Quinolones and Macrolides Resistance-Associated Mutations in *Chlamydia trachomatis* in Women Endocervical Samples in the West Region of Cameroon

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ABSTRACT

Chlamydia trachomatis infection is a public health problem worldwide. Although antibiotic resistance of this strict intracellular bacterium is rare, it is important to monitor the appearance of resistance genes to available efficient antibiotics. This study aimed to screen for mutations in some of these genes in *C. trachomatis* clinical isolates, which may be associated to resistance to quinolone and macrolide antibiotics. Thirty-five endocervical samples were collected from women aged between 18 and 49 in five district hospitals in the Western Region of Cameroon. The mutations in quinolones (*parC* and *gyrA*) and macrolides (L4, L22 and 23S rRNA) resistance domains were detected by PCR followed by sequencing on positive samples to *C. trachomatis*. The overall mutation rate for the studied genes was 60% in the studied samples. Seven (20%) and twelve (34%) samples presented mutations in the *parC* and *gyrA* gene respectively. Mutations in L4 (11.42%) and L22 (60%) were detected in ours samples, while no mutation was found in 23S rRNA gene. Seven clinical samples (20%) presented mutations to both macrolide and quinolone resistance genes. This study revealed a relatively high rate of mutations in the resistance genes to macrolides and quinolones in *C. trachomatis* in the West Cameroon. This rate of mutation calls for the competent authorities for better surveillance of *C. trachomatis* infection in West Cameroon to avoid a sudden increase in resistance to antibiotics in the years to come.

Keywords: *Chlamydia trachomatis*, genes, mutations, resistance, macrolide, quinolone.

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I. INTRODUCTION

Chlamydia trachomatis is the most common sexually transmitted disease and causative agent of a variety of disease situations including ocular trachoma leading to blindness; oculo-genital diseases causing infertility, pelvic inflammatory disease; reactive arthritis in adults; lymphogranuloma venereum and perinatal infections [1]. *C. trachomatis* infection affects 92 million people around the world each year [2]. This infection and their sequelae are major public health problems, resulting in high morbidity and economic losses linked to low worker performance and

treatment costs [3]. These costs increase significantly when the disease is not diagnosed and treated on time, leading to complications [4]. The first line antibiotic used for the treatment of *C. trachomatis* infection include doxycycline, erythromycin, azithromycin, ciprofloxacin, and ofloxacin [5], [6]. In women, bacteriological cure does not confirm anatomical and functional cure. Indeed, irreversible tubal or pelvic lesions may have formed and persist after eradication of microbial agents.

Chlamydia trachomatis, which exhibits natural resistance to antibiotics active on the cell wall such as β -lactams or glycopeptides, is sensitive to only a few antibiotics which

target the bacterial cell wall. On the other hand, this strict intracellular bacterium is more sensitive to antibiotics which have good cellular penetration, which explains why macrolides (erythromycin, azithromycin), tetracyclines (doxycycline) and quinolones (ciprofloxacin, ofloxacin) are generally used for the treatment depending on clinical situations [7], [8]. Some cases of azithromycin treatment failure for *C. trachomatis* infections have been noted and are explained by high MICs and by a positive correlation between MICs and *C. trachomatis* loads [9].

The principal targets of the quinolones are DNA gyrase and topoisomerase IV [10]. Quinolones act on bacteria by inhibiting these two enzymes which have two different subunits, GyrA/GyrB and ParC/ParE. Quinolone resistance most commonly occurs after point mutations in quinolone resistance determining regions (QRDRs) of subunit genes [11]. The resistance of various microorganisms to macrolides is often associated with mutations in ribosomal protein genes, particularly in L4 and L22, as well as with mutations in the peptidyl transferase region of the 23S rRNA gene [12]. At present, antimicrobial resistance is a barrier to the effective treatment of a growing range of infections and requires actions, especially as we face the emergence of new resistance mechanisms that are rapidly spreading around the world [13]. It is necessary to update the recommendations on antibiotic treatment due to the development of resistance to the molecules generally used. To achieve this objective, the response of a germ or a group of germs to an antibiotic must be known in time and space to adapt the therapies to updated data. This is all the truer as the epidemiological data are constantly changing. In a recent study conducted in five hospitals in the West Cameroon region, using PCR detection, we found that the prevalence of *C. trachomatis* infection was 11.5% and the circulating serotypes were found to be mainly D (49%), E (29.4%) and G (21.6%) (Unpublished results). As this prevalence can be considered high, we are wondering whether in the same area, resistant strains of *C. trachomatis* are circulating amount women due to mutations on some genes in the bacteria, which could complicate the management of patients and the epidemiological control of the disease. Although antibiotic resistance in *C. trachomatis* is rare, it is important to stay alert to detect any changes in the sensitivity of this microorganism to prescribed antibiotics. The objective of this study was to detect mutations in certain genes of *C. trachomatis* and its distribution in West Cameroon, which may be associated with the resistance of this microorganism to antibiotics quinolone and macrolide families.

