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Fluoroquinolone-resistant *Streptococcus agalactiae* isolates from Argentina

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ABSTRACT

Fluoroquinolone resistance is a growing problem that has only recently emerged in *S. agalactiae*. Between 2005-2007, WHONET - Argentina network evaluated levofloxacin susceptibility in 1128 clinical *S. agalactiae* isolates, 10 (0,9%) of which proved to be resistant . Nine of them had come from 5 hospitals (in Buenos Aires City and 4 Argentinean provinces) and recovered from urine (n = 7) and vaginal screening cultures (n = 2). Three strains were also resistant to macrolides, lincosamides and B streptogramins due to the *erm*A gene. All nine fluoroquinolone-resistant isolates bore the same two mutations, Ser79Phe in ParC and Ser81Leu in GyrA proteins. Genetic relationships were analyzed by *Apa*I-PFGE and two clones were determined, A (n = 6) and B (n = 3). To our knowledge, these are the first fluoroquinolone-resistant *S. agalactiae* isolates detected in Latin America.

Key Words: *Streptococcus agalactiae*; fluoroquinolone resistance; Argentina.

RESUMEN

Aislamientos de *Streptococcus agalactiae* resistentes a fluoroquinolona en Argentina. La resistencia a fluorquinolonas es un problema creciente y recientemente ha emergido en aislamientos de *S. agalactiae*. Entre los años 2005-2007 la Red WHONET - Argentina evaluó la sensibilidad a levofloxacina en 1128 aislamientos clínicos de *S. agalactiae*. Se detectaron 10 cepas resistentes (0,9%). Nueve de estos aislamientos fueron derivados de 5 hospitales (4 de provincias, 1 de Ciudad de Buenos Aires) y habían sido recuperados de muestras de orina (n = 7) y de cultivos vaginales (n = 2) en evaluaciones de tamizaje. Tres de estos aislamientos también fueron resistentes a macrólidos, lincosamidas y estreptograminas B, y presentaban el gen *erm*A. Los nueve aislamientos contenían las mismas dos mutaciones, Ser79Phe en la proteína ParC y Ser81Leu en la proteína GyrA. La relación genética fue analizada mediante *Apa*I-PFGE y se determinó la presencia de dos clones, A (n = 6) y B (n = 3). Estos representarían los primeros aislamientos de *S. agalactiae* con resistencia a fluoroquinolonas detectados en América Latina.

Palabras clave: *Streptococcus agalactiae*, resistencia a fluoroquinolonas, Argentina

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Streptococcus agalactiae usually colonizes gastrointestinal, respiratory and urogenital human tracts causing different kinds of infections. Urogenital colonization of pregnant women with *S. agalactiae* is a critical risk factor for invasive neonatal disease, being antimicrobial prophylaxis recommended during delivery (2, 11). *S. agalactiae* is traditionally considered to be a neonatal pathogen, although recent increasing incidence of infections in adults was observed in the USA, especially in patients with underlying medical conditions (12). Fluoroquinolone resistance is a growing problem in human pathogens and *S. agalactiae* isolates with this phenotype have recently emerged in a few countries. The main resistance mechanisms known today are mutations in the quinolone resistance- determining region (QRDR) of ParC protein in positions Ser79 and Asp83 (1, 6, 14, 15). Additional mutations in Ser81 and Glu85 of GyrA protein are also related to fluoroquinolone resistance (1, 6, 15).

