EXTENDED REPORT

Detection by broad-range real-time PCR assay of *Chlamydia* species infecting human and animals

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Background: Tests available for molecular diagnosis of chlamydial infections detect *Chlamydia trachomatis*, but do not find other *Chlamydia* species associated with genital, ophthalmic, cardiovascular, respiratory or neurological diseases. The routine detection of all *Chlamydia* species would improve the prognosis of infected people and guide therapeutic choices.
Aim: To design and validate a sensitive, specific, reproducible, inexpensive and easy-to-perform assay to quantify most *Chlamydia* species.
Methods: Primers and probe were selected using the gene coding for the 16S rRNA. The detection limits were assessed for suspensions of *Chlamydia trachomatis*, *Chlamydia psittaci and Chlamydia pneumoniae*. The performance of this test was compared with that of two commercial kits (Amplicor-Roche and Artus) on 100 samples obtained from children with trachoma.
Results: The detection capacities for *Chlamydia trachomatis* of the broad-range real-time polymerase chain reaction (PCR) were similar or slightly better than those obtained with commercial kits (0.2 copies of DNA/μl). Only the broad-range PCR identified specimens containing *Chlamydia psittaci and Chlamydia pneumoniae*. The commercial kits and the broad-range assay detected *Chlamydia* species in 5% and in 11%, respectively, of samples from children with trachoma.
Conclusions: This new real-time PCR offers a sensitive, reproducible assay that produces results in <3 h. With panels of quantified *Chlamydia* species, this real-time PCR can be run with all real-time PCR equipment. Larger trials are needed to confirm the utility of this test in diagnosis and for therapeutic follow-up.

The Chlamydiaceae (*Chlamydia*)—two distinct Gram-negative obligate intracellular bacterial lineages that branch into nine separate clusters—cause cervicitis, urethritis, rectitis, endometritis and salpingitis, inclusion conjunctivitis and cardiorespiratory infections, and are responsible (or are cofactors) for trachoma.[1–4] The Chlamydiaceae, reclassified according to their 16S and 23S ribosomal gene sequences, which were previously known only by the genus *Chlamydia*, were divided into two genera, Chlamydia and *Chlamydophila* gen nov. The *Chlamydophila* gen nov assimilates the current species *Chlamydia pecorum, Chlamydia pneumoniae* and *Chlamydia psittaci*, to form *Chlamydophila pecorum* comb nov, *Chlamydophila pneumoniae* comb nov and *Chlamydophila psittaci* comb nov. Three new *Chlamydophila* species were derived from *Chlamydia psittaci*: *Chlamydophila abortus* gen nov, sp nov, *Chlamydophila caviae* gen nov, sp nov and *Chlamydophila felis* gen nov, sp nov.[5]

Chlamydia trachomatis is the most common sexually transmitted agent in non-gonococcal urethritis and may cause epididymitis.[6–9] Infants may develop eye diseases (inclusion conjunctivitis), pneumonia, and pharyngeal and enteric complications.[1 10–12] *Chlamydia* associated with poverty triggers trachoma, the prime cause of preventable infectious blindness.[13 14 12 15] *Chlamydia psittaci* shed by avian species and by domestic mammals may also cause conjunctivitis and severe pulmonary diseases.[16 17 12] *Chlamydia pneumoniae* can induce proliferation of smooth muscle cells and contribute to the aggravation of primary pulmonary symptoms,[18 19 4] and is associated with pharyngitis, bronchitis, pneumonia and endothelial disorders.[20–22]

Various molecular approaches have been developed in the nucleic acid amplification tests (NAATs) used for diagnosis of chlamydial infections:

1. Qualitative PCR (Amplicor Roche-USA), which amplifies a cryptic plasmid DNA from *Chlamydia trachomatis*
2. Quantitative PCR assay (Artus-Germany), which amplifies a sequence of a major structural protein (ompA)
3. The AC2 assay (Gen-Probe-USA), which targets the *Chlamydia trachomatis* rRNA and uses a technology based on the transcription-mediated amplification of isolated target sequences[23]
4. The Becton Dickinson ProbeTec (Becton Dickinson Diagnostic Systems-USA), which targets a sequence within a cryptic plasmid DNA of *Chlamydia trachomatis* using DNA polymerase and a specific restriction-enzyme strand-displacement amplification.[24]

As the commercial diagnostic tests for these entities are able to detect only *Chlamydia trachomatis*, we designed a set of primers and a probe that recognises Chlamydiaceae using the real-time technology. The goal of this work was to develop a sensitive, inexpensive and easy-to-perform assay that was able to detect and quantify most of the species of *Chlamydia* infecting humans and animals. The detection limits for this new assay were assessed with strains of *Chlamydia trachomatis*, *Chlamydia psittaci* and *Chlamydia pneumoniae*. In addition, the detection capacities of this assay were studied in parallel with two commercial kits (Amplicor and Artus), with samples obtained from children with trachoma.