II. METHODOLOGY

A. Participants

This study was conducted on 35 endocervical swab samples positive for *C. trachomatis* PCR amplification of the following genes: parC, gyrA, L4, L22 and 23S rRNA. These samples were collected from women in five district hospitals of the West Cameroon region between May 2018 and November 2019 as follows: Dschang (09), Bafang (03), Mbouda (13), Bafoussam (05) and Bangangté (05) (Fig. 1). The participants involved in this study were 18-49 years old.

They came to the hospital seeking prenatal consultation, prenuptial consultation or contraception. This study was conducted with ethical clearances obtained from the Cameroon National Committee for Ethics in Human Health (2018/05/1022/CE/CNERSH/ SP) and from Post Graduate Institute of Medical Education and Research, Chandigarh (PGI/IEC/2020/001586). An administrative authorization was also obtained from the West Regional Delegation of Public Health and from each hospital. Persons who fulfilled the following criteria were included: be a volunteer, provided a signed informed consent, be more than 18 years, be sexually active, must not have sexual intercourse for at least 48 hours, not menstruating.

Fig. 1. Map representing the West Region of Cameroon and showing the study areas.

B. Procedure

The endocervical swabs samples were obtained from women using a non-lubricated speculum and an abrasive swab of the “bactopick” type by the trained health care workers at the study sites. The swab was then placed into a storage tube containing 1 ml of sterile saline (0.9% NaCl) and stored at -20 °C until further processing [14].

The obtained endocervical samples were submitted to DNA extraction, using the QIAamp mini kit according to the manufacturer’s instructions [15]. Briefly, the tubes containing the endocervical sample were removed from the freezer, left at room temperature for one hour and centrifuged at 15,000 rpm for 10 minutes. The supernatant was removed while preserving the pellet, ATL buffer and Proteinase K were added to it and it was homogenized with the help of a vortex mixer (iwx-VT Neutrition), and then incubated at 56 °C for 3 hours while homogenizing during incubation. Once the incubation was complete, 200 µL of AL buffer was added and incubated at 70 °C for 10 minutes. Then 200 µL

absolute ethanol was added and homogenized. The mixture was then transferred into minicolumns and centrifuged at 8,000 rpm for 1 minute. After extensive washing with 500 µL of buffer AW1 and then with 500 µL of AW2, 50µL of buffer AE was added, left at room temperature for 5 minutes. The tubes were centrifuged, and the extracted DNA was stored at -20 °C until further analysis. Nucleic acids concentration was estimated after the extraction by measuring the absorbance at 260 nm using a Nanodrop spectrophotometer (Thermo Scientific NanoDrop 2000 Spectrophotometer). Then, the ratio of spectrophotometric absorbance of extracted sample at 260 nm to that of 280 nm was used to determine the purity of DNA. A260/A280 ratio ≥ 1.8 indicates pure DNA extraction [16].

