Research Square

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Validation of a duplex qPCR for detection of Chlamydia trachomatis DNA in ocular samples and comparison with a direct immunofluorescence method using samples from endemic and non-endemic areas in Brazil

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Abstract

Trachoma, caused by the bacteria Chlamydia trachomatis, is the world leading infectious cause of blindness. The disease is associated with poor living conditions, especially in developing countries. In these countries, diagnosis is usually based on clinical evaluation, although laboratory confirmation is necessary. Serological-based tests for trachoma laboratory confirmation are cheaper than molecular-based tests, but the later are more sensitive and specific. Among the available molecular tests, qPCR has the best cost-benefit. With this in mind, the present study developed a new duplex qPCR reaction, which concomitantly detects C. trachomatis cryptic plasmid and the human 18S rRNA gene, as an internal reaction control. The new qPCR was validated using 50 previously qPCR-characterized samples for trachoma infection, and showed 95% specificity and 100% sensitivity, with an estimated LOD95 of 600 ag/µL. Next, 50 samples from an endemic area (Marajó, Pará) and 12 from a non-endemic area (Curitiba, Paraná) were investigated using direct immunofluorescence assay (DFA) or the new duplex qPCR. Among the 50 endemic samples, three were positive by clinical evaluation (6%), 18 by DFA (36%) and 48 by qPCR (96%). All samples positive by the clinic evaluation were also positive by qPCR. From the 18 DFA-positives, qPCR identified 16 as positives as well. On the other hand, 32 samples that were DFA-negative due to the low number of elementary bodies (<5 per slide) were positive by qPCR. The results show that the new duplex qPCR has sensitivity and specificity in similar levels to commercial qPCR tests available, and that qPCR indeed is more sensitive than clinical evaluation or DFA, thus allowing earlier treatment start. The ubiquitous presence of C. trachomatis DNA in samples from the endemic region confirms that constant monitoring is necessary. Additionally, effective measures for the elimination of trachoma and the detection of bacterial DNA in the active infection are of fundamental importance, and this newly developed duplex qPCR is an important tool towards this goal.

Background

Trachoma is a chronic and contagious disease caused by the bacteria *C. trachomatis*. It is considered the leading cause of preventable blindness in developing countries, being associated with poor infrastructure and hygiene conditions (www.trachomacoalition.org). In Brazil, trachoma is present in most of the country, but especially where life quality conditions are low [1].

C. trachomatis can be found as 19 serovars with distinct characteristics, with trachoma being caused by serovars A, B, Ba, or C [2]. Clinical symptoms include acute inflammation of the conjunctiva and cornea, high mucus production, presence of follicles with retinal bleeding and reduced sight [3]. Depending on the intensity, the infection can evolve to a spontaneous cure or to produce tissue fibrosis after repeated infections [4, 5], resulting in trichiasis, damage to the cornea and, ultimately, blindness [4, 6]. In Brazil, trachoma diagnosis relies on direct clinical evaluation [1]. However, although a gold standard method has not yet been defined, some protocols allow for laboratory-based diagnostic confirmation of the infection.

Serological methods are more common than molecular-based methods, mainly for the easiness of use and cost, but each has its own set of advantages and disadvantages. Direct immunofluorescence assay

(DFA) is the closest to a laboratory gold standard method for trachoma diagnosis, although qPCR has become more prevalent and has greater sensitivity. DFA uses fluorescent monoclonal antibodies to detect the presence of a *C. trachomatis* specific protein (MOMP) or polyclonal antibodies against lipopolysaccharide (LPS) [7]. However, despite displaying high specificity, DFA's sensitivity is low [8–10], possibly because trachoma sampling is affected by several factors, such as previous recent use of eyewash by the patient, associated infections or even the stage of the infection. Micro immunofluorescence methods use serovars-specific monoclonal antibodies, which increases the sensitivity for *C. trachomatis* detection but is a laborious technique that requires a trained technician to analyze the data, thus limiting its use [11].

Molecular-based protocols are faster, more sensitive and more specific than serological-methods for detecting *C. trachomatis*, real time PCR (qPCR) being the most common. Several qPCR protocols have been published, mostly targeting urogenital infection [12–16]. However, fully or semi-automated commercial tests aiming the urogenital infection such as the GeneXpert CT/NG (Cepheid, USA), the Aptima Combo2 CT/NG assay (Hologic, USA) or the Amplicor CT/NG (Roche Molecular Systems, USA) have been shown to be very sensitive and specific for the diagnosis of trachoma [14, 17–21]. The Cepheid CT assay was also evaluated in field settings, and showed very robust results, confirming that qPCR is a good tool for monitoring the incidence of trachoma in low-infrastructure laboratories [20].

The present study aimed to develop and validate a new research duplex qPCR reaction for concomitant detection of *C. trachomatis* cryptic plasmid and the human 18S rRNA gene in ocular swab samples. We also compared the performance of the new qPCR to DFA or clinical evaluation for the diagnosis of *C. trachomatis* infection and found that, as expected, qPCR is more sensitive and specific.

