Multi-centric validation of an in-house-developed beacon-based PCR diagnostic assay kit for *Chlamydia* and *Neisseria* and portable fluorescence detector

Divya Sachdev,1† Kirti Wasnik,2† Achchhe Lal Patel,1 Subash C. Sonkar,1,3 Parul Desai,2 Jayanti Mania-Pramanik,4 Shilpa Kerkar,4 Sunil Sethi,5 Nandita Sharma,5 Pratima Mittal,3 Priti Ghope,3 Ajay Khandhari2 and Daman Saluja1,∗

Abstract

**Objective.** The development of an accurate, sensitive, specific, rapid, reproducible, stable-at-room-temperature and cost-effective diagnostic kit, and a low-cost portable fluorescence detector to fulfil the requirements of diagnostic facilities in developing countries.

**Methods.** We developed the ‘Chlamy and Ness CT/NG kit’ based on molecular beacons for the detection of *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG). Multi-centric evaluation of the CT/NG kit was performed using the commercially available nucleic acid amplification test (NAAT)-based FTD Urethritis basic kit for comparison from December 2014 to November 2016. The stability of the kit reagents at 4 and 37 °C and the inter-day reproducibility of results were also analysed.

**Results.** The sensitivity and specificity of the kit were found to be 95.83 and 100.00 % for the detection of *C. trachomatis* and 93.24 and 99.75 % for *N. gonorrhoeae*, respectively, when tested against the commercial kit. The positive predictive value (PPV) was 100.00 and 98.57 %, whereas the negative predictive value (NPV) was 99.54 and 98.79 % for *C. trachomatis* and *N. gonorrhoeae*, respectively. Analysis of the kappa statistics enhanced the ‘inter-rater’ $\kappa=0.976$ for *Chlamydia* and $\kappa=0.943$ for *Neisseria*.

**Conclusion.** Our kit was found to be as sensitive and specific as commercially available kits. Its low cost and ease of use will make it suitable for the routine diagnosis of *C. trachomatis* and *N. gonorrhoeae* in the resource-limited settings of developing countries.

INTRODUCTION

Nucleic acid amplification test (NAAT)-based assays for the detection of *Chlamydia* and *Neisseria* have enhanced sensitivity, specificity and ease of detection and reduced turnaround times as compared to traditional detection methods, including culture, the direct fluorescent antibody (DFA) test and microscopy [1–9]. The use of NAATs for the diagnosis of *Chlamydia* in various high-income countries, as recommended by the Centers for Disease Control (CDC), has led to a decrease in the incidence of pelvic inflammatory disease (PID) [6, 9, 10]. Nonetheless, the high cost of NAAT-based assays discourages their routine use in developing countries, including India, where the infection load is maximal [11, 12]. The syndromic management implemented by the World Health Organization (WHO) in developing countries has been found to have low sensitivity and specificity, leading to overtreatment, the misuse of antibiotics and missed treatment [13–17].

This underscores the urgent need for a rapid NAAT-based diagnostic test that is suitable for middle- and low-income
group countries like India, with resource-limited facilities in a majority of the healthcare settings. Therefore, we have developed a molecular beacon (fluorescently labeled probe)-based PCR (MB-PCR) detection kit for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, as well as a portable detection reader, and evaluated the performance against a commercial kit. We also lyophilized and stabilized the reagents of kit at 4 and at 37 °C for several months. The diagnostic kit and the detector were subjected to multi-centric evaluation of their reproducibility, sensitivity and specificity.

**METHODS**

**Sample collection and processing**

A total of 482 female subjects (≥18 years) were recruited at four different centres between December 2014–November 2016. The study was a random blindfolded clinical evaluation of the diagnostic kit, as the technicians performing the assay were unaware of the presence of any kind of clinical observations. Dry endocervical swabs were collected and processed as per the ethical guidelines of the Indian Council of Medical Research, India and adopted by the Institutional Ethical Committee at different research institutes, namely PGIMER, Chandigarh (188 subjects); NIRRH, Mumbai (92 subjects); Safdarjung Hospital, New Delhi (159 subjects); and ACBR, Delhi–110007 (42 subjects). The swabs were immersed in PBS (1 ml) for 10 min before processing and total genomic DNA (gDNA) was isolated using the MN Nucleospin Tissue kit (Macherey-Nagel GmbH & Co. KG) as per the manufacturer’s instructions.