The extracted DNA was amplified using Cryptic plasmic primer KL1-KL2 (KL1: 5'-TCCGGAGCGAGTTACGAAGA-3'; KL2: 5'-AATCAATGCCCGGGATTGGT-3') [17] and MOMP primer (forward: 5'-CCT GTG GGG AAT CCT GCT GAA-3'; Reverse: 5' GTC GAA AAC AAA GTC ACC ATA GTA 3') [18]. The amplification reaction was performed in a final volume of 25 µl containing 1X PCR buffer (10 mMTris-HCl; 50 mM KCl; 1.5 mM MgCl₂), 0.5 µM of each pair of primer, 200 µM of dNTPs; 1U of Taq DNA polymerase, 5 µl of DNA. The PCR conditions consisted of initial denaturation step at 95 °C for 10 minutes; 50 cycles of 95 °C for 30 seconds, 60 °C for 1 minute, and 72 °C for 1 minute; and a final extension at 72 °C for 7 minutes. The amplicons were electrophoresed through an agarose gel 2% and the visualized on a UV transilluminator (Agilent Technologies Sure Cycle 8800).

All PCRs were performed in a final volume of 25 µL containing 1X PCR buffer (10 mMTris-HCl; 50 mM KCl; 1.5 mM MgCl₂), 0.5 µM of each pair of primer (Table 1) [19,20], 200 µM of dNTPs; 1U of Taq DNA polymerase and 5 µl of DNA. The PCR conditions were: 95 °C for 10 minutes; 40 cycles of 95 °C for 1 minute, 56 °C and 60 °C for 23 rRNA for 1 minute, 72 °C for 2 minutes, and a final extension at 72 °C for 7 minutes.

PCR products were sequenced in a BigDye Terminator sequencing kit (Applied Biosystems, USA) on a 3500 Dx sequencing machine available in the Department of Medical Microbiology, PGIMER, Chandigarh. The obtained nucleotide sequences of *parC*, *gyrA*, *L4*, *L22* and *23S rRNA* were compared to the nucleotide sequences in the GenBank nucleotide sequence databases to detect any modification. The following accession numbers were used: AF044267 for the *gyrA* sequences, AF044268 for the *parC* sequences, AE001273 for the ribosomal protein L4 and AE001273 for the ribosomal protein L22 and NR103960 for the 23S rRNA.

TABLE I: PRIMERS USED FOR PCR AMPLIFICATION AND SEQUENCING

Primer target	Sequence 5' to 3'	Size (bp)
CT <i>gyrA</i> F	AAGGACTCTCGTTAGAGTCTC	362
CT <i>gyrA</i> R	CGATRCCTGAGGAGCCATTAC	
CT <i>parC</i> F	GATACAGAAACCCTSGACACAC	201
CT <i>parC</i> R	CATGGAAGGTCATGAGATCCG	
CT L4 protein F	GAAGTTTGAATTGCCTGATGC	250
CT L4 protein R	GGCTTAGGACCGAAAACAATC	
CT L22 protein F	AGTGCAGGATTGATGAGAAA	230
CT L22 protein R	GTTAGATGACTCGTGCGCTTC	
CT 23S rRNA F	AAGTTCGACCTGCACGGTGG	725
CT 23S rRNA R	TCCATTCCGGTCTCTCGTAC	

bp: base pair.

III. RESULTS

Obtained PCR products of *parC*, *gyrA*, *L4*, *L22* and *23S rRNA* genes (Fig. 2) were sequenced to detect mutations possibly associated to quinolone and macrolides resistance in *C. trachomatis*. Mutations were identified in 21(60%) of the 35 analysed samples and varied with the genes in consideration.

Fig. 2. Gel electrophoresis profiles of PCR amplified from A) *parC* gene: positive samples showed a 201 bp band as positive control (lane 20 and 26); negative control lane 28. B) *gyrA* gene: positive samples showed a 362 bp band as the positive controls (lane 20 and 28), negative control (lane 29). C) PCR amplification of L22 protein from *C. trachomatis*-positive patients: lane 1 to 9, 11, 12 (250bp), positive samples; lane 14, positive control; lane 13, negative control; lane 10, DNA ladder (100bp). D) PCR amplification of L4 protein from *C. trachomatis*-positive patients: lane 1 to 4, 6, 9, 10, 13, 15, 16, 17, 18 (230bp), positive samples; lane 19, positive control; lane 14, negative control; lane 20, DNA ladder (100bp).