Methods

Samples. Different sets of samples were used. Commercial DNA: Commercial DNA for *Chlamydia trachomatis* serovar D or serovar J were purchased from ATTC (VR-885D and VR-886D , respectively). Lyophilized DNA was diluted in water to 10 ng/µL, aliquoted and stored at -80 °C until use. Trachoma-negative DNA was extracted from whole blood of non-endemic area volunteers using the High Pure PCR Template Preparation Kit (Roche Applied Sciences, Germany), aliquoted and stored at -80 °C until use. Each commercial DNA was 1:10 serially diluted in human trachoma-negative extracted DNA for the dynamic linear range investigation. Johns Hopkins University's samples: Fifty samples were collected using dry swabs from trachoma patients in Tanzania [22, 23] and saved in the bio-repository at the Johns Hopkins University's Chlamydia Research Laboratory (Baltimore, USA. Samples were characterized for the presence of *C. trachomatis* DNA using the Aptima Combo2 CT/NG assay (Hologis, USA). Instituto Evandro Chagas' samples: Fifty samples were collected in an endemic area (Marajo Island, Brazil and Ananindeua, Brazil) and twelve were collected in a non-endemic area (Curitiba, Brazil). Each eyelid was analyzed by a nurse trained in the WHO simplified classification scheme, who collected samples of individuals irrespective of clinical signs of trachoma. Samples were placed in the specific collection tube,

according to the manufacturer's instructions (Digene "Sample Collection of Female Swabs" kit, Qiagen, Germany).

DNA extraction. Archived de-identified patient samples from Johns Hopkins University or Instituto Evandro Chagas were vortexed to homogenize the sample collection gel. Two hundred microliters of the gel were aliquoted into a clean tube for processing with QIAmp DNA Blood Mini kit (Qiagen, Germany). Samples were also processed with the DNA extraction kit “High Pure PCR Template Preparation kit” (Roche Applied Sciences, Germany), showing indistinguishable results. Extracted DNA was aliquoted and stored in -80 ˚C freezer.

qPCR conditions. Detection of *C. trachomatis* cryptic plasmid DNA[24] was performed using the Multiplex PCR Mastermix (IBMP, Curitiba, Brazil) containing 9 mM Mg-acetate, 5 µL of extracted DNA, oligonucleotides cryp05F (0.2 µM, 5’ GGCGTCGTATCAAAGATATGG 3’), cryp06R (0.2 µM, 5’ CGATGATTTGAGCGTGTGTA 3’), and cryp11P (0.1 µM, 5’ FAM-TCTCGGGTTAATGTTGCATGATGCTT-BHQ1 3’) in a reaction volume of 25 µL. Specific oligonucleotides were designed for concomitant detection of human 18S rRNA gene. Hence, the reaction also contained the oligonucleotides 18S2F (0.1 µM, 5’ GAAACTGCGAATGGCTCATTAAATCA 3’), 18S2R (0.1 µM, 5’ AGAGCTAATACATGCCGACGGG 3’), and 18S2P (0.05 µM, 5’ HEX- TGGTTCCTTTGGTCGCTCGCTCC-BHQ1 3’). All six oligonucleotide sequences were tested for unspecific reaction against 55 common parasites and bacteria but no amplification was observed (a list of the organisms is shown in **Supplemental Table 1**). Reactions were performed on the ABI7500 Standard instrument (Thermo Fisher Scientific – Waltham, USA), with the following cycling conditions: 95˚C/10 min, and 45 x [95˚C/15 sec + 60˚C/1 min]. Baseline was set from 3 to 15 for both targets. For *C. trachomatis* DNA, threshold was set to 0.1, and quantification cycle (C_q) values between 19 and 41.50 were considered positive detection. For human DNA, threshold was set to 0.1 and detection was considered positive and free of contaminants if C_q values were between 14 and 37.

Direct Immunofluorescence detection (DFA). Samples were methanol-fixed in glass slides, transported at 4-8 ˚C and stored at -20 ˚C until analyzed. DFA was performed according to the manufacturer’s instructions of the kit “Chlamydia T” (Biocientífica S.A., Buenos Aires, Argentina). Briefly, fixed samples were exposed to fluorescent-labelled monoclonal antibodies against anti-MOMP from *C. trachomatis*. Slides were analyzed in a fluorescence microscope, and were considered valid when containing a minimum of 100 cells and 5 clearly visible *Chlamydia* antigenic structures. Positive and negative controls were performed in all DFA assays.

Statistical analysis. All qPCR assays in the ABI7500 system were performed in technical triplicates, except for limit of detection (LOD) determination (8-12 replicates). Results are expressed as mean ± standard deviation of the quantification cycle (C_q) values. Paired Student’s t-test (95% confidence level) were calculated using GraphPad Prism v5.0 software (Graph Pad Prism Inc, USA). The 95% limit of detection ($LOD_{95\%}$) for detection of the *Chlamydia* genomic target was calculated by Probit regression analysis[25]. Kappa coefficient was calculated between the results obtained with the new duplex qPCR and the results of the pre-characterization of the same samples by the commercial Aptima Combo2

CT/NG assay (taken as gold standard), as well as between the new duplex qPCR and the DFA assay or the clinical evaluation. The coefficient was used to test agreement between the diagnostic methods, and Kappa results were interpreted according to [26, 27]: 1.00–0.81 almost perfect, 0.80–0.61 substantial, 0.60–0.41 moderate, 0.40–0.21 fair and ≤0.20 poor agreement. STARD guidelines were followed in the course of this work.