MATERIAL AND METHODS

Human samples

Sampling was carried out after obtaining the informed consent of parents or guardians during epidemiological surveys, and in accordance with the Declaration of Helsinki and the electroencephalogram human experimentation guidelines.

Abbreviations: CFU, colony forming units; NAAT, nucleic acid amplification test; PCR, polymerase chain reaction

A total of 100 clinical samples obtained from children (boys and girls aged 1–10 years) living in the Kankan area (Haute-Guinea) who had trachoma diagnosed by experienced ophthalmologists (according to the World Health Organization grading card: ⩾5 follicles in a specific area of the upper eye lid) were obtained by vigorous scraping of the upper conjunctiva with Dacron swabs. To avoid cross contamination, the doctor in charge of sampling was not allowed to handle pens, torches or clinical files, or to register any data. Independent assistants managed the identification and seating of the children and the registration of data. Disposable material was used, and paper covers on the seats were changed after each patient had used them; equipment and surfaces were cleaned with disinfectant and 1 N hydrochloric acid. The operators wore disposable shirts, masks and glasses, and two pairs of gloves, one of which was changed for each new patient. Once clinical diagnosis was confirmed, the upper tarsal conjunctiva was swabbed intensely with Dacron swabs, and the samples were placed in a sterile tube, which was closed tightly. Before introduction of the swabs into the containers, the outside tube surfaces were decontaminated with sterile gauze lightly dampened with 1 N hydrochloric acid. The tubes were kept dry in a container (4–8℃) for <8 h after sampling and then stored at −20℃ for DNA extraction. The aliquots were blind tested with the three assays. The Amplicor and Artus PCRs were carried out according to the manufacturers' instructions.

Nucleic acid extraction

Specimens in the Dacron swabs were thawed; 500 µl of phosphate-buffered saline was added and vortexed intensively. DNA was extracted using the Magnapure (Roche Applied Sciences) from 200 µl of specimens using total nucleic acid isolation. The extracts were recovered in 50 µl of water and analysed by Amplicor and by Artus.

A separate extraction with Magnapure was carried out for the broad-spectrum *Chlamydia* PCR. To monitor the extraction and amplification processes, 5 µl of a whole virus preparation of seal herpes virus (a gift from G J van Doornum, Department of Virology Erasmus MC, Rotterdam, The Netherlands) was added to each sample before extraction (final concentration of about 1000 viral particles/ml).

Primers and probe

Alignments of the chlamydial genes coding for the 16S rRNA (rDNA) were carried out to identify the highest conserved regions in *Chlamydia pneumoniae, Chlamydia trachomatis and Chlamydia psittaci* sequences exhibiting >90% homology.[25–27 4] The sequences were selected using the primer Express 1.0 software (Applied Biosystems, France). The forward primer (5'TCGAGAATCTTTCGCAATGGAC) and the reverse primer (5'CGCCCTTTACGCCCAATAAA) were BLAST searched using the NCBI Blastn FAQs (ncbi.nlm.nih.gov/Genbank). These primers bracket a highly conserved sequence (multicopy gene) coding for the ribosomal RNA in most sequences of *Chlamydia trachomatis, Chlamydia psittaci, Chlamydia pneumoniae, Chlamydia felis, Chlamydia pecorum, Chlamydia caviae, Chlamydia suis* and *Chlamydia muridarum*.[28 5 26 27 4] The reporter (5'fluor, 6-FAM) and the quencher (TAMRA) dyes were attached to the 5' and 3' ends of the probe, respectively (FAM-AAGTCTGACGAAGCGACGCCGC). No cross reactivity with genes of other microorganisms, or with any other mammalian gene was detected for the primers or for the probe.

