Development, validation and testing costs of an in-house real-time PCR assay for the detection of *Chlamydia trachomatis*

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Abstract

Purpose. To improve the screening of *Chlamydia trachomatis* (C. trachomatis) in Brazil, an accurate and affordable method is needed. The objective of this study was to develop and assess the performance and costs of a new in-house real-time PCR (qPCR) assay for the diagnosis of C. trachomatis infection.

Methodology. Asymptomatic women aged 14–25 years who attended primary health services in Manaus, Brazil, were screened for C. trachomatis using the Digene Hybrid Capture II CT-ID (HCII CT-ID) DNA test. A subset of cervical specimens were tested using an in-house qPCR and a commercial qPCR, Artus C. trachomatis Plus RG qPCR kit (Artus qPCR) kit, as a reference test. A primer/probe based on the sequence of cryptic plasmid (CP) was designed. An economic evaluation was conducted from the provider’s perspective.

Results. The primers were considered specific for C. trachomatis because they did not amplify any product from non-sexually transmitted bacterial species tested. Overall, 292 specimens were tested by both the commercial kit (Artus qPCR) and the in-house qPCR. Of those, one resulted in no amplification and was excluded from the analysis. The sensitivity, specificity, and positive and negative predictive values of the in-house qPCR were 99.5 % [95 % confidence interval (CI): 97.1–100], 95.1 % (95 % CI: 89–98.4), 97.4 % (95 % CI: 94–99.1) and 99.0 % (95 % CI: 94.5–100), respectively. The cost per case of C. trachomatis was £0.44 ($0.55) for HCII CT-ID, £1.16 ($1.45) for Artus qPCR and £1.06 ($1.33) for in-house qPCR.

Conclusion. We have standardized an in-house qPCR to detect cervical C. trachomatis targeting CP. The in-house qPCR showed excellent accuracy and was more affordable than the commercial qPCR kit.

INTRODUCTION

*Chlamydia trachomatis* is the most commonly diagnosed curable sexually transmitted infection worldwide [1, 2]. In Brazil, it is estimated that 9.4 % of women in the general population [3] and 9.8 % of parturient women under 25 years of age [4] are infected by C. trachomatis. Genital C. trachomatis infection remains unnoticed in 50–88 % of women [5]. Given the frequent asymptomatic nature of the infection, and the importance of early treatment to reduce transmission and prevent C. trachomatis-related morbidity, many developed countries are offering opportunistic screening to all sexually active people under the age of 25 years [1, 6, 7]. Screening has been shown to be effective for the identification of asymptomatic infected women, although there is some controversy about its impact to reduce the incidence of pelvic inflammatory disease [8]. Importantly, although the cost-effectiveness of screening is largely determined by the rates of complications prevented, studies indicate that shares in total savings and in quality-adjusted life-years (QALY) gains due to prevented cases of tubal infertility are 6–24 and 33 %, respectively [9].

In Brazil, the Digene Hybrid Capture II CT-ID (HCII CT-ID) DNA test (Qiagen) for identifying C. trachomatis is the only molecular test approved in the public health system for C. trachomatis screening. This nucleic acid hybridization assay is no longer recommended by the USA Centers for Disease Control and Prevention for routine use based on performance [10]. This test has shown sensitivity ranging from 93.8–97.7 % and specificity ranging from 95.9–100.0 % compared to that of...
culture [11–14]. However, up to 27.7% of negative results generated by this test were reported to be false among asymptomatic young women in Manaus when compared with a commercial real-time PCR (qPCR) kit [15].

qPCR has been developed, and offers both high sensitivity and specificity when compared to traditional culture-based, commercial and in-house amplification methods [16, 17]. However, qPCR assays present disadvantages such as being relatively time consuming, having a high cost, and requiring specialized equipment and trained laboratory staff [18]. Therefore, a reliable and affordable new diagnostic method would be extremely valuable for clinicians and for the public health system. Importantly, the development of new in-house qPCR approaches that allow the minimization of the need for reagents, time and costs can help the scaling up of national C. trachomatis screening programmes in resource-constrained countries. Several studies have employed a composite reference standard, which combines the result of a more sensitive test with culture as a standard test in order to define a better standard against which a new test can be compared [19]. Culture of Chlamydia is not available routinely in laboratories in Brazil and its use carries a high labour cost. Therefore, the Artus C. trachomatis Plus RG qPCR 96 CE (Artus qPCR) kit, which has shown high sensitivity and specificity [20], was used alone as a standard for evaluating the accuracy of the new in-house qPCR assay. The objective of this study was to describe the development, and assess the performance and costs, of a new in-house qPCR assay for the diagnosis of genital chlamydial infection.

