Genotyping Ocular Strains of *Chlamydia trachomatis* by Single-tube Nested PCR

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A single-tube nested PCR able to discriminate between ocular infection with *Chlamydia trachomatis* of serovar A, B, or C is described. The method uses genotype-specific primers labeled with different fluorochromes, "drop-in/drop-out" PCR amplification, and product analysis on an Applied Biosystems 373A DNA Sequencer using GENESCAN 672 software. The system readily detected mixed infection with serotypes A and B within the same eye. This strategy provides a rapid genotyping method applicable to a wide variety of epidemiological studies.

Chlamydia trachomatis is an obligate intracellular bacterium of major clinical significance. Worldwide, *C. trachomatis* is a frequent cause of sexually transmitted disease and has important sequelae, for example, infertility and extrauterine pregnancy. *C. trachomatis* serotypes A, B, and C are the etiological agents of trachoma, one of the major causes of preventable blindness in the world. As chlamydiae are difficult to grow in cell culture, PCR-based detection has obvious advantages. Antibodies to the surface-exposed loops on the major outer membrane protein (MOMP), encoded by variable segments (VS) I, II, and IV of the MOMP gene, are protective. Details of the genotype and variant distribution are crucial for chlamydial vaccine design and epidemiological studies.

Genotyping of *C. trachomatis* has been achieved by restriction endonuclease cleavage of PCR-amplified MOMP DNA,⁽¹⁻³⁾ or nested PCR based on type-specific regions of the MOMP gene.⁽⁴⁾ This nested PCR requires tube opening to decrease the concentration of the outer primers and to add the inner (nested) primers. The procedure is inconvenient and significantly increases the risk of cross-contaminating the sec-

ondary reactions with amplified products from the first reaction.

An attractive alternative is to mix the outer and inner primers together in a single reaction. A thermal profile is chosen that initially allows only the outer primers to take part in amplification; subsequently, the inner primers amplify the nested fragment. This technique has been termed "drop-in/drop-out" nested PCR.⁽⁵⁾ The original use was to amplify a single human immunodeficiency virus (HIV) template in the background of genomic DNA from 70,000 cells.

We have developed a single-tube "drop-in/drop-out" nested PCR able to discriminate between ocular infection with *C. trachomatis* of serotype A, B, or C. Genotype-specific PCR primers are labeled with different fluorochromes, and the reaction products are analyzed by denaturing polyacrylamide gel electrophoresis on an Applied Biosystems 373A DNA Sequencer using GENESCAN 672 software.

MATERIALS AND METHODS

Sample Collection and Preparation

The study was based in the villages of Jali and Berending, The Gambia, West Af-

TABLE 1 Primers Used for Drop-in/Drop-out Chlamydial Genotyping PCR

	Melting temperature (°C) ⁽⁸⁾	Sequence (5' → 3')
Outer primers		
FLS	59	CTCTTGAAATCGGTATTAGTATTTGCCGCT
FLA	61	TTAGAAICGGAATTGTGCATTTACGTGAGC
Nested primers		
ABC-1	46	ACA / TGATGTGAATAAAGAA
A-1/FAM	46	FAM - CAGTGTTAGGAACAATA
B-1/JOE	48	JOE - CTTTAGTCTGGTTCTCA
C-1/TAMRA	48	TAMRA - CAGTGTTAGGAATAAGC

FIGURE 1 Schematic of primer selection based on the MOMP gene of *C. trachomatis*. Outer primers FLS and FLA were chosen from a region conserved sequence in *C. trachomatis* serotypes. For the nested primers, ABC-1 was selected from the conserved sequence downstream from FLS, and the genotyping primers A-1, B-1, and C-1 were selected from variable segment II. These genotype-specific primers were labeled with fluorescent dyes (FAM, JOE, or TAMRA). All six primers were used in each drop-in/drop-out PCR.