Results

The performance of the new qPCR was evaluated using commercial genomic extracted DNA from two distinct *C. trachomatis* serovars, D and J. Figure 1 shows linear dynamic range for detection of the *C. trachomatis* genomic target in serovar J. Insert in Figure 1 shows representative traces of the reactions used to calculate the linear regression (red lines a-f) obtained with concomitant detection of the human 18S rRNA gene (green lines). Reaction parameters for *C. trachomatis* DNA detection were efficiency of 92% (slope –3.52), R^2 of 98.6%, and Y-intercept of -32.79. Table I shows average ± SD of the quantification cycle (C_q) for each DNA concentration used in Figure 1. Data show that the reaction is more sensitive towards the detection of serovar J, since the lowest concentration (100 ag/µL) was not detected for serovar D DNA.

Using data from Figure 1, a Probit analysis determined an $LOD_{95\%}$ of 600 ag/µL when assessed with commercial genomic DNA serovar J. When a synthetic double strand DNA containing the same genomic sequence was used as template, $LOD_{95\%}$ reached 1.61 copies/µL, which translates to 8 copies per reaction (Supplemental Figure S1).

Next step was to validate the new duplex qPCR with previously characterized samples. DNA from fifty samples previously characterized by the Aptima Combo2 assay was analyzed by the duplex qPCR described in Figure 1. Table II compares the results obtained with both protocols. Samples 1-30 were previously characterized as “Positive” and samples 31-50 were characterized as “Negative” for *C. trachomatis* DNA. Both assays yielded the same result for all samples except for one. “Negative” sample (#34) was classified as “Inconclusive” by the qPCR because the reaction failed to detect the human DNA marker, thus invalidating the results. This sample would have to be re-processed in a clinical setting because failure to detect the human DNA might be attributed to degraded DNA template. These data yield a true-positive rate (sensitivity) of 100% and a true-negative rate (specificity) of 85%, with no false-negatives or false-positive detections, and one inconclusive (Table III). Cohen’s kappa coefficient was calculated to be 0.657, which means that both assays have substantial agreement.

Next, we decided to compare the new duplex qPCR protocol to a DFA protocol that uses a monoclonal anti-MOMP antibody. Figure 2A shows the evaluation of 50 samples from a trachoma-endemic endemic region (Marajo Island, Brazil), while Figure 2B shows the evaluation of 12 samples from a non-endemic region (Curitiba, Brazil). Green lines represent the detection of the human 18S rRNA gene. Blues lines represent the detection of the *C. trachomatis* cryptic plasmid DNA. It can be observed that the specific bacterial DNA sequence can be found in most of the samples from the endemic area, while no detection

was observed in the samples from the non-endemic area. *C. trachomatis* DNA could be identified in 48 of the 50 endemic-area samples, displaying 96% positivity. However, only three of these samples were clinically positive for *C. trachomatis* (Table IV), confirming an already known strong disparity between the two diagnostic techniques. Figure 2C shows the dispersion of the C_q for each sample detected in Figure 2A plotted over the linear regression of the detection of serially diluted *C. trachomatis* serovar J DNA. It can be observed that the samples have high C_q, highlighting the importance of the linear detection of 100 ag/µL (Table I) and non-linear detection of up to 10 ag/µL (Figure 2C).

Table IV shows that samples 1-50 (endemic region) were primarily negative for the clinical evaluation as well as for immunodetection of MOMP, whereas 48 of those were classified as “Positive” by qPCR. Samples 51-62 (non-endemic region) were classified as “Negative” for all three diagnostic techniques (clinical evaluation, DFA, or qPCR). There was no correlation between the clinical evaluation, the number of elementary bodies (EBs) as observed by DFA, or the results of the direct immunodetection protocol with the qPCR results. Indeed, Kappa coefficient analysis showed poor correlation between clinical evaluation and qPCR or between DFA and qPCR (less than 0.2 for both cases).

Discussion

The work presented here shows the development and validation of a new duplex qPCR test for detection of *C. trachomatis* DNA in ocular samples. To reach the necessary sensitivity, we chose a target in the cryptic plasmid, which can have up to ten copies per bacterial genome [24, 28].