Real-time TaqMan broad-spectrum PCR assay

Different temperatures and annealing or extension times for each step of the reactions were studied in preliminary assays to determine the best experimental conditions in which at least 0.5 copies/µl of *Chlamydia trachomatis* DNA could be detected. PCR reactions were carried out in a final volume of 50 µl containing 2× TaqMan Universal Mastermix (MNL 430449, Applied Biosystems), forward primer (0.5 µmol/l), reverse primer (0.5 µmol/l), FAM-TAMRA probe (0.4 µmol/l) and 25 µl of the isolated DNA eluted in distilled water. After incubation for 2 min at 50℃ with uracil N-glycosylase to neutralise potential PCR contaminants from previous reactions, the microtubes were incubated for 10 min at 95℃. The PCR cycling programme consisted of 50 two-step cycles of 10 s at 95℃, and 65 s at 60℃. The amplification and detection were carried out with the ABI Prism 7000 sequence detector system (Applied Biosystems). Retest and second derivative analysis were carried out with the SmartCyclerII (IL Laboratory, Cepheid-Sunnyvale, California, USA). The Ct value for each sample was determined according to the fluorescence signal exceeding the background limit of 0.20. Each run contained negative controls with no template.

Calibrated samples containing *Chlamydia trachomatis,* purchased from the European Union Concerted Action on Quality Control of Nucleic Acid Amplification Program (EQCP, Glasgow, UK), were tested pure and diluted in distilled water before DNA extraction to assess the linearity, sensitivity, reproducibility and detection limits. The introduction into each sample before DNA extraction of a non-human calibrated virus served to monitor the DNA extraction and to assess PCR inhibition. Dilutions of *Chlamydia trachomatis*, *Chlamydia psittaci* and *Chlamydia pneumoniae* were retested using the SmartCyclerII system, which enabled us to follow the second derivative of the growth curve (rates of change for the curve slopes) in realtime. Here, the highest peak of the second derivative curves representing the point of maximum curvature of the signal curves or the transition from non-specific signals and background to amplified product fluorescence was used to validate the true relevance of the signals.

RESULTS

Table 1 shows that the broad-range real-time PCR assay is able to detect and semiquantify *Chlamydia trachomatis*. The broad-range assay detection capacities for *Chlamydia trachomatis* are similar or slightly better than those obtained with two commercial kits, and as few as 0.2 copies of DNA/µl were detected with this assay in three different experiments. Table 2 shows that only the broad range assay for *Chlamydia* was able to detect three different species of *Chlamydia*, whereas the commercial kits did not detect any of the dilutions of either *Chlamydia psittaci* or *Chlamydia pneumoniae*. The broad range test was positive for the three samples containing the equivalent of 0.5 CFU/1000 µl or more of *Chlamydia psittaci and Chlamydia penumoniae*. The linearity and reproducibility of the assay were studied with supernatants of cells infected with *Chlamydia psittaci or Chlamydia pneumoniae* (commercial quality control panels are not yet available), and the Ct values generating positive signals (confirmed by analysing the second derivative) were similar when tested in different experiments (dispersion always <1.2 Ct).

Table 3 shows that when comparing the performances of the Amplicor, the Artus and the broad-spectrum real-time PCR assay with samples obtained from children with trachoma, the rates of positivity were similar for Amplicor and Artus, but greater for the broad-range assay (11 positive *v* 5 positive for the commercial assays). The six positive samples detected as positive with the broad-range assay should have been further investigated to determine the sequences of the amplicons, but the specimens were tested in a routine laboratory in which tubes containing amplified products were discarded, suggesting that in future studies additional steps should be carried out to detect the

Table 1 Detection capacities of the broad-range real-time PCR assay and two commercial kits on *Chlamydia trachomatis* elementary bodies

Results (three different assays)			
Concentration (elementary bodies of *Chlamydia* trachomatis/µl*)	**Amplicor†**	**Artus†**	**Broad-range assay**
50	+++	+++	+++
25	+++	+++	+++
12.5	+++	+++	+++
3.2	+++	+++	+++
0.8	+++	+NN	+++‡
0.2	+N+	NNN	+++¶
0.1	NNN	NNN	NN+§

N, negative; +, positive.
*Dilutions before DNA extraction.
†According to manufacturer's instructions.
‡Ct values were 35.0, 35.7 and 35.8, respectively.
¶Ct values were 37.2, 37.5 and 37.0, respectively.
§Ct = 39.

implication of *Chlamydia* species other than *Chlamydia trachomatis* in trachoma.