**Primer and probe**

The previously designed primers and a novel molecular beacon probe 5'-GGGAGGGCGGTAGGGATGGCAACAAAATACCTGCC-3' [melting temperature (Tm)=69.7 °C] with either TYE 563 Iowa Black RQ-Op or Cy3-BHQ2 fluorophore-quencher pairs were designed by us (see [18]), custom-synthesized by Integrated DNA Technologies (IDT), Singapore against the gyrA gene (gene ID: 88941), and evaluated for the detection of *C. trachomatis* [18, 19]. The presence of genital gonococcal infection was detected as described previously [20].

**Clinical evaluation of MB-PCR for CT**

Different concentrations of beacon, ranging from 0.2 to 1.6 µM, were used to identify the optimal concentration with the maximum signal-to-noise ratio. The sensitivity of the beacon was estimated using serially diluted gDNA (1 ng to 10 fg). To analyse the specificity of the beacon, competition assays were performed with an unlabelled specific probe (1:1 to 1:50) and an unlabelled non-specific probe (1:1 to 1:50). Amplified products probed with the beacon were diluted with 1 x PBS (1:2), and fluorescence was monitored using an enzyme-linked immunosorbent assay (ELISA) reader (M200, Tecan Pvt Ltd) at 554 nm excitation and 568 nm emission, in addition to an indigenous fluorescence detector. Further, clinical evaluation of MB-PCR for CT was performed using a commercial kit (FTD Urethritis kit, Fast Track Diagnostics, Luxembourg) for comparison and patient samples (n=150, selected randomly from the previous study). Since Tye563 is a brighter substitute for Cy3, we switched to a Tye563-based beacon for all further analysis.

**Design and evaluation of the fluorescence detector**

A portable fluorescence detector was developed (DSS Imagtech Pvt Ltd, India) for end point detection (Fig. 1). The performance of the indigenous fluorescence detector was evaluated against an ELISA reader and agarose gel electrophoresis using molecular beacon-probed amplicons obtained from the PCR amplification of serially diluted plasmid DNA (1 ng to 1 fg) as the template. To monitor the fluorescence using the indigenous fluorescence detector, PCR tubes were directly placed under the slot provided in the fluorescence detector (with a fluorophore-specific filter) and images were captured. The performance of the detector was also evaluated using clinical samples.

**Lyophilization of PCR pre-mix**

A PCR pre-mix (14 rxn of 25 µl each) comprising the molecular beacons and primers (CT beacon primer mix, CTBP-MIX; NG beacon primer mix, NGBP-MIX) was lyophilized and its stability was monitored at 37 and 4 °C for 12 months. A positive control for CT/NG (a mix of 10 ng of TA clones of gyrA and orf1 gene each) was also prepared and lyophilized. Different tubes of the lyophilized mix were used to perform the PCR assay at regular time intervals (in triplicates).

**Multi-centric evaluation of the in-house diagnostic kit in comparison with the commercial FTD Urethritis basic kit**

The analytical sensitivity and clinical performance characteristics [sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV)] of the molecular beacon-based detection system were analysed by comparing the detection results with those of the commercial kit, which was considered to be the gold standard. A total of 482 clinical samples were analysed at the four centres using different thermal cyclers and real-time PCR instruments for the in-house assay and FTD urethritis kit (at PGIMER, the Agilent Sure Cycler 8800/LightCycler 480 II; at NIRRH, the ABI Verity thermal cycler/CFX96; at Safdarjung Hospital, the Bio-Rad S1000 thermal cycler/QuantaStudio 6 Flex; and at ACBR MJ Research, the PTC-200/QuantaStudio 6 Flex thermal cycler/Real-time instrument).