The development of the new reaction was performed using commercial DNA from two serovars, D and J, which exhibited different LOD (Table I). However, for diagnostic purposes, the difference observed between the detection of the two serovar’s DNA is irrelevant since 1 genome of *C. trachomatis*, containing up to 10 copies of the targeted cryptic plasmid, is roughly equal to 1 fg DNA [28], which is detected for both serovars. Other PCR protocols, either nested, real time or digital, manual or semi-automated, have been published in the last years [12–15, 17, 18, 29, 30]. Although most were not developed for ocular samples, they have been validated for such use, with sensitivity and specificity similar to its original sample matrix (i.e., urovaginal fluids). This is a possible explanation for their limit of detection around 10 genomes of *C. trachomatis*, which is not the most suitable LOD for detection of these bacteria in ocular samples [28]. However, much like the reaction presented herein, some published tests do reach the necessary sensitivity of 1 genome of *C. trachomatis* to be able to detect its low abundance in ocular swab samples [13, 14, 29].

The new duplex qPCR was validated using ocular samples previously characterized for the presence of *C. trachomatis* DNA using the Aptima Combo2 CT/NG assay [23, 31]. The results reached substantial agreement as per the calculated kappa coefficient. However, when the qPCR results was compared to the clinical evaluations or DFA, the agreement was considered poor very likely because each technique evaluates a different biological marker of the disease.

Examination and laboratory tests are frequently discordant, possibly due to infection kinetics [22] and age-dependent manifestations of infection [24]. The gold standard method for diagnostic of *C. trachomatis* still is, in many countries, clinical inspection of the patient's inverted eyelid [32, 33], while some use DFA [9, 10]. Very few diagnostic facilities perform molecular tests for trachoma determination. Thus, given the distinct nature of each test, it is important to identify and differentiate each of their biological targets. Clinical inspection measures the number of lesions in the inverted eyelid of both eyes, which requires a skilled health practitioner. DFA uses antibodies to recognize the presence of *C. trachomatis* proteins in swab samples of the inverted eyelids. DFA requires a fluorescence microscope and it is prone to inconclusive results due to high levels of background fluorescence, thus also requiring a skilled professional for accurate readings. Moreover, the DFA test threshold for positivity of >5 EBs per analysis could be questioned, since the presence of EB is indicative of cellular adaptation for infection [34]. Molecular tests such as qPCR detects and amplifies sequences of the bacterial genome in a very specific pattern, thus being more reliable than DFA. Therefore, despite recent advances, DFA still is less sensitive than qPCR [8–10, 31, 35–38]. Indeed, since the clinical signs of trachoma infection are not so unique [39], clinical evaluations correlate poorly with PCR detection levels in low prevalence areas or after mass antibiotic treatments [40].

Our results corroborate the differences between the all three techniques. When 62 uncharacterized samples were probed for the presence of *C. trachomatis* antigens (DFA) or for *C. trachomatis* DNA (qPCR), almost opposite results emerged (Table IV). Clinical evaluation or detection of EB by DFA showed very few positives amongst the endemic samples, while 96% were positive by qPCR (48 out of 50 samples). This means that, although the patients did not show clinical signs of active infections or scars from repetitive infections, they have been recently infected because the bacterial DNA was still present, possibly in tissue that was not yet removed by the body. Indeed, latent class analyses of clinical examination versus qPCR detection suggest that qPCR positivity is a better predictor for determining chlamydial infection than clinical inspections [41].

However, limitations of the qPCR technique must not be understated. Molecular assays for trachoma diagnosis might present a false-negative rate as high as 20% [41], possibly because of the infection timing or poor DNA extraction efficiencies [42–45]. Second, the assay presented herein relies on the presence of the cryptic plasmid inside the bacteria, which could be missing [46]. Some authors have also suggested that other species of *Chlamydia* can also cause trachomatous inflammation [47]. Other limitations that were not evaluated in our study include differences between conjunctival and epithelial specimens, human conjunctival cell yield, DNA extraction efficiency, and thorough removal of molecular inhibitors that may also affect test performance [42–45].

Conclusions

This work shows the development and validation of a new duplex qPCR that concomitantly detects *C. trachomatis* cryptic plasmid DNA and a human endogenous control target. The newly developed reaction was validated against qPCR-pre-characterized samples, showing high sensitivity and specificity. It was

also compared with traditional techniques for trachoma diagnosis such as clinical evaluation of the eyelid or direct immunofluorescence assay, showing greater overall performance. The presented qPCR may be an important research tool for monitoring patients and diagnosing trachoma in ocular samples in endemic regions as part of the effective measures for the elimination of the disease.

Abbreviations

qPCR – polymerase chain reaction

DFA – direct immunofluorescence assay

LOD – limit of detection

EB – elementary bodies

MOMP – major outer membrane porin

Declarations

a. Ethics approval and consent to participate

The Johns Hopkins University's Institutional Review Board approved the samples collection in Tanzania under the number IRB00096404, which were then stored at the bio-repository at the Chlamydia Research Laboratory (Baltimore, USA). The Ethics Committee of the Instituto Evandro Chagas, under the CAEE number 48053415.3.0000.0019, approved all samples collection in Brazil. All participants from both collection sites consented to participate in the site's respective research study.

b. Consent for publication

Not applicable.

c. Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

d. Competing interests

Instituto de Biologia Molecular do Paraná (IBMP) produces the PCR mastermix and oligonucleotides used in the qPCR experiments. Several authors received grants or fellowships from the Brazilian funding agencies CNPq and CAPES. This work was partially funded by a grant from the Banco Nacional de Desenvolvimento Econômico e Social (BNDES), contract no. 15.2.0473.1 (Operation #4.816.864). However, IBMP, BNDES, CAPES or CNPq had no participation whatsoever in the present study's design, data collection, analysis or interpretation, or writing of the report and decision to submit for publication.

e. Funding

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f. Authors' contributions

JFRF supervised all Brazilian samples collection procedures, analyzed the patients' eyelids, performed the DFA experiments and wrote the first draft of the manuscript. KKL performed all DNA extractions of the Brazilian samples, performed and analyzed most of the qPCR experiments, performed the specificity tests, and wrote the first draft of the manuscript. STMG participated in the Brazilian samples collection and helped analyze the patients' eyelid as well as the DFA results. GDM and MPMM participated in the Brazilian samples collection and analysis of the patients' eyelids. PHCS and WT designed the oligonucleotides and standardized the qPCR reaction. TJ performed qPCR experiments with the synthetic positive control and all statistical analyses. ABF performed the qPCR validation experiments using the Johns Hopkins University's samples and helped analyzed all qPCR results. RCPR participated in the DNA extraction of the Brazilian samples, in the standardization of the qPCR, and in the execution of the specificity tests. CAG and SW coordinated the samples collection in Tanzania, and were responsible for their integrity at the Chlamydia Research Laboratory. ADTC supervised the oligonucleotides design, the standardization and validation of the qPCR, all statistical analyses and wrote the final version of the manuscript. AJLAC oversaw the sample collection as well as the clinical evaluation and DIF analyses in Brazil, and helped to write the final version of the manuscript.

g. Acknowledgements

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Tables

Table I. Quantification cycle (C_q) for detection of different concentrations of *C. trachomatis* DNA, serovar J and serovar D. Data are shown as mean C_q ± SD were obtained with more than 5 independent experiments for each serovar. ND, not determined.

Concentration (per µL)	C_q (mean ± SD) for Serovar J	C_q (mean ± SD) for Serovar D
10 pg	18.54 ± 0.19	19.03 ± 0.62
1 pg	22.00 ± 0.29	22.77 ± 0.20
100 fg	25.99 ± 0.22	27.63 ± 0.89
10 fg	30.14 ± 0.04	31.53 ± 0.22
1 fg	34.32 ± 0.65	34.29 ± 0.33
100 ag	36.76 ± 0.91	ND

Table II. Validation of qPCR shown in Figure 1 with pre-characterized patient samples. Samples were pre-characterized by the Aptima Combo2 CT/NG assay (Hologic, USA). Extracted DNA was then analyzed by the NAT Trachoma kit described in the present paper.

Sample #	Pre-characterization by the CT/NG assay	Mean C_q ± SD for CRYP target	Detection of reaction internal control	Classification by this paper's qPCR
1	POSITIVE	22,60	YES	POSITIVE
2	POSITIVE	30,36	YES	POSITIVE
3	POSITIVE	29,65	YES	POSITIVE
4	POSITIVE	25,75	YES	POSITIVE
5	POSITIVE	30,75	YES	POSITIVE
6	POSITIVE	39,28	YES	POSITIVE
7	POSITIVE	38,99	YES	POSITIVE
8	POSITIVE	32,56	YES	POSITIVE
9	POSITIVE	27,45	YES	POSITIVE
10	POSITIVE	25,07	YES	POSITIVE
11	POSITIVE	28,10	YES	POSITIVE
12	POSITIVE	29,83	YES	POSITIVE
13	POSITIVE	37,59	YES	POSITIVE
14	POSITIVE	25,48	YES	POSITIVE

15	POSITIVE	27,43	YES	POSITIVE
16	POSITIVE	35,39	YES	POSITIVE
17	POSITIVE	29,26	YES	POSITIVE
18	POSITIVE	28,82	YES	POSITIVE
19	POSITIVE	37,21	YES	POSITIVE
20	POSITIVE	32,56	YES	POSITIVE
21	POSITIVE	31,50	YES	POSITIVE
22	POSITIVE	40,08	YES	POSITIVE
23	POSITIVE	37,51	YES	POSITIVE
24	POSITIVE	26,01	YES	POSITIVE
25	POSITIVE	29,12	YES	POSITIVE
26	POSITIVE	26,88	YES	POSITIVE
27	POSITIVE	31,41	YES	POSITIVE
28	POSITIVE	30,85	YES	POSITIVE
29	POSITIVE	33,11	YES	POSITIVE
30	POSITIVE	27,99	YES	POSITIVE
31	NEGATIVE	ND	YES	NEGATIVE