In 2005, WHONET - Argentina group (67 Hospitals) started the national surveillance of fluoroquinolone resistance in *S. agalactiae*. Levofloxacin susceptibility was evaluated by disk diffusion in 1128 *S. agalactiae* clinical isolates between 2005-2007, detecting fluoroquinolone resistance in 10 (0.9%) isolates (3). Nine of them were submitted to the National Reference Laboratory (INEI) in order to characterize both the molecular mechanisms involved in fluoroquinolone resistance and their genetic relationship. Isolates were recovered from 5 hospitals located in Buenos Aires City and in the provinces of Santa Fe, Santa Cruz, Entre Ríos and Buenos Aires. Seven *S. agalactiae* isolates were recovered from urine and two from vaginal screening cultures. Minimal inhibitory concentration (MIC) was determined by the agar dilution method according to CLSI guidelines for penicillin, cefotaxime, erythromycin, azithromycin, clindamycin, norfloxacin, ofloxacin, ciprofloxacin, levofloxacin, gatifloxacin and moxifloxacin (3). Briefly, plates containing Mueller-Hinton agar plus 5% sheep blood and two-fold antibiotic dilutions were inoculated and incubated during 18-24 h at 35 °C in a 5% CO_2 atmosphere (3). *Streptococcus pneumoniae* ATCC 49619 and *Staphylococcus aureus* ATCC 29213 were used as control strains. Macrolide resistance phenotypes were evaluated by disk diffusion positioning erythromycin (15 µg) and clindamycin (2 µg) disks 12 mm edge to edge (3). Expression of efflux pump was evaluated comparing the MIC of ciprofloxacin alone and supplemented with 20 mg/l of reserpine. Standard PCR was employed to detect the presence of *erm*A, *erm*B and *mef*A genes (13). QRDR sequences of *par*C and *gyr*A genes were determined using primers described previously (6). The *S. agalactiae* 2603V/R sequence (NC_004116) was used for DNA and protein sequence comparisons. Clonal relationships among the isolates were evaluated digesting genomic DNA with *Apa*I enzyme. DNA fragments were resolved on 1% agarose gel using a CHEF-DR III apparatus (Bio-Rad Laboratories, Hercules, CA) applying a 2 to 20 second-switch time, using 6 V/cm during 20 h at 14 °C. DNA patterns were analyzed with Bionumerics software using Dice coefficient and 1% tolerance. Isolates were considered genetically indistinguishable when they showed identical PFGE profiles and were assigned the same clonal subtype (e.g. subtype A1). PFGE profiles with 1-6 bands of difference were considered possibly related and were assigned to the same clonal type but different clonal subtype (e.g. subtype A1, A2). Isolates whose PFGE profiles differed by more than 6 bands were considered to be unrelated and were assigned different clonal types (e.g. clone A, B).

All nine *S. agalactiae* isolates showed susceptibility to penicillin and cefotaxime (Table 1). Three out of nine strains were resistant to macrolides, erythromycin and azithromycin, but susceptible to clindamycin (Table 1). However, by erythromycin/clindamycin double disk assay, an inducible resistance phenotype to macrolides, lincosamides and streptogramines ($iMLS_B$) was observed. These three isolates showing $iMLS_B$ phenotype were positive for the *erm*A gene and negative for *erm*B and *mef*A genes. All nine *S. agalactiae* isolates were resistant to all the fluoroquinolones assayed; however, different *in vitro* activities were observed (MIC range): norfloxacin (32-64 mg/l) = ofloxacin (32-64 mg/l) = ciprofloxacin (32-64 mg/l) < levofloxacin (16-32 mg/l) < gatifloxacin (4 mg/l) < moxifloxacin (2 mg/l) (Table 1). Differences were not observed among ciprofloxacin MICs in the presence or in the absence of reserpine, discarding a possible efflux pump activity affected by reserpine (Table 1). The same Ser79Phe and Ser81Leu mutations were detected in all nine isolates in ParC and GyrA proteins respectively (Table 1). Analysis of fluoroquinolone-resistant *S. agalactiae* isolates by *Apa*I-PFGE established the presence of 2 clonal types named A and B (Figure 1). Six isolates were genetically related and were differentiated in five clonal subtypes, A1 to A5, sharing more than 85% similarity (Table 1; Figure 1). The remaining three isolates showed undistinguishable DNA patterns and were classified as clone B (Figure 1; Table 1).

Resistance to penicillin and cephalosporins are still uncommon in *S. agalactiae,* being amino acidic modifications in *pbp* genes the main resistance mechanism (4). Macrolide resistance is mainly mediated by Erm methylase or Mef efflux pump. The isolates studied herein and expressing $iMLS_B$-phenotype harboured the *erm*A gene. Macrolide and lincosamide resistance in *S. agalactiae* is growing in our country and in 2007 reached 11.3% and 5.2%, respectively (WHONET - Argentina network data). Previous studies from Argentina reported the presence of *erm*A and *erm*B methylase genes, MefA efflux pump and more interestingly the emergence of lincosamide

Table 1. Sources, susceptibility (MIC), resistance mechanism and genetic relationship data of fluoroquinolone-resistant *S. agalactiae*.

