

Short Communication

Restriction endonuclease patterns of the *omp1* gene of reference *Chlamydia trachomatis* strains and characterization of isolates from Cameroonian students

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Eighteen reference strains of *Chlamydia trachomatis* were differentiated by *omp1* PCR- and nested PCR-based RFLP analysis, using two restriction digestions, one with *AluI* and the other with the three enzymes *HpaII*, *EcoRI* and *HinfI*. *AluI* digestion allowed the differentiation of 12 different profiles after CT1/CT5 PCR and 13 different profiles after the nested PCR. The triple hydrolysis permitted the identification of 15 different patterns. In all, 16/18 reference strains were clearly identified. These reference patterns were successfully used to genotype 34 of 35 (28 strains and 7 clinical specimens) samples from infected students, collected during a screening programme in Yaounde (Cameroon). Genotypes D, Da, E, F, G and J were found. The most prevalent *omp1* genotype was E ($n = 14$; 40%), followed by F ($n = 7$; 20%). As RFLP patterns of reference strains are essential for typing clinical isolates, they will greatly facilitate *C. trachomatis* characterization in many resource-limited laboratories.

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Introduction

Chlamydia trachomatis is the causative agent of a variety of diseases and syndromes, including trachoma, urogenital infections and lymphogranuloma venereum, depending on the biovar and serovar of the strains involved. Currently, 19 human serovars and numerous variants have been identified with the use of polyclonal and monoclonal antibodies against the major outer-membrane protein (MOMP). Serovars A–C are most often associated with trachoma and serovars L1–L3 are associated with lymphogranuloma venereum, while serovars D–K are primarily associated with urogenital and neonatal infections (Morré *et al.*, 1998a; Ossewaarde *et al.*, 1994; Sturm-Ramirez *et al.*, 2000). Various studies have shown the feasibility of typing clinical isolates by PCR-based RFLP analysis of the amplified *omp1* gene encoding MOMP (Frost *et al.*, 1991; Rodriguez *et al.*, 1991; Sayada *et al.*, 1991), avoiding the costly production of monoclonal antibodies. This method was originally performed on cell cultures of cervical and urethral specimens. Nowadays, the direct typing

of cervical specimens by a nested *omp1* PCR-RFLP assay is used for the differentiation of isolates into genotypes (Lan *et al.*, 1993, 1994). Nevertheless, typing of *C. trachomatis* is not routine in many African laboratories. Up to now, only one study showing the genotypic diversity of genital *C. trachomatis* in sub-Saharan Africa has been performed (Sturm-Ramirez *et al.*, 2000). Although RFLP analysis of an unknown strain consists of comparing the patterns with those of reference strains, patterns of all reference strains have not yet been published. Therefore, the aim of this study was to present RFLP patterns of 18/19 *omp1*-amplified reference strains, in order to promote typing of *C. trachomatis* by RFLP analysis in many laboratories with limited resources. In addition, this study was intended to describe the distribution of *C. trachomatis* genotypes in urogenital specimens, collected from Cameroonian students residing in Yaounde.

Methods

Bacterial strains. Eighteen *C. trachomatis* reference strains were used: A/Har-13, B/Har-36, Ba/Apache-2, C/TW-3, D/UW-3/Cx, Da/TW-448H-11, E/VR-348B, F/IC-Cal-3, G/UW-57/Cx, H/UW-43/cx, I/UW-

Abbreviation: MOMP, major outer-membrane protein.

12/Ur, Ia/UW-202H14, J/UW-36/Cx, K/UW-31/Cx, L1/440/901B, L2/434/902B, L2a/UW-396 and L3/404/903. Clinical strains were collected after screening 1267 students in Yaounde between May and July 2001 (Ngandjio *et al.*, 2003).

omp1 amplification. Eighteen reference and 28 student strains propagated in McCoy cells were pre-treated with proteinase K (200 µg ml⁻¹) in lysis buffer containing 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 4.5 mM MgCl₂, 0.45 % NP-40 (w/v) and 0.45 % Tween 20 in 2 ml microtubes. Tubes were incubated at 56 °C for 90 min and then at 95 °C for 15 min to inactivate the proteinase K. The one-step PCR was performed in a final volume of 50 µl, with primers CT1 and CT5 (Table 1). The program was 95 °C for 5 min, 35 cycles of 1 min at 95 °C, 1 min at 57 °C and 2 min at 72 °C and one last cycle of 72 °C for 10 min.

The nested PCR was performed on the 18 reference strains and on seven Cobas Amplicor PCR-positive clinical specimens kept at -80 °C in 2-sucrose phosphate (2SP) medium. The 2SP samples were pre-treated with the lysis reagent used for the PCR Cobas Amplicor test, according to the manufacturer's instructions (Roche Diagnostics). Briefly, 50 µl 2SP specimen was added to 50 µl lysis buffer and incubated for 10 min at room temperature, and then diluted in 100 µl specimen diluent and heated at 95 °C for 10 min. The primers used were NLO and NRO in the first step and then PCTM3 and SERO2A (Table 1) in the second PCR, performed with 1 µl of the amplified product from the first step. The program of the nested PCR was 5 min at 95 °C, 45 cycles of 1 min at 95 °C, 90 s at 45 °C and 150 s at 72 °C and one cycle of 10 min at 72 °C. Amplified products were analysed by electrophoresis on a 2 % (w/v) agarose gel and visualized by ethidium bromide staining under UV fluorescence.