32	NEGATIVE	ND	YES	NEGATIVE
33	NEGATIVE	ND	YES	NEGATIVE
34	NEGATIVE	ND	NO	INCONCLUSIVE
35	NEGATIVE	ND	YES	NEGATIVE
36	NEGATIVE	ND	YES	NEGATIVE
37	NEGATIVE	ND	YES	NEGATIVE
38	NEGATIVE	ND	YES	NEGATIVE
39	NEGATIVE	ND	YES	NEGATIVE
40	NEGATIVE	ND	YES	NEGATIVE
41	NEGATIVE	ND	YES	NEGATIVE
42	NEGATIVE	ND	YES	NEGATIVE
43	NEGATIVE	ND	YES	NEGATIVE
44	NEGATIVE	ND	YES	NEGATIVE
45	NEGATIVE	ND	YES	NEGATIVE
46	NEGATIVE	ND	YES	NEGATIVE
47	NEGATIVE	ND	YES	NEGATIVE

48	NEGATIVE	ND	YES	NEGATIVE
49	NEGATIVE	ND	YES	NEGATIVE
50	NEGATIVE	ND	YES	NEGATIVE

Table III. Absolute numbers and percentage of detections: true-positive, true-negative, false-positive, and false-negative rates. Results were calculated from data shown in Table II.

Category	Detections	Total	Rate (%)
Positive	30	30	100,0
Negative	19	20	95,00
Inconclusive	1	50	2,00
False-negative	0	30	0,00
False-positive	0	20	0,00

Table IV. Comparison of duplex qPCR results to the characterization of samples from endemic and non-endemic areas clinical evaluation (CE) or direct immunofluorescence (DFA). The number of elementary bodies (EBs) observed in each sample is shown.

Sample #	Clinical Evaluation (CE)	C_q (mean ± SD)	Duplex qPCR classification	Agreement CE x qPCR	EBs	DFA classification	Agreement DFA x qPCR
1	NEGATIVE	36.59 ± 0.59	POSITIVE	NO	0	NEGATIVE	NO
2	NEGATIVE	-	NEGATIVE	YES	8	POSITIVE	NO
3	NEGATIVE	36.18 ± 0.54	POSITIVE	NO	0	NEGATIVE	NO
4	NEGATIVE	41.62 ± 0.00	POSITIVE	NO	0	NEGATIVE	NO
5	NEGATIVE	38.91 ± 3.36	POSITIVE	NO	0	NEGATIVE	NO
6	NEGATIVE	35.93 ± 1.49	POSITIVE	NO	0	NEGATIVE	NO
7	NEGATIVE	36.06 ± 1.73	POSITIVE	NO	5	POSITIVE	YES
8	NEGATIVE	43.05 ± 1.78	POSITIVE	NO	2	NEGATIVE	NO
9	NEGATIVE	39.12 ± 2.67	POSITIVE	NO	3	NEGATIVE	NO
10	NEGATIVE	42.20 ± 4.22	POSITIVE	NO	0	NEGATIVE	NO
11	POSITIVE	36.82 ± 0.91	POSITIVE	YES	7	POSITIVE	YES
12	NEGATIVE	38.31 ± 0.00	POSITIVE	NO	2	NEGATIVE	NO
13	NEGATIVE	37.89 ± 1.43	POSITIVE	NO	0	NEGATIVE	NO
14	NEGATIVE	33.90 ± 0.49	POSITIVE	NO	1	NEGATIVE	NO
15	POSITIVE	36.86 ± 1.49	POSITIVE	YES	6	POSITIVE	YES
16	NEGATIVE	37.07 ± 2.49	POSITIVE	NO	0	NEGATIVE	NO
17	NEGATIVE	37.83 ± 1.46	POSITIVE	NO	2	NEGATIVE	NO

18	NEGATIVE	35.08 ± 0.00	POSITIVE	NO	6	POSITIVE	YES
19	NEGATIVE	37.61 ± 2.79	POSITIVE	NO	2	NEGATIVE	NO
20	NEGATIVE	37.53 ± 2.81	POSITIVE	NO	0	NEGATIVE	NO
21	NEGATIVE	33.58 ± 0.89	POSITIVE	NO	2	NEGATIVE	NO
22	NEGATIVE	34.55 ± 0.37	POSITIVE	NO	0	NEGATIVE	NO
23	NEGATIVE	35.94 ± 0.21	POSITIVE	NO	4	NEGATIVE	NO
24	NEGATIVE	34.83 ± 0.16	POSITIVE	NO	5	POSITIVE	YES
25	NEGATIVE	37.07 ± 1.46	POSITIVE	NO	0	NEGATIVE	NO
26	NEGATIVE	37.05 ± 1.51	POSITIVE	NO	6	POSITIVE	YES
27	NEGATIVE	37.78 ± 0.37	POSITIVE	NO	3	NEGATIVE	NO
28	NEGATIVE	33.10 ± 0.45	POSITIVE	NO	6	POSITIVE	YES
29	NEGATIVE	36.73 ± 0.59	POSITIVE	NO	2	NEGATIVE	NO
30	NEGATIVE	37.40 ± 1.19	POSITIVE	NO	5	POSITIVE	YES
31	NEGATIVE	35.24 ± 0.30	POSITIVE	NO	6	POSITIVE	YES
32	NEGATIVE	35.26 ± 1.04	POSITIVE	NO	7	POSITIVE	YES
33	NEGATIVE	34.98 ± 0.76	POSITIVE	NO	5	POSITIVE	YES
34	NEGATIVE	34.06 ± 0.00	POSITIVE	NO	6	POSITIVE	YES
35	NEGATIVE	35.23 ± 1.08	POSITIVE	NO	8	POSITIVE	YES
36	POSITIVE	38.28 ± 0.50	POSITIVE	YES	9	POSITIVE	YES
37	NEGATIVE	34.23 ± 0.48	POSITIVE	NO	2	NEGATIVE	NO