To perform the MB-PCR, lyophilized CTBPMIX and NGBPMIX were reconstituted with 70 µl of reconstitution buffer [0.3 mM MgCl2 (Bangalore Genei, Pvt Ltd, India) and 0.003 % Triton X-100 (Sigma Aldrich, USA)]. Five microlitres of reconstituted CTBPMIX or NGBPMIX was added to the PCR tube containing PuReTaq Ready-To-Go PCR beads (GE Healthcare, USA), along with 3 µl template DNA (gDNA isolated from clinical samples as
described above) and 17 µl water. PCR amplification followed by beacon hybridization was performed as described previously [19, 20]. The end products were visualized using a fluorescence detector. A quantitative PCR assay was also performed using the FTD Urethritis basic kit as per the manufacturer's instructions.

Cases with positive results from both tests were considered to be true positive and those with negative results from both tests were considered to be true negative. Samples that tested positive by the beacon assay but negative by qPCR were considered to have given false positive results, whereas samples that were positive by qPCR but negative by molecular beacon were considered to have given false negative results.

**Statistical analysis**

All statistical analysis was performed using Medcalc software (https://www.medcalc.org/calc/diagnostic_test.php). The true situation was calculated using kappa statistics, which signified perfect agreement between the two tests [21, 22].

**RESULTS**

In the current study, we focused on the development of MB-PCR for CT and translating the detection assays for *C. trachomatis* and *N. gonorrhoeae* into a kit, the ‘Chlamy and Ness CT/NG detection kit’, along with the development of a portable low-cost fluorescence detector. The detection assay for *N. gonorrhoeae* was previously evaluated by our group [20].

In the present samples, the prevalence of *C. trachomatis* was 9.98 % (95 % confidence interval (CI): 0.944 to 1.000) and that of NG was 15.35 % (CI 95 %: 0.944 to 1.000). We also found that 98/482 (20.33 %) subjects were positive for either *C. trachomatis* or *N. gonorrhoeae* and 16/98 (16.32 %) were co-infected with *C. trachomatis* and *N. gonorrhoeae*.

**Clinical evaluation of CT-MB-PCR**

The optimal concentration of beacon was standardized at 1.2 pmol (Fig. 2a) and was found to detect up to 50 fg of gDNA (Fig. 2b). The observation of a decrease of approximately 75 % in fluorescence on the addition of 50-fold unlabelled specific probe and a negligible change on the addition of non-specific probe proved the specificity of the beacon (Fig. 2c). Clinical evaluation of CT-MBPCR was performed against agarose gel electrophoresis and the commercial kit. The fluorescence of the probed amplicons was monitored using an ELISA reader and a fluorescence detector (Fig. 2d, e).

Out of 150 samples, 31 tested positive by gel electrophoresis as well as by different methods of fluorescence detection. Two samples that were borderline in the gel electrophoresis results were considered to be true positive through monitoring of the fluorescence. One sample that was found to be positive by agarose gel electrophoresis was determined to be negative by the beacon and the commercial kit and was thus...

![Fig. 1. (a) Picture of hand-held in-house-developed fluorescent reader. (b) Diagrammatic representation of the arrangement of the optical elements of the fluorescent reader: the light-emitting diode (LED), excitation filter (green for Chlamydia and blue for Neisseria) and sample tube holder are arranged linearly, whereas the emission filter (orange for Chlamydia and yellowish green for Neisseria) is positioned at a right angle (90 degrees) with respect to the objective. The light emitted by the LED passes through the specific excitation filter, allowing a specific wavelength to pass through the sample and thus excite the fluorophore. The emitted fluorescence is then passed through the emission filter and captured by the attached camera.](https://www.microbiologyresearch.org/jmm/67/1287-1293.png)
considered to be true negative. This suggested that using the molecular beacon improved both the sensitivity and specificity of the detection assay.

**Evaluation of the fluorescence detector**

The detection limit of our fluorescent reader was found to be 5 fg for *N. gonorrhoeae* gDNA and 50 fg for *C. trachomatis* gDNA (Fig. S1, available in the online version of this article). A concordance was observed among the results for clinical samples tested with the fluorescence detector, agarose gel electrophoresis and the ELISA reader (Fig. 3).

