. It is believed that the single tube assays using techniques like hybridization capture for the detection of wide range of resistance genes would allow their application beyond the laboratory directly in clinical practice\textsuperscript{128}. Efforts should be made to design common assay for detection as well as to investigate the drug resistance profile of \textit{N. gonorrhoeae} so that infected patients can be treated immediately. Such methods would facilitate an early treatment with the most effective drugs as well as will control the transmission of infection.

The single dose approach has also contributed to the ability of \textit{N. gonorrhoeae} to develop resistance to commonly used, inexpensive and effective drugs. The capacity and ease for genetic recombination and high transmissibility of resistant genes has made the development of new antibiotics a challenging area of research. The choice of antibacterial agent must take into account the data generated by laboratory based surveillance of susceptibility. This surveillance would be useful not only in deciding the correct treatment but also in helping to detect the emergence of new antibiotic resistant traits and to monitor the effectiveness of prescribed treatment. To make this information reliable, the laboratories need to adopt and use standardized laboratory procedures and take part in external quality assessment programme\textsuperscript{129}. Studies addressing the effect of continued suppression of bacteria by an antimicrobial agent (post-antibiotic effect) and the effect exerted at sub-inhibitory concentrations, known as the post-antibiotic sub-MIC effect (PA-SME) need to be carried out for the rational use of antibiotics in \textit{N. gonorrhoeae} infection. Global initiatives are imperative to integrate diagnostics, disease management and control of antimicrobial resistance.

It has also been observed that the problem of antibiotic resistance is greatly influenced by poverty and the factors related to it. Due to the cost involved, treatment of gonorrhoea relies on syndromic management in developing countries including India. The problem of drug-resistance in these countries, though an important issue, is thus not properly addressed, as there are other issues such as basic health care of higher priority. Formulation and implementation of policies related to understanding of the problem and the consequences of lack of control, both by users and policy makers will help in reducing transmission of resistant strains.

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