38	NEGATIVE	35.84 ± 0.84	POSITIVE	NO	2	NEGATIVE	NO
39	NEGATIVE	36.93 ± 0.00	POSITIVE	NO	3	NEGATIVE	NO
40	NEGATIVE	36.59 ± 0.75	POSITIVE	NO	2	NEGATIVE	NO
41	NEGATIVE	-	NEGATIVE	YES	5	POSITIVE	YES
42	NEGATIVE	35.12 ± 1.11	POSITIVE	NO	4	NEGATIVE	NO
43	NEGATIVE	40.70 ± 0.00	POSITIVE	NO	5	POSITIVE	YES
44	NEGATIVE	34.10 ± 0.77	POSITIVE	NO	7	POSITIVE	YES
45	NEGATIVE	34.50 ± 0.36	POSITIVE	NO	3	NEGATIVE	NO
46	NEGATIVE	35.23 ± 1.42	POSITIVE	NO	2	NEGATIVE	NO
47	NEGATIVE	38.71 ± 1.58	POSITIVE	NO	0	NEGATIVE	NO
48	NEGATIVE	33.72 ± 0.50	POSITIVE	NO	1	NEGATIVE	NO
49	NEGATIVE	34.53 ± 0.34	POSITIVE	NO	2	NEGATIVE	NO
50	NEGATIVE	36.38 ± 0.00	POSITIVE	NO	1	NEGATIVE	NO
51	NEGATIVE	-	NEGATIVE	YES	0	NEGATIVE	YES
52	NEGATIVE	-	NEGATIVE	YES	0	NEGATIVE	YES
53	NEGATIVE	-	NEGATIVE	YES	0	NEGATIVE	YES
54	NEGATIVE	-	NEGATIVE	YES	0	NEGATIVE	YES
55	NEGATIVE	-	NEGATIVE	YES	0	NEGATIVE	YES
56	NEGATIVE	-	NEGATIVE	YES	0	NEGATIVE	YES
57	NEGATIVE	-	NEGATIVE	YES	0	NEGATIVE	YES

58	NEGATIVE	-	NEGATIVE	YES	0	NEGATIVE	YES
59	NEGATIVE	-	NEGATIVE	YES	0	NEGATIVE	YES
60	NEGATIVE	-	NEGATIVE	YES	0	NEGATIVE	YES
61	NEGATIVE	-	NEGATIVE	YES	0	NEGATIVE	YES
62	NEGATIVE	-	NEGATIVE	YES	0	NEGATIVE	YES

Figures

Figure 1.

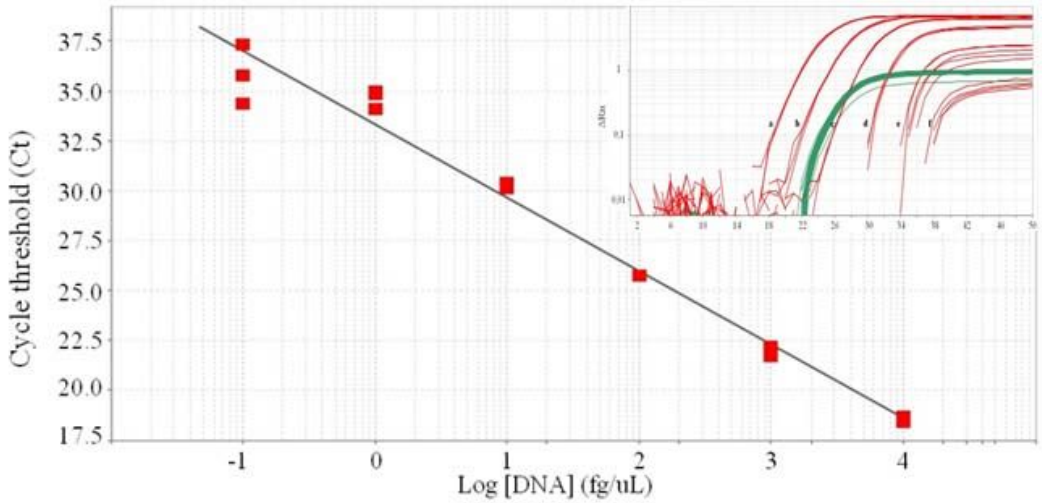


Figure 1

Linear dynamic range for detection of the C. trachomatis genomic target in serovar J DNA. C. trachomatis DNA was serially diluted in human DNA and evaluated using the new duplex qPCR. The reaction shows efficiency of 92% and R2 of 98.6%. Insert in Figure 1 shows representative traces of the reactions used to calculate the linear regression (red lines a-f), obtained with concomitant detection of the human 18S

rRNA gene (green lines). Traces and linear regression are representative of more than 10 independent experiments with 3-12 replicates per run for each concentration.