**Evaluation and inter-day reproducibility of lyophilized reagents of the detection kit**

Positive PCR amplification using 1 ng of plasmid DNA (lyophilized) and 500 fg of gDNA of the pathogen (stored at −20 °C) showed that the lyophilized master mix for both *C. trachomatis* and *N. gonorrhoeae* was stable for 75 days at 37 °C and for up to 345 days at 4 °C (Fig. S2). Comparison of the MB-PCR detection for 50 samples at day one and a repeated assay on the eighth day showed complete concordance, demonstrating the 100 % reproducibility of the in-house kit (Fig. S3).

**Multi-centric clinical evaluation of in-house diagnostic assay**

The analytical performance of the kit was compared with that of the commercial qPCR kit. A comparable decrease in the fluorescence intensity of the beacon with the increase in the cycle threshold (Ct) of the commercial kit on decreasing gDNA concentration for PCR suggested the semi-quantitative nature of the assay (Fig. 4). The multi-centric evaluation of the kit was performed by using the FTD Urethritis basic NAAT (qPCR) kit as the gold standard and gDNA isolated from 482 patient samples. Comparative analysis of PCR by the two methods for *C. trachomatis* showed that out of 482 samples, 46 (9.54 %) were true positive, while 434 (90.04 %) samples were true negative, as both methods showed concordant results. Two samples (0.41 %) were determined to be negative by our assay and positive by the commercial kit, and thus were considered to be false negatives. No samples were considered to be false positives for *C. trachomatis*.

Similarly, for *N. gonorrhoeae*, 69 (14.31 %) samples were true positive, while 407 (84.43 %) samples were true negative. Our assay detected one (0.20 %) false positive, whereas five (1.03 %) samples gave false negative results for *N. gonorrhoeae*.

Based on the composite reference standard method, the sensitivity and specificity of our Chlamy and Ness CT/NG detection kit were comparable with those of the commercial kit (Table 1). The inter-repeatability between the two tests, as rated by kappa statistics, was more than 0.80, and signifies the acceptable range and good agreement between the two tests in diagnosis. Moreover, κ=0.976 (95 % CI: 0.944 to 1.000) for Chlamydia and κ=0.943 (95 % CI: 0.900 to 0.985) for Neisseria further strengthen the repeatability of the agreement (Table 1).
DISCUSSION

The high cost of commercially available NAAT-based kits (about $25 to $60 per test in India) and the need for expensive infrastructure and expertise to carry out quantitative PCR make these unaffordable for routine diagnosis of sexually transmitted infections (STIs) in developing countries.

Thus in this study we developed an MB-PCR-based CT/NG detection kit, along with the portable fluorescence detector, for the diagnosis of *C. trachomatis* and *N. gonorrhoeae*. We then performed multi-centric validation for the developed kit. We found that MB-PCR for NG [20] and CT (current study) was able to detect gDNA at levels as low as 5 fg and 50 fg, respectively, which could easily be visualized using a hand-held detector (Figs 3 and S1). Use of molecular beacons and the detection of results directly in the PCR tube using a fluorescence detector enhances the sensitivity and specificity, as well as eliminating the need for EtBr staining of the DNA using agarose gel for visualization of the amplicons. The elimination of agarose gels makes the detection convenient, rapid and suitable for laboratories where bio-safety and the containment of bio-hazardous materials are rudimentary, as well as reducing the chance of cross-contamination and carry-over contamination [18, 20, 23–25]. Features such as low cost (~$8), small size, light weight, the use of light-emitting diodes (LEDs; also low cost) and easy-to-use specific filters in various combinations make the fluorescent reader economical, portable, affordable and suitable for remote laboratories as compared to expensive ELISA readers.

The collection of dry swabs and the stability of lyophilized pre-mix at ambient temperature (even at 37 °C for 75 days) enhances the portability and the ease of storage and transport for clinical samples and the kit at resource-limited laboratories without deep freezers and with intermittent electricity failure. The use of thermostable lyophilized pre-mix for the PCR assays also eliminates the requirement for a trained micro-pipetting technician, as well reducing the chance of cross-contamination and individual errors [20, 24, 26–28]. Further, our PCR assay can be performed on any thermal cycler (~$2.5K), which is far less expensive.
Table 1. Performance characteristics of the Chlamy and Ness detection kit

Comparison of the sensitivity, specificity, PPV, NPV, PLR, NLR and accuracy of the different methods calculated with 95 % CI.