RFLP assay. RFLP analysis of PCR and nested PCR products was carried out as described previously (Rodriguez *et al.*, 1991). Briefly, 17 µl amplified DNA product was digested separately overnight, in one reaction with 10 U *AluI* (Promega) and in a second reaction with 10 U of each of the three enzymes *HpaII*, *EcoRI* and *HinI* (Promega).

Analysis of digested DNA was performed by electrophoresis of the total reaction on an 8 % polyacrylamide gel with ethidium bromide staining; patterns were compared visually.

Results and Discussion

RFLP patterns of the reference strains after the one-step PCR and nested PCR are shown in Figs 1 and 2. The patterns of each strain were not exactly the same after one-step and nested amplification, due to differences in the length of the amplified fragments (about 1200 bp in the one-step and 1000 bp in the nested PCR). Thus, we decided to present two kinds of RFLP patterns, based on the one-step amplification

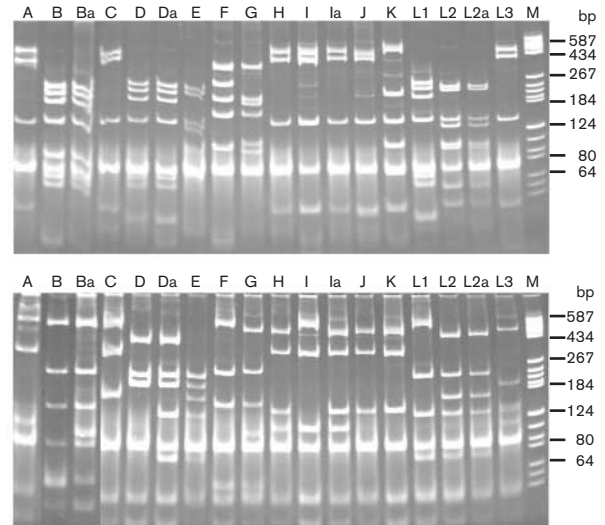


Fig. 1. Reference patterns from CT1/CT5 PCR-based RFLP genotyping of 18 *C. trachomatis* reference strains cleaved with the endonuclease *AluI* (top) and with *HpaII*+*EcoRI*+*HinI* (bottom). Lanes M, size markers (pBR322 DNA digested with *HaeIII*).

performed on strains or the nested PCR performed on clinical specimens. *AluI* digestion allowed the differentiation of 12 different profiles after the CT1/CT5 PCR and 13 different profiles after the nested PCR. The triple hydrolysis permitted the identification of 15 different patterns, after either CT1/CT5 or nested PCR. In all, 16/18 reference strains were clearly identified by the two methods. Genotypes L2 and L2a could not be separated. Better identification of strains of these two genotypes can be obtained after *NlaIII* digestion (Rodriguez *et al.*, 1993). The differences between genotypes H and Ia were minor. Successful identification of these two genotypes requires an additional digestion with the enzyme *DdeI* (Rodriguez *et al.*, 1993). Genotype I should be included in the analysis because of the identity of its pattern with that of Ia in the *DdeI* digestion. Genotype Ga was not included in our study. However, genotypes G and Ga can be identified after *BstUI* digestion (Morré *et al.*, 1998a). The genotypes involved in the three main diseases caused by *C. trachomatis* (A, B, Ba and C in trachoma, D, Da, E, F, G, H, I, Ia, J and K in urogenital tract infection and L1, L2/L2a and L3 in lympho-

Table 1. Primers used for one-step and nested PCR of *omp1*

Primer	Strand	Sequence (5'–3')	Position*	Reference
CT1	Sense	GCCGCTTTGAGTTCGTTCCTC	34–56	Dutilh <i>et al.</i> (1989)
CT5	Antisense	ATTTACGTGAGCAGCTCTCAT	1142–1164	Rodriguez <i>et al.</i> (1991)
NLO	Sense	ATGAAAAAACTCTTGAAATCG	1–21	Lan <i>et al.</i> (1994)
NRO	Antisense	CTCAACTGTAAGTGCATTTT	1108–1128	Lan <i>et al.</i> (1994)
PCTM3	Sense	TCCTTGCAAGCTCTGCCTGTGGGAATCCT	58–84	Lan <i>et al.</i> (1993)
SERO2A	Antisense	TTTCTAGA(T/C)TTCAT(T/C)TTGTT	1045–1064	Frost <i>et al.</i> (1991)

*According to the *omp1* nucleotide sequence of *C. trachomatis* serovar strain D/UW-3/Cx (Stephens *et al.*, 1998).