rica. A total of 1364 conjunctival swabs were obtained in the villages by Dr. R. Bailey and colleagues from both clinically negative individuals and trachoma cases. Swabs were taken from the upper subtarsal conjunctiva and immediately expressed into 1 ml of chlamydial antigen detection transport medium (IDEIA, Novobiolabs, Cambridge, UK). All cells and bacteria suspended in the transport medium were pelleted by centrifugation (13,000 rpm, 30 min) and then incubated with 2 μ g of proteinase K in 50 μ l of 0.5% Tween 20/0.5% NP-40 at 60°C for 60 min. Proteinase K was inactivated by heating the samples to 100°C for 10 min.⁽⁶⁾

The *C. trachomatis* strains A/SA-1/OT, B/Jali-20/OT, and C/UW-1/OT were used as positive controls for genotyping. Elementary bodies (EBs), prepared by the method of Salari and Ward,⁽⁷⁾ were tested at concentrations of 10^3 – 10^5 /ml.

Oligonucleotide Design and Synthesis

Six oligonucleotides were selected based on the nucleotide sequence of the MOMP gene (Table 1). The outer primers, FLS and FLA, were chosen from a region of sequence conserved in all *C. trachomatis* serotypes. For nested primers, ABC-1 was selected from the conserved sequence downstream of FLS and the three genotyping primers A-1, B-1, and C-1 from variable segment II (Fig. 1). The outer primers, 30 bp in length, were predicted to exhibit higher melting temper-

FIGURE 2 Genotype PCR specificity. All combinations of *C. trachomatis* serotypes A, B, and C were tested in single-tube PCR. Genotypes A+B or B+C were readily distinguished by size difference in the PCR product. The electrophoretograms from single-tube A+C or A+B+C reactions are presented as individual tracks to separate genotypes A and C, which are distinguished by fluorochrome label, not size [Note that in this presentation the red size marker tracks are removed.] In all tests the genotyping primers A-1/FAM, B-1/JOE, and C-1/TAMRA showed complete specificity for DNA from the corresponding serotype.

atures than shorter, inner primers (Table 1). All primers were designed to avoid both regions of secondary structure and primer-dimer formation at their 3' ends. Oligonucleotide synthesis was performed on an Applied Biosystems 381A DNA Synthesizer. The nested primers A-1, B-1, and C-1 were derivatized with a free primary amino group at their 5' end using AminoLink 2 phosphoramidite (Applied Biosystems).

Fluorescent Labeling of Oligonucleotides

Genotype-specific primers were labeled with fluorescent dyes: primer A-1 with FAM-NHS ester, B-1 with JOE-NHS ester, and C-1 with TAMRA-NHS ester, according to the manufacturer's instructions (Applied Biosystems). The fluorescently labeled primers were purified by gel filtration followed by reverse-phase HPLC. The purified oligonucleotide primers were stored at -20°C in the dark.

PCR Amplification Reaction

PCR amplification reactions were performed in a total volume of 50 μl . Each reaction contained 2 μl of extracted DNA from conjunctival swabs or 2 μl of control DNA from *C. trachomatis* serotype A, B, or C; the outer primers FLS and FLA (0.5 pmole each); inner primers ABC-1, A-1/FAM, B-1/JOE, C-1/TAMRA (10 pmoles each); dATP, dCTP, dGTP, dTTP (10 nmoles each); KCl (2.5 μmoles); Tris-HCl (pH 8.3) (0.5 μmole); MgCl_2 (75 nmoles), and *Taq* DNA polymerase (1 unit; Promega, Southampton, UK). AmpliWax beads (Perkin-Elmer Cetus) were used to facilitate the "hot start" technique.⁽⁵⁾ Following the hot start, samples were subjected to 15 cycles each, consisting of 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min. This was followed by 2 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 1.5 min, then 28 cycles each consisting of 85°C for 1 min, 48°C for 1 min, and 72°C for 1 min and 30 sec followed by an additional 7 min of extension at 72°C . The samples were stored at 4°C following PCR, prior to analysis.