**METHODS**

**Study population**

The specimens were collected from 1187 women presenting at primary care services in Manaus for cervical cancer screening, between October 2012 and December 2013. We included asymptomatic women aged 14 to 25 years who agreed to sign the consent form. We excluded pregnant women and those who had used antibiotics during the previous 15 days.

For C. trachomatis screening, a single cervical specimen per participant was collected using the Digene cervical sampler brush (Qiagen) and was placed into a tube containing 1.0 ml Digene sample transport medium. Samples were stored at room temperature until the end of the week, when they were transferred to the Fundação Alfredo da Matta (FUAM) laboratory and stored at −20 °C until processed.

Specimens were tested using the HCII CT-ID according to the manufacturer’s instructions [11]. Of the total 1187 women screened, 1169 participants had a HCII CT-ID test result (10 were not tested and 8 were excluded because they had an indeterminate test result). Among those, 153 tested positive for C. trachomatis [15].

**Validation study**

A subset of stored cervical specimens was used to evaluate the performance of the in-house qPCR assay against the Artus qPCR kit (Qiagen) according to the assay package instructions. A laboratory technician, blind to the HCII CT-ID results, tested specimens that were positive by HCII CT-ID and a randomly selected equal number of samples that tested negative. A total of 292 specimens were tested, given that 7 out of 153 HCII CT-ID specimens with a positive result could not be identified.

**Artus qPCR**

A 300 µl aliquot was transferred from each selected specimen to a 1.5 ml tube before the HCII CT-ID test was performed. DNA was extracted using a QIAamp DNA mini kit (Qiagen) and the Artus qPCR performed on the Rotor-Gene 3000 instrument (Corbett Research). The interpretation of all Artus qPCR results followed the algorithms in the manufacturer’s package insert [21]. The only sample identifiers were specimen numbers.

**In-house qPCR primer and probe design**

Based on gene alignment of omp1 (all genotypes) and cryptic plasmid (CP) conserved sequences available in the GenBank database (www.ncbi.nlm.nih.gov/GenBank/), specific primers and probes were designed using the Applications of Integrated DNA Technologies tool (IDT tools – www.idtdna.com/scitools/Applications/RealTimePCR/) (Table 1). OligoAnalyzer 3.1 (IDT tools – www.idtdna.com//calc/analyzer) was used to evaluate primer and probe parameters, including the melting temperature (Tm), difference in melting temperatures for primer pairs (ΔTm), G+C content (G+C mol%), self-dimers, hairpins, hetero-dimer structures, repetitive sequence and Gibbs free energy (ΔG). Primers with acceptable parameters were selected and aligned with the sequences from the National Center for Biotechnology Information database using the Basic Local Alignment Search Tool (BLAST) to test for possible nonspecific interactions. The primers and probes selected did not have nonspecific interactions with the microorganisms present in the vaginal cavity or human genes. The designed primer set was a combination of two primer pairs that amplify CP target regions of C. trachomatis. The same methods described

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>GenBank ID*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L2b/CS784/08</td>
<td>L2b</td>
<td>NZ_CP009926.1</td>
</tr>
<tr>
<td>Ia/CS190/96</td>
<td>Ia</td>
<td>NZ_CP001572.1</td>
</tr>
<tr>
<td>Sweden2</td>
<td>E</td>
<td>FM865439.1</td>
</tr>
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<td>Sweden3</td>
<td>E</td>
<td>FM865440.1</td>
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<tr>
<td>Sweden4</td>
<td>E</td>
<td>FM865441.1</td>
</tr>
<tr>
<td>weden5</td>
<td>E</td>
<td>FM865442.1</td>
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</tbody>
</table>