<table>
<thead>
<tr>
<th>Results for clinical specimens (n=482)</th>
<th>Chlamydia trachomatis</th>
<th>Neisseria gonorrhoeae</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Molecular beacon assay (GyrA gene target)</td>
<td>Molecular beacon assay (OrfI gene target)</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
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<tr>
<td>---------------------------------------</td>
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</tr>
<tr>
<td>FTD Urethritis Positive</td>
<td>46</td>
<td>2</td>
</tr>
<tr>
<td>FTD Urethritis Negative</td>
<td>0</td>
<td>434</td>
</tr>
<tr>
<td>Sensitivity (95 % CI)</td>
<td>95.83 % (85.75 to 99.49 %)</td>
<td>93.24 % (84.93 to 97.77 %)</td>
</tr>
<tr>
<td>Specificity (95 % CI)</td>
<td>100.00 % (99.15 to 100.00 %)</td>
<td>99.75 % (98.64 to 99.99 %)</td>
</tr>
<tr>
<td>PPV (95 % CI)</td>
<td>100.00 %</td>
<td>98.57 % (90.68 to 99.80 %)</td>
</tr>
<tr>
<td>NPV (95 % CI)</td>
<td>99.54 % (98.24 to 99.88 %)</td>
<td>98.79 % (97.22 to 99.48 %)</td>
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<tr>
<td>PLR– (95 % CI)</td>
<td>0.04 (0.01 to 0.16)</td>
<td>0.07 (0.03 to 0.16)</td>
</tr>
<tr>
<td>NLR– (95 % CI)</td>
<td>–</td>
<td>380.43 (53.66 to 2696.92)</td>
</tr>
</tbody>
</table>

than any real-time PCR instrument ($20K–30K). Hence the infrastructure costs for the assay are low and affordable. Our assay will cost one-third to one-fifth of the price of the existing diagnostic kits available in India.

The Chlamy and Ness CT/NG detection kit was validated against the commercially available in vitro diagnostics (IVD) approved (CE 0123) qPCR-based FTD Urethritis basic kit at multiple diagnostic laboratories. This kit is available in India and is used by several diagnostic laboratories. It is sensitive and specific. The use of kappa statistics strengthened the reliability of the diagnostic tests [21]. The 100 % reproducibility of the test results further supported their authenticity.

Thus, the outcome of this study provides an opportunity for clinicians to deliver treatment to patients based on definite diagnosis rather the syndromic approach.

Conclusion

In a nutshell, our Chlamy and Ness CT/NG detection kit is a step forward towards the development of point-of-care diagnostics for use in resource-limited settings. Its high sensitivity and specificity, low cost and ease of use make it suitable to meet the majority of the WHO’s proposed ‘ASSURED’ (Affordable, Sensitive, Specific, User-friendly, Robust and rapid, Equipment free and Deliverable) guidelines [29]. These features collectively reduce the cost of our diagnostic assay as compared to the currently available commercial kits, making the test affordable to the majority of the Indian population, as well those in other developing countries. The kit has the potential to make it possible to implement the diagnostic assay for routine screening programs in the developing world, replacing syndromic management and providing an economical strategy to not only control infection by these two pathogens, but also reduce the overuse and misuse of antibiotics. The detection of infection amongst asymptomatic patients will further avert the spreading of infection and its long-term sequelae.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

Dry endocervical swabs were collected as per the guidelines of the Indian Council of Medical Research, India and as adopted by the Institutional Ethical Committee and processed at different research institutions from the four different centres, namely the Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh–160012, the Infectious Diseases Biology National Institute For Research In Reproductive Health (NIRRH), Mumbai–400012, Obstetrics and Gynaecology, Vardhaman Mahavir Medical College and Safdarjung Hospital, New Delhi–110029 and the Dr. B R Ambedkar Center For Biomedical Research (ACBR), Delhi–110007. The results obtained were not used to influence treatment by the clinician.

References


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