RESULTS AND DISCUSSION

During the development of this PCR system for genotyping ocular strains of *C. trachomatis*, the temperature conditions

and number of cycles for each step were systematically modified to obtain optimal sensitivity and selectivity. The electrophoretogram (Fig. 2) demonstrates that the developed method accurately identified any combination of serotypes A, B, and C within a single PCR. The direct application of genotyping PCR to conjunctival swabs from trachoma patients is shown in Figure 3. In this gel picture, generated by GENESCAN software, the internal size standards appear red and the genotype-specific amplification products from serotypes A, B, and C are blue, green, and yellow, respectively. The blue and yellow fragments are generated by primers to the same segment of the MOMP gene and exhibit identical sizes (314 bp). The green fragments specific for serotype B represent amplifica-

tion products of 278 bp. In patients with mixed A and B ocular chlamydial infection, both fragments are present in the same track; this is most easily seen in the electrophoretogram (Fig. 4).

Of the 1363 villagers screened for *C. trachomatis* ocular infection, genotype A was detected in 136 samples, genotype B in 31, and 12 had mixed A and B infections. Genotype C was not observed. To confirm the specificity of PCR genotyping, we sequenced both strands of variable segments I, II, and IV of PCR-amplified MOMP gene from all positive clinical samples. There was complete concordance between A and B genotyping and the DNA sequence analysis data. The sequence results also confirmed the cases of mixed A plus B infections. At sites where the A and B sequences differ

FIGURE 3 Six percent polyacrylamide-urea gel of fluorescently labeled PCR products. The GENESCAN-2500P internal standards appear as red bands (size in bp at left) in all tracks. The positive controls for serotype A (blue, 314 bp), serotype B (green, 278 bp), and serotype C (yellow, 314 bp) were loaded in the three tracks at the right (labeled A, B, and C). The remaining tracks were loaded with PCR products derived from clinical samples and demonstrate either no detectable infection with *C. trachomatis*, infection with serotype A, infection with serotype B, or mixed infection with serotypes A and B (the latter marked by ●).

FIGURE 4 Example electrophoretogram derived from *C. trachomatis* serotype A and B mixed infection. The electrophoretogram, produced by the GENESCAN 672 software, represents a single track of the gel image (Fig. 3). The horizontal axis denotes fragment size, and the vertical axis denotes band intensity. Internal standards are shown in red, serotype A-specific bands in blue, and serotype B-specific bands in green. The accompanying table gives information on calculated fragment sizes and areas. The presence of both a green (278 bp) peak and a blue (314 bp) peak indicates the mixed infection.

FIGURE 5 DNA sequences and chromatograms of variable segment I derived from PCR-amplified MOMP gene from two conjunctival swabs. The graphic representation was generated by ABI 373 Analysis Software (v. 1.2.0), with each base represented by a different color. (a) An example of *C. trachomatis* serotype A infection; (b) mixed serotype infection. The latter is demonstrated by the inability to accurately base-call at certain positions (N) owing to chromatogram ambiguities.

(N in Fig. 5b), the DNA analysis software detects two bases.

To assess the potential value of this nested genotype PCR for clinical and epidemiological studies it was necessary to establish the sensitivity of the test. We analyzed all 1363 clinical samples by PCR for *C. trachomatis* plasmid genes.⁽⁹⁾ Ostergaard et al.⁽⁹⁾ established that this plasmid PCR detected 10^{-17} g of chlamydial DNA and showed 100% sensitivity and 99% specificity when compared with cell culture plus a commercial EIA kit (IDEA). In all of our ocular samples where plasmid DNA was detected by PCR, the nested genotyping PCR gave a positive result indicating comparable levels of sensitivity.

We developed this drop-in/drop-out nested PCR for studies on trachoma in The Gambia. Advantages include the use of a transport medium that permits other tests, for example, antigen detection, a simple nontoxic DNA extraction procedure, and the feasibility of handling large numbers of clinical samples. The ability to accommodate >40 samples on a single GENESCAN run and to achieve two runs in a 24-hr period means that analysis of PCR products is not the rate-limiting step.

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