These mutations associated to quinolones resistance were detected in 7 (20%) samples for *parC* gene and in 12 (34%) for *gyrA* gene. Sequence analysis of *ParC* gene showed that these mutations are located at position 83, resulting in a substitution of an Arginine by Glycine (01 sample in Dschang and 01 sample in Bafoussam) and at position 94 resulting in substitution of Threonine by Alanine (01 in Dschang, 02 in Mbouda and 02 in Bangangté) (Table II). On *gyrA* gene, 06 mutations were identified respectively at position 137 characterised by substitution of Glutamine by Leucine (01 sample in Bafang and 01 sample in Mbouda); at position 148, substitution of Histidine with Arginine (01 Dschang), at position 136, substitution of Threonine with Isoleucine (01 at Mbouda); at position 98, substitution of Leucine with Arginine (01 in Dschang and 03 in Mbouda); at position 167, substitution of Isoleucine with Threonine (01 in Bafang and 01 in Mbouda) and at position 174, substitution of Valine with Alanine (02 in Bafang) (Table II).

No mutation was detected in 23S rRNA in all the samples analysed. Four (11.42%) samples showed mutations in ribosomal protein L4 gene while 21(60%) samples showed

mutations in *L22* gene. The mutation observed in the *L4* gene was at position 129, resulting in replacement of Glutamine by Lysine (01 in Bafoussam, 01 in Dschang and 02 in Bafang) in four clinical samples (Table 2). In the gene *L22*, mutations were observed at position 77, substitution of Valine by Alanine (01 in Bafoussam, 06 in Dschang, 01 in Bafang, 07 in Mbouda and 07 in Bangangté); at position 52, replacement of Arginine by Cysteine (05 in Dschang, 02 in Bafang, 05 in Mbouda and 02 in Bangangté); at position 65, replacement of Glycine by Serine (08 in Dschang, 01 in Bafang, 07 in Mbouda and 02 in Bangangté) (Table II).

Some mutations were observed simultaneously in genes responsible for resistance to quinolones and macrolides. Seven samples (20%) were concerned: 02 with mutations in *parC*, *gyrA* and *L4* genes (01 in Dschang and 01 in Mbouda); 03 mutations with mutations in both *gyrA* and *L22* genes (01 in Dschang, 01 in Bafang and 01 in Mbouda); 01 with mutation in *parC* and *L22* genes (01 in Bafang) and 01 with mutation in *parC* and *L22* genes in Bafang (Table II).

TABLE II: DISTRIBUTION OF MUTATIONS IN *PARC*, *GYRA*, *L4* AND *L22* GENES AND AMINO ACID CHANGES IN *C. TRACHOMATIS* CLINICAL SAMPLE IN DISTRICT HOSPITALS IN WEST CAMEROON

Resistance to quinolones					District hospitals				
Mutation	NC	AC	p	n (%)	b	d	f	m	t
<i>parC</i> mutation	A→G	T to A	94	5 (14.28)	0	1	0	2	2
	A→G	R to G	83	2 (5.71)	1	1	0	0	0
	A→T	Q to L	137	2 (5.71)	0	0	1	1	0
<i>gyrA</i> mutation	C→T	T to I	136	1 (2.86)	0	0	0	1	0
	A→G	H to R	148	1 (2.86)	0	1	0	0	0
	C→T	L to R	98	4 (11.43)	0	1	0	3	0
	T→A	I to T	167	2 (5.71)	0	0	1	1	0
	T→C	V to A	174	2 (5.71)	0	0	2	0	0
Resistance to macrolides									
<i>L4</i> mutation	C→A	Q to K	129	4 (11.42)	1	1	2	0	0
	G→A	G to S	52	14 (40)	0	5	2	5	2
<i>L22</i> mutation	C→T	R to C	65	18 (51.42)	0	8	1	7	2
	T→C	V to A	77	19 (54.29)	1	6	1		7
Multiple Resistance to quinolones and macrolides									
Mutation			n(%)		1	2	3	4	5
<i>gyrA</i> and <i>L22</i> mutation			3 (8.57)		0	1	1	1	0
<i>gyrA</i> , <i>parC</i> and <i>L22</i> mutation			2 (5.71)		0	1	0	1	0
<i>parC</i> and <i>L4</i> mutation			1 (2.85)		0	0	0	0	1
<i>parC</i> and <i>L22</i> mutation			1 (2.85)		0	0	0	0	1

b: Bafoussam; d: Dschang; f: Bafang; m: Mbouda; t: Bangangté.
n(%): number of sample with mutation (percentage of the 35 sample analysed); NC: Nucleotide change; AC: Amino acid change.