					MIC (mg/l)												Mutation in QRDR		*Apa* I-PFGE
Isolate	Hospital	Province	Sample	Resistance profile	NOR	OFL	CIP	C+R	LEV	GAT	MOX	PEN	CTX	ERY	AZI	CLI	PCR	ParC	GyrA	type
M6395	HCU	Santa Fe	Urine	FQ, TET	32	64	32	32	32	4	2	0,06	0,06	0,12	0,25	0,12	-	S-79-F	S-81-L	A1
M6196	JAF	CABA	Vaginal	FQ	64	64	32	32	32	4	2	0,06	0,06	0,12	0,25	0,12	-	S-79-F	S-81-L	A1
M6530	EVI	Bs As	Urine	FQ	64	64	64	32	32	4	2	0,06	0,12	0,12	0,25	0,12	-	S-79-F	S-81-L	A2
M6405	HCU	Santa Fe	Urine	FQ	64	64	64	32	32	4	2	0,06	0,06	0,12	0,25	0,12	-	S-79-F	S-81-L	A3
M6459	HRR	Santa Cruz	Vaginal	FQ	64	32	32	32	16	4	2	0,06	0,06	0,12	0,25	0,12	-	S-79-F	S-81-L	A4
M6497	DCM	Entre Ríos	Urine	FQ	64	64	32	32	32	4	2	0,06	0,06	0,12	0,25	0,12	-	S-79-F	S-81-L	A5
M6394	HCU	Santa Fe	Urine	FQ, iMLSb, TET	64	32	32	32	16	4	2	0,06	0,06	1	4	0,12	*erm*A	S-79-F	S-81-L	B
M6396	HCU	Santa Fe	Urine	FQ, iMLSb, TET	32	64	32	32	16	4	2	0,06	0,06	4	32	0,12	*erm*A	S-79-F	S-81-L	B
M6397	HCU	Santa Fe	Urine	FQ, iMLSb, TET	64	64	32	32	16	4	2	0,06	0,06	4	32	0,12	*erm*A	S-79-F	S-81-L	B

Abbreviations. HCU, Htal. Cullen, Santa Fe; JAF, Htal. Fernández, CABA; EVI, Hosp Interzonal General de Agudos "Evita", Buenos Aires; HRR, Htal. Regional Río Gallegos, Santa Cruz; DCM, Htal. Masvernat, Entre Ríos; FQ, fluoroquinolones; TET, tetracycline; iMLSb, inducible phenotype of resistance to macrolides, lincosamides and streptogramins B; NOR, norfloxacin; OFL, ofloxacin; CIP, ciprofloxacin; C + R, ciprofloxacin plus reserpine; LEV, levofloxacin; GAT, gatifloxacin; MOX, moxifloxacin; PEN, penicillin; CTX, cefotaxime; ERY, erythromycin; AZI, azithromycin; CLI, clindamycin. *Apa*I-PFGE type, restriction patterns by pulse-field gel electrophoresis using *Apa*I enzyme.

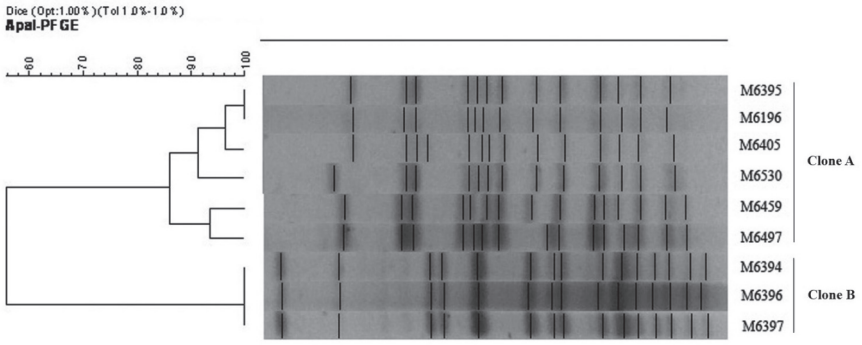

Figure 1. Pulsed field gel electrophoresis (PFGE) and genetic relation analysis of fluoroquinolone-resistant *S. agalactiae* isolates. Genetic relationship analysis among fluoroquinolone-resistant *S. agalactiae* from Argentina using Dice algorithm and Bionumerics Software. Scale only shows similarity percentage.

nucleotidyl-transferase LnuB enzyme in *S. agalactiae* (5, 7-9). The mutations associated with fluoroquinolone resistance detected in this study are the most frequently described (1, 6, 10, 14, 15).

A report from Japan has recently analyzed 189 *S. agalactiae* invasive isolates recovered from 97 medical institutions (10). Forty-five isolates (23.8%) were resistant to fluoroquinolones and showed the same ParC and GyrA mutations described herein. *Apa*I-PFGE analysis of those isolates revealed that the emergence of fluoroquinolone resistance in *S. agalactiae* was mainly associated with the dissemination of one epidemic clone (10). Although a visual comparison would not be appropriate because of inter-laboratory and pulse time differences, we observed that there was a high *Apa*I-PFGE similarity between the Japanese dominant clone and the clonal type A described here. The Japanese isolates were from invasive infectious diseases while our isolates were recovered from urine and vaginal screening cultures, alerting us about the potential of this clone to cause invasive infectious diseases in our country.

In conclusion, this is the first report of fluoroquinolone-resistant *S. agalactiae* isolates detected in Latin America due to mutation in both *par*C and *gyr*A genes, denoting the possible dissemination of a dominant clone. In Argentina, the prevalence of fluoroquinolone resistance in *S. agalactiae* is still low (0.9%) and demands a real need for continued surveillance in order to detect and be on the alert for the emergence of this phenotype in other cities.

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