DISCUSSION

The NAATs are known for being the most sensitive methods for the diagnosis of *Chlamydia trachomatis* infections and can detect as few as one organism per assay, whereas the limit of detection for the conventional tests is ⩾10 organisms;[28–30] previous studies with urine samples showed that commercial kits are not significantly different in their ability to detect *Chlamydia trachomatis*.[23 24]

The commercial NAATs have monospecies spectrums and fail to meet the expectations of the clinical microbiology services. It has been reported that these assays may be effective to varying degrees for the detection of *Chlamydia trachomatis* in samples from patients with conjunctivitis or trachoma, but they may produce negative results for other species or if the plasmid (detected by Amplicor and by the Becton Dickinson Probe Tec test) that is required for chlamydial growth in vitro is altered or is absent.[31 32] Hence, the conclusions drawn from amplifying narrow sequences (in the plasmid and only for the species *Chlamydia trachomatis*) or regions coding for structural proteins that may be altered (Artus and Gen Probe AC2 assays) may underestimate the levels of infection.[13 14 33 34 31 32] In the present study, the amplification of the plasmid (Amplicor) and the amplification of the momp-gene (Artus) showed identical rates of positivity in patients with trachoma, suggesting that the low detection levels may not be due to the loss of the plasmid or due to mutations in the mono-gene.[31 35 27]

Previous trials carried out in areas with endemic trachoma showed discrepancies between the clinical diagnosis and PCR (Amplicor), with positivity levels ranging from <10% to 70%. As an example, in high-prevalence communities with active trachoma in Nepal, 70% of clinically active cases were positive by PCR, whereas only 8% were positive in the low-prevalence areas. In a hyper endemic region in Africa, PCR was positive in <57%.[13 34 35 15 36–38] In the present trial, where the prevalence of clinical follicular trachoma was 30%, only 5% were positive using commercial kits, suggesting the need for a comprehensive review of the predictive value of the commercial NAATs.

It was reported that the persistent *Chlamydia psittaci* infection may have contributed to the development of lymphomas, as was supported by the clinical responses observed with antibiotic treatment[39]; in patients with ocular adnexal lymphoma (higher prevalence of *Chlamydia psittaci* infection in both tumour and peripheral blood mononuclear cells), this new broad-spectrum assay should be a complementary tool for routine diagnosis. In addition, *Chlamydia pneumoniae* was associated with chronic forms of follicular conjunctivitis, ophthalmia neonatorum and with human

Table 2 Comparison of the detection capacities of the broad-range real-time PCR for *Chlamydia* with two commercial kits on dilutions of semiquantified culture supernatants of *Chlamydia psittaci and Chlamydia pneumoniae*

Results (three different assays)					
				Broad-range assay	
CFU (equivalent)/200 µl		**Amplicor***	**Artus/***	**Result**	**Ct †¶**
Chlamydia psittaci ‡	1000	NNN	NNN	+++	23
	100	NNN	NNN	+++	26
	10	NNN	NNN	+++	28.7
	1	NNN	NNN	+++	31.8
	0.1	NNN	NNN	+++	34.9
	0.01	NNN	NNN	NNN	>45
C. pneumoniae‡	1000	NNN	NNN	+++	26
	100	NNN	NNN	+++	28.8
	10	NNN	NNN	+++	31.7
	1	NNN	NNN	+++	34.9
	0.1	NNN	NNN	+++	37.8
	0.01	NNN	NNN	NNN	N>45

N, negative; +, positive.
*According to manufacturer's instructions.
†The detection limits using the ABI PRISM 7000 and the SmartCyclerII were identical. Samples testing positive by real-time PCR were analysed by the multicomponent algorithm. The results express the pure dye component obtained with ABI PRISM 7000.
‡Flasks (25 cm^2) with a monolayer of receptive cells were infected with purified strains of *Chlamydia psittaci* or *Chlamydia pneumoniae*. After 3 days, the supernatants were divided into two aliquots; the first was centrifuged (3000 *g*, 30 min, 4°C) and frozen (−80°C), and the second was serially diluted to assess the concentration of *Chlamydia* (number of CFU/ml). For DNA extraction, the frozen supernatants were thawed, resuspended and diluted to obtain the equivalent of 0.5×10^4 CFU/ml phosphate-buffered saline. Cts were determined according to the signals exceeding the background of 0.20: baseline starts at 6 and ends at 15.
¶The software for the analysis of the second derivative (SmartCycler) confirmed the positive results, and the variations of the slopes during the amplification processes for each dilution of each of the species were significant. The dispersion of the Ct values (intra-assays) were <1.2 cycles.