*Sequence submission ID.
Table 2. In-house qPCR primer and probe sequences for the CP region

<table>
<thead>
<tr>
<th>Primer/probe</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>Forward CP</td>
<td>5'-CTAGGCGTGTAGCTGGCT-3'</td>
</tr>
<tr>
<td>Probe CP</td>
<td>5'-FAM-TTGCAGCTTGAGTCTGCTGAGA-3'</td>
</tr>
<tr>
<td>Reverse CP</td>
<td>5'-TGCTCTGTAACACTGCTCC-3'</td>
</tr>
</tbody>
</table>

FAM, 6-Carboxyfluorescein.

were used to design the primers and probe for the omp1 gene. The names and sequences of all primers are shown in Table 2.

In-house qPCR assay

The in-house qPCR reaction was performed in a 10 µl volume, using 200 µM each primer and probe (Invitrogen), 1× TaqMan Universal PCR Master Mix NO UNG (Applied Biosystems) and 1 µl purified DNA [the same samples that were tested with Artus qPCR, which were extracted using a QIAamp DNA mini kit (Qiagen)]. The cycling conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 °C. This reaction was performed with the StepOnePlus real-time PCR system (Applied Biosystems). This reaction was used with the CP primer set and the omp1 target.

All samples that showed no amplification for C. trachomatis were tested for the human β-actin constitutive gene to verify that DNA extraction had been performed satisfactorily and the presence of viable DNA. The reaction for β-actin was performed in a 5 µl volume, using 1× TaqMan Universal PCR Master Mix NO UNG (Applied Biosystems), 300 µM each primer (Invitrogen), 100 µM probe (Invitrogen) and 1 µl DNA. The cycling conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. This reaction was performed with the StepOnePlus real-time PCR system (Applied Biosystems). This reaction was also optimized in order to reduce an excessive waste of reagents, since the normal reaction was performed in 25 µl final volume, but was standardized for this project in a 5 µl volume. Both reaction volumes resulted in the same results. Specificity of the in-house qPCR was tested using DNA extracted from Candida sp. and Neisseria gonorrhoeae, as well as human DNA. All reactions were tested in triplicate, alongside positive and negative controls.

Economic analysis

We compared the costs of screening and managing C. trachomatis using the different tests. Incremental recurrent costs only were estimated, assuming all other costs would be equal. Costs of infrastructure, training and supervision were not included. Direct costs were labour, diagnostic supplies and drugs, which were estimated using the ingredients approach in which the total quantities of goods and services used were estimated and multiplied by their respective unit prices [22]. Cost of labour was determined through an observational time allocation study and time units were multiplied by the relevant salary units of staff (laboratory technicians) performing various tasks. The costing exercise was conducted from the provider’s perspective, considering FUAM as the sole provider. Financial and economic costs were the same because there were no donated goods or services. With regards to HCII CT-ID, costs of tests included the costs when procured by the public Unified Health System (SUS) in Brazil for the year 2015, and converted into US dollars ($) using the fixed exchange rate of 3.49 reais per $. All research-related costs were excluded. Costs of diagnostic inputs, as well as formulas for economic evaluation can be found in Table 3. To calculate the cost per person screened, the retesting cost of indeterminate results was considered zero because in standard care such women are assumed to be positive and therefore treated. Total testing costs were divided by the number of people tested to obtain the cost per case identified by test. Cost values in the text are shown in pounds Sterling (£), using the exchange rate $1=£0.8.

Statistical analysis

Data were analysed using STATA/SE version 11.2 (Stata). The performance characteristics of the in-house qPCR test compared to that of Artus qPCR (sensitivity, specificity, and positive and negative predictive values) were calculated by standard methods, and are presented with the 95% confidence intervals (CI). To calculate the sample size of the validation study, we assumed a sensitivity of the in-house qPCR to be equal to that of the Artus qPCR (98 %) and a precision of 10 %. The number of infections needed to test the accuracy of the in-house qPCR was eight. Given that the prevalence of C. trachomatis in young women is approximately 9.6 %, the number of specimens to be tested was 83. However, it was further increased to obtain more precise estimates.