VI. DISCUSSION

Quinolone resistance results from mutations in genes encoding DNA gyrase and topoisomerase IV, which are the main targets of these antibiotics [10]. Only 02 mutations (5.71%) were detected on the *parC* gene while 06 were detected on *gyrA* gene (17.64%). These mutation rates are

high when compared to 0.66% and 2.66% respectively for *parC* and *gyrA* obtained for 300 endocervical samples analyzed in Iran [1]. Mutations identified in the quinolone resistance determining regions (QRDR) in this study were as follow: Arg83Gly, Thr94Ala, Ile167Thr, Val174Ala, Leu98Arg, Thr136Ile, Gln137Leu, and His148Arg in *parC* and *gyrA* genes. Mutations identified in *L4* and *L22* genes includes Gly52Ser, Arg65Cys, Val77Ala, and Gln129Lys. The amino acid changes observed in this study were the same as those described by Torabizadeh *et al.*, Takashi *et al.* and Misyurina *et al.* [1], [19], [20]. But in some of our samples, these mutations differ in their positions compared to those described by these authors and could be due to the presence of endemic strains of *C. trachomatis* in the West Cameroon region or Cameroon in general. Quinolone resistance is explained by point mutations in the region determining resistance to these antibiotics and in *C. trachomatis*, this mutation mainly concerns the *gyrA* gene [11] as observed in the present study. Some authors attribute these mutations to prolonged exposure of the microorganism to subinhibitory concentrations of these antibiotics [21]. The level of self-medication in Cameroon is very high and could justify the rates of mutations observed [22].

In accordance with the results of Vica *et al.* no mutation was observed in 23S rRNA gene [23]. However, Misyurina *et al.* described in 2004, samples with mutations in the peptidyl transferase region of the 23S rRNA gene in *C. trachomatis* [20]. Resistance to antibiotics of the macrolide family is mainly due to mutations in ribosomal proteins L4 and L22 [12]. As demonstrated by Misyurina *et al.* the mutations that we observed with macrolides were reside in a nonconserved region of the L22 gene in *C. trachomatis*, which is the case for several other microorganisms. In the present study, although the number of mutations was low, the number of samples containing them was relatively high, 25 samples over 35.

Although the number of mutations on the studied genes were relatively low, their rate in our samples may be considered high. This may be explained by the predominance of probabilistic prescriptions of antibiotics in the hospitals surveyed rather than based on antibiograms. This is due to a lack of adequate infrastructure and / or qualified laboratory personnel. Elsewhere, self-medication and street drugs may have contributed to this situation, the health-care system in the country being relatively weak [22].

Mutations in resistance genes to both quinolones and macrolides were observed in 7 samples in 3 of 5 district hospitals (Bangangté, Mbouda and Bafang). This could therefore represent a probable alert of emerging multidrug resistance to *C. trachomatis* in the west region of Cameroon. In fact, Jyoti *et al.* and Jones *et al.* have described in the USA, *C. trachomatis* isolates resistant to both doxycycline, azithromycin and ofloxacin on the one hand and on the other hand resistant to tetracycline associated with multidrug resistance to doxycycline, minocycline, erythromycin, clindamycin, and sulfamethoxazole respectively [24], [25].

V. CONCLUSION

This work uncovered few mutations in the samples analysed and the rate of these mutations potentially

associated to resistance to quinolones and macrolides was high (60%). Also, some cases of multidrug resistance were identified. These results suggest that special attention should be paid to the surveillance of *C. trachomatis* infection in West Cameroon to avoid the occurrence of multi-resistance to antibiotics in the population.

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