Figure 2.

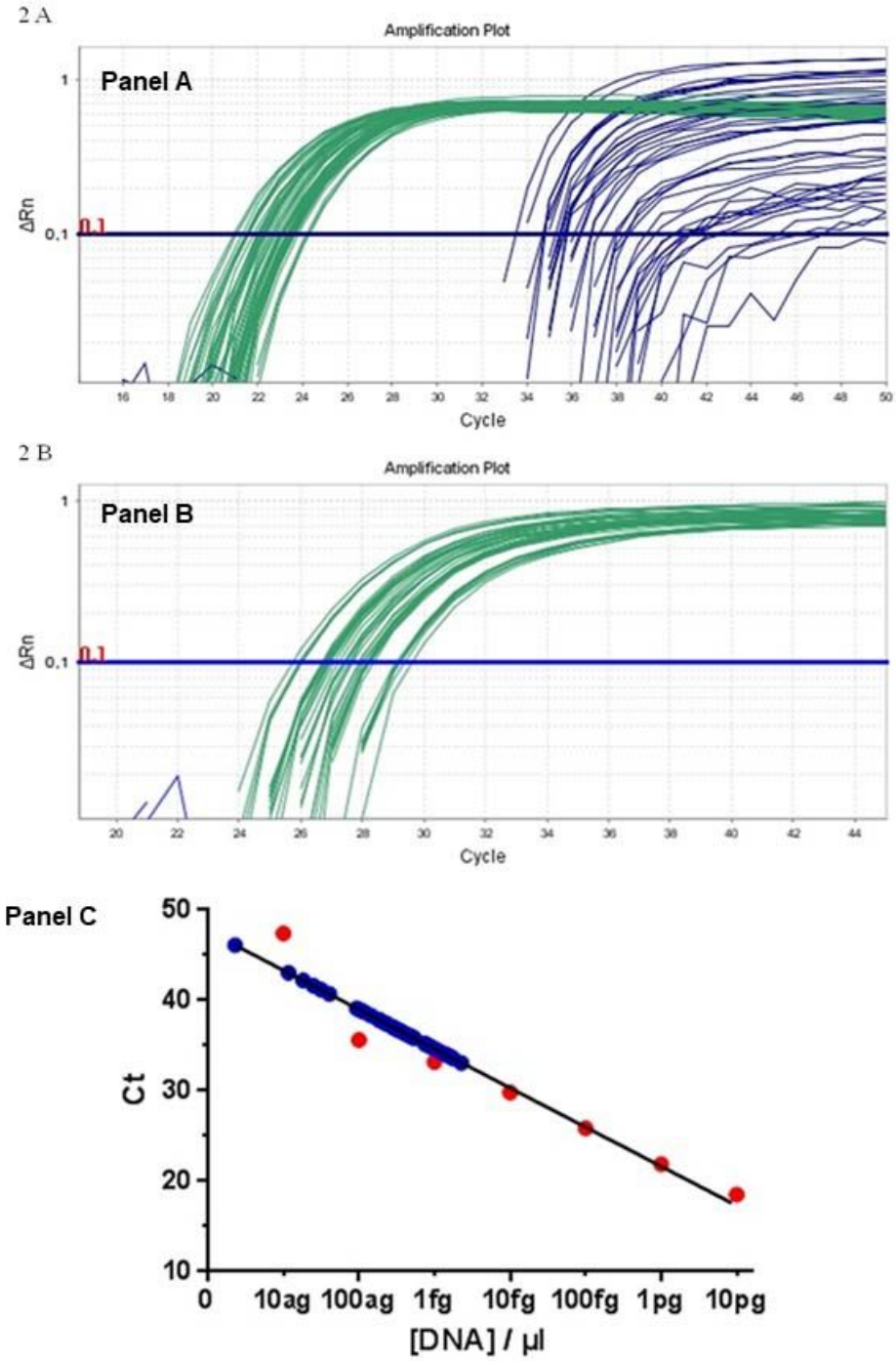


Figure 2

Detection of C. trachomatis DNA in human samples. Samples from an endemic and a non-endemic region in Brazil were collected and their DNA extracted as described in Methods. Panel A shows the detection of C. trachomatis DNA (blue lines) parallel to the detection of human DNA (green lines) in

samples from the endemic area. Panel B shows the detection of human DNA (green lines) but no C. trachomatis DNA in samples from the non-endemic area. Panel C shows the quantification of C. trachomatis DNA present in each of the positive samples (blue circles) based on the linear regression obtained with serial dilutions of C. trachomatis serovar J DNA (red circles).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalTableS1.docx
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