Table 3 Comparison of the detection capacities of the broad-range real-time PCR for *Chlamydia* with two commercial kits on 100 clinical samples obtained from children with trachoma

Technique	Amplicor*	Artus*	Broad-range assay
Total tested	100	100	100
Negative samples	95	95	89
Positive samples	5†	5†	11†
	+	+ (20)	+ (23)
	+	+ (28)	+ (24)
	+	+ (32)	+ (33)
(Ct) values for the	+	+ (36)	+ (35)
samples testing	+	+ (29)	+ (31)
Positive by real-time PCR	N	N (>40)‡	+ (33)‡ #
	N	N (>40)‡	+ (37)‡¶
	N	N (>40)‡	+ (33)‡¶
	N	N (>40)‡	+ (30)‡¶
	N	N (>40)‡	+ (32)‡¶
	N	N (>40)‡	+ (31)‡¶

N: negative; +: positive.
*According to manufacturer's instructions.
†The five samples detected as positive by Amplicor were detected positive by the other two methods. Reaction volumes were 50, 20 and 50 µl, respectively.
‡The signals obtained for the internal controls (plasmid supplied in the Artus Abbott kit and amplified simultaneously in the same reaction tube or seal herpes virus for the broad-range assay amplified separately) showed no inhibition. The yield of recovery of DNA after elution was acceptable (Ct values of the controls for each sample compared with the Ct obtained with the blanks and controls were never delayed by more than 1.5 cycles).
¶The second derivative of the plots indicates that the peaks are relevant (positive).

choroidal neovascular membranes,[19 16 17] suggesting that by inducing chronic inflammation and pro-angiogenic cytokines it may contribute to the pathogenesis of age-related macular degeneration[20 40] (chlamydial genes coding for cytokines, growth factors and signalling pro-inflammatory molecules are upregulated as early as 2 h post-infection).[10 41]

The load of *Chlamydia pneumoniae* is also higher in people with aortic stenosis in the calcified and fibrotic regions of the aortic valve,[21] and this infection was associated with acute myocardial infarction in young men in the US military. Nevertheless, its direct involvement is under discussion as clinical trials carried out with macrolides do not seem to alter the risk of cardiac events,[42] and no benefits for survival in patients with peripheral arterial disease have yet been reported.[43–46]

Presently, the NAATs carried out to detect *Chlamydia psittaci* show poor performance, and the sensitivity of this method could have been enhanced by adding amplification steps (''nested PCR'') or by hybridisation of the amplicons with specific probes, but this technology, useful for research purposes, is impractical in a clinical routine microbiology setting.[16 30] Regarding the broad-range NAATs for *Chlamydia* species, one PCR assay was reported, but the amplified products were electrophoresed through 2% agarose and the amplicons hybridised (which is reagent-consuming and time-consuming) before the results could be assessed as positive or negative by ultraviolet transillumination.[25] Because *Chlamydiae* are not isolated in any of the media used by microbiology laboratories, the NAATs are becoming the reference methods.[26 4 47 29] However, we should warn of the risk of misdiagnosis (false negative) when drawing conclusions from results obtained with mono-specific assays. A broad-spectrum biological assay should be of great help in understanding the pathophysiology and the association of clinical signs with *Chlamydia* (*trachomatis* and others), which may guide the choice towards agents with appropriate activity against intracellular bacteria.

In conclusion, this study shows the results of the first broad-range real-time PCR assay, targeting sequences of a conserved region of a bacterial multi copy gene coding for the 16S rRNA shared by most *Chlamydia* species. This assay is reproducible and results can be obtained within 3 h at considerably lower cost than of those on the market. If quality control panels of quantified *Chlamydia* are available, the broad-range real-time PCR can be easily adapted to different equipments and can be run as a routine test.

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