Table 3. Economic evaluation of C. trachomatis screening testing using the HCII CT-ID assay, the Artus qPCR assay or the in-house qPCR in an asymptomatic young (14–25 years) female population in Manaus, Brazil

<table>
<thead>
<tr>
<th></th>
<th>HCII CT-ID</th>
<th>Artus qPCR</th>
<th>In-house qPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test cost (A)</td>
<td>$8.98/£7.18</td>
<td>$17.15/£13.72</td>
<td>$5.11/£4.09</td>
</tr>
<tr>
<td>Testing time cost (B)</td>
<td>$94.32/£75.46</td>
<td>$254.66/£203.73</td>
<td>$245.23/£196.18</td>
</tr>
<tr>
<td>Total test and testing cost (C)</td>
<td>$100.30/£82.64</td>
<td>$271.81/£217.45</td>
<td>$250.34/£200.27</td>
</tr>
<tr>
<td>Cost per person screened (D)</td>
<td>$0.36/£0.29</td>
<td>$0.93/£0.74</td>
<td>$0.86/£0.69</td>
</tr>
<tr>
<td>Cost per case identified by test</td>
<td>$0.55/£0.44</td>
<td>$1.45/£1.16</td>
<td>$1.33/£1.06</td>
</tr>
</tbody>
</table>

Test cost (A) = unit price of purchasing tests. Testing time cost (B) = (no. time units) × (cost per time unit) (time unit: monthly lab staff salary expressed hourly; divided by 60 min). Testing cost (C) = unit price of purchasing test (A) + testing time cost (B). Cost per person screened (D) = testing cost of entire population + retesting cost of intermediate = C/N + r-retest)/N. Cost per case identified by test = D divided by number of tests positive.
ethical approval

The study protocol was approved by the Research Ethics Committee of FUAM (approval number 028/2011) and by the Ethics Committee of the Health Secretariat in Manaus. All participants signed a written consent form that was approved by the Ethics Committee. Testing with qPCR was approved by the Research Ethics Committee of FUAM (approval number 742.410).

Results

Primers and probes

Primers and probes for the CP and omp1 targets were designed. The primers were tested at different concentrations (0.2, 0.5 and 0.9 µM) to identify the best candidate with the same probe concentration (0.2 µM). The best primer concentration was 0.2 µM, which was tested with CP and omp1 gene primers and probes in separate reactions. Amplifications were only obtained with the CP primers and probe. Because omp1 is a very polymorphic gene, we selected its most conserved part in order to detect all different C. trachomatis genotypes and used degenerate primers. With these omp1 primers we achieved amplification in half of the specimens only, probably because of the presence of mutation(s) or a variable area in the remaining ones or, alternatively, a reaction inhibitor present in the in-house qPCR mix. Our reaction results are only using the CP target. The primers and probe designed for the CP target showed specificity, because no cross-reaction was observed for other sexually transmitted species tested or with the human DNA. We were able to standardize this reaction in 10 and 5 µl volumes for the CP and β-actin primer sets, respectively.

Performance of the in-house qPCR

Overall, 292 specimens were tested by Artus qPCR. Of those, one resulted in no amplification and was excluded from the evaluation of the performance analysis. The specimens were tested by the in-house qPCR. When compared to the commercial test, the sensitivity of the in-house qPCR was 99.5% (95% CI: 97.1–100) (187/188), the specificity was 95.1% (95% CI: 89–98.4) (98/103), the positive predictive value was 97.4% (95% CI: 94–99.1) (187/192) and the negative predictive value was 99.0% (95% CI: 94.5–100) (98/99). The in-house qPCR test detected 99.5% (187/188) of C. trachomatis cases with a positive Artus qPCR result. The in-house qPCR retrieved one out of 103 false-negative results and five out of 103 (4.9%) false-positive results (98/103).