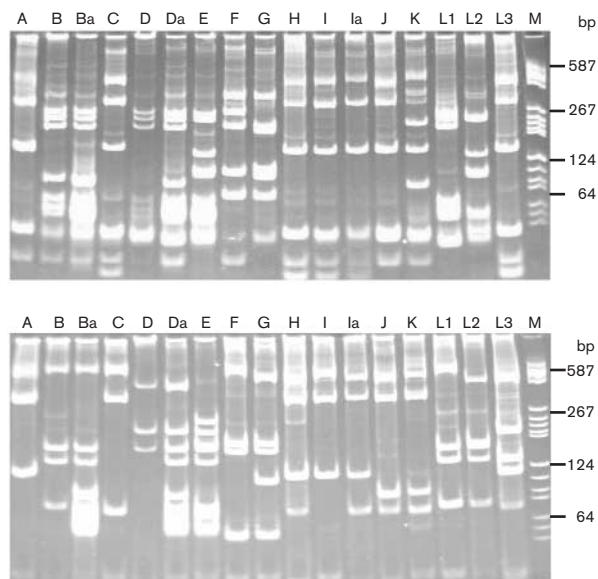


Fig. 2. Reference patterns from PCTM3/SERO2A PCR-based RFLP genotyping of 17 *C. trachomatis* reference strains (genotypes L2 and L2a had similar profiles) cleaved with *AluI* (top) and with *HpaII*+*EcoRI*+*HinfI* (bottom). Lanes M, size markers (pBR322 DNA digested with *HaeIII*).

granuloma venereum) were clearly identified. In addition, the genotypes corresponding to the three main groups based on genetic relatedness and serological reactivity (Yuan *et al.*, 1989) were clearly differentiated (genotypes B, Ba, D, Da, E, L1 and L2 in the B complex, genotypes F and G in the intermediate group F/G and genotypes C, A, H, I, Ia, J, K and L3 in the C complex).

The accessibility of reference patterns facilitated the typing of strains circulating among Cameroonian students. The prevalence of urogenital chlamydial infection in the screening study was 3.78% (48/1267). Thirty-five Cameroonian positive specimens were used in this typing study, of which 28 strains were isolated on McCoy cells and typed by PCR-based RFLP analysis. The remaining seven specimens were subjected to direct *omp1* amplification by nested PCR and typed. The isolation of strains in cell culture is expensive and not easy to perform, but allows good amplification of *omp1*. Direct amplification of *omp1* from specimens by nested PCR is not always successful. In our study, of the 20 non-cultured specimens, only seven could be typed after nested-PCR *omp1* amplification. In all, of the 35 strains from students from which the *omp1* gene was successfully amplified, RFLP analysis allowed genotyping of 34 (97.1%). The genotypes found were E ($n = 14$; 40%), F ($n = 7$; 20%), G ($n = 5$; 14.3%), D ($n = 3$; 8.6%), Da ($n = 3$; 8.6%) and J ($n = 2$; 5.7%). One strain could not be identified. Sequence analysis found it to be a genotype J strain, with three nucleotide substitutions, in one case involving an *AluI* restriction site. The *omp1* sequence of our triple-mutant J strain was not identical to those of variants Ja and Jv, respectively described

by Dean *et al.* (2000) and Morr e *et al.* (1998a). The patterns were very reproducible and comparison between clinical and reference patterns was easy. The same size markers have to be used for good comparison with reference patterns. PCR-based RFLP analysis has been shown in many studies, including ours, to be a simple, sensitive, inexpensive and reproducible method for typing of urogenital *C. trachomatis* in clinical specimens (Ikehata *et al.*, 2000; Morr e *et al.*, 1998a).

This study was the first to characterize Cameroonian *C. trachomatis* isolates. RFLP analysis found a heterogeneous population of *C. trachomatis* in this student population. These results are in agreement with those reported in other areas (Brunham *et al.*, 1996; Sturm-Ramirez *et al.*, 2000; van de Laar *et al.*, 1996; van Duynhoven *et al.*, 1998). The predominance of class B genotypes (57%) in this study and in many others may suggest a real biological advantage over other genotypes (Sturm-Ramirez *et al.*, 2000). The distribution of *C. trachomatis* genotypes was comparable in males and females, with genotype E (respectively 43.8 and 36.8%) predominating. The most frequent types, E and F, have been described previously (Jurstrand *et al.*, 2001; Morr e *et al.*, 1998b; Poole & Lamont, 1992). Genotypes D/Da, F and G were also common in both sexes. The observed distribution of genotypes has also been described in many studies (Batteiger *et al.*, 1989; Morr e *et al.*, 2000; van de Laar *et al.*, 1996; van Duynhoven *et al.*, 1998).

The interest of our study was to obtain reference RFLP patterns after amplification of the *C. trachomatis omp1* gene either from cell culture isolates or directly from clinical specimens. With the availability of these reference patterns, PCR-based RFLP typing could be used in laboratories with limited resources to characterize *C. trachomatis* circulating in such areas, since ordering reference strains is expensive and performing reference patterns is time-consuming. It is up to each laboratory to choose the best method, depending on its resources.

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