Costs of C. trachomatis screening methods

Table 3 shows the costs of C. trachomatis detection using the three available screening tests. The cost per case of C. trachomatis was £0.44 ($0.55) for the HCII CT-ID, £1.16 ($1.45) for the Artus qPCR and £1.06 ($1.33) for the in-house qPCR.

Discussion

Our evaluation of the in-house qPCR for C. trachomatis screening using cervical samples showed an excellent sensitivity and specificity compared to the commercial Artus qPCR kit in young women from the general population in Manaus. These results are consistent with previous studies in which in-house qPCR showed higher sensitivity and specificity versus Roche Cobas Amplicor [17, 23, 24]. Interestingly, the in-house qPCR identified almost all C. trachomatis cases among asymptomatic women, missing only one positive sample. A possible explanation for this false-negative result was a very small quantity of C. trachomatis DNA in that sample and/or some degree of degradation of the DNA during storage and freezing. In our study, 4.9% of the in-house qPCR samples had a false-positive result. This finding was lower than that reported in previous studies, in which between 5.3 and 8% of the in-house qPCR positive results were reported as false [23, 24]. The accuracy of a positive result is essential in order to avoid psychosocial issues, overtreatment and unnecessary partner notification. The HCII CT-ID described here showed a sensitivity of 72.3% and specificity of 91.3% compared to that of Artus qPCR [15].

Testing by qPCR allows simultaneous achievement of amplification, specific hybridization and detection. Specific and sensitive gene quantification occurs with a minimal contamination risk. A drawback of the study was that we were not able to standardize a reaction for the omp1 gene and we could amplify half of the samples that were tested. This target was used by Jalal and colleagues [25], resulting in 96% of positive samples detected. However, six samples that were positive did not amplify omp1. They justified the absence of a positive result because the omp1 gene needs at least three copies to be amplified C. trachomatis DNA. On the contrary, when CP is targeted, only one copy is needed to achieve amplification, which could reduce the sensitivity of the test. In our in-house qPCR, only the C. trachomatis-specific CP target was amplified in the reaction. However, all C. trachomatis strains are known to harbour a species-specific CP, and their DNA sequences are highly conserved, even between different strains [26]. By using a species-specific C. trachomatis sequencing primer and validating it, we could confirm the molecular diagnosis of C. trachomatis in the endocervical samples that showed a positive result in the in-house qPCR.

C. trachomatis screening is not routinely available in the primary health care system in Brazil, although the prevalence of C. trachomatis in the general population is high and screening has been claimed to be crucial for decreasing the burden of the infection. One of the main reasons is budget constraints. With a calculated cost of £1.38 ($1.72), the in-house qPCR test may represent a more affordable solution for C. trachomatis infection detection in Brazil than a commercial qPCR. Compared with the HCII CT-ID DNA test, it showed a better performance and cost effectiveness. In addition, one distinct advantage of the in-house qPCR
versus the commercial qPCR from the laboratory technician perspective is a much simpler preparation step before amplification, as well as a shorter time needed thereafter. This difference becomes important for laboratories that need to process a large number of samples.

This study had a few limitations. The detection of C. trachomatis by use of the HCII CT-ID DNA test is dependent on the number of organisms present in the specimen and may be affected by patient factors, such as the presence of symptoms [27]. Although the qPCR assay is highly sensitive and specific [28], it can be affected by contamination or inhibitors [29]. Both Artus qPCR and the in-house qPCR were performed on the same source of stored DNA. The justification was to save costs and labour incurred to collect samples. As all samples were coded before the HCII CT-ID DNA test, the use of data and samples collected for a previous study was justified.

In conclusion, we have developed an in-house qPCR to detect cervical C. trachomatis targeting CP. The in-house qPCR showed excellent accuracy the commercial qPCR. An important additional advantage of the in-house qPCR method was its lower cost. Findings from this study can help to revise the national recommendation of using the HCII CT-ID test, which is the only test available to detect C. trachomatis in the Brazilian public health system. The in-house qPCR should be considered as a good candidate for a preferred diagnostic method for screening programmes in Brazil towards reducing the burden of C. trachomatis infection and its secondary transmission.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References


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