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Molecular characterization of *Streptococcus agalactiae* from vaginal colonization and neonatal infections: a 4-year multicenter study in Greece ☆

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ABSTRACT

A multicenter collection comprising of 171 *Streptococcus agalactiae* isolates from pregnant women recovered between 2007 and 2010 and 46 from unmatched neonates with invasive infections was subjected to antimicrobial susceptibility testing and genetic characterization. High rates of erythromycin resistance (20.47%) were observed only in isolates from pregnant women. ST1 was dominant in the vaginal colonization, whereas the hypervirulent ST-17 clone was detected in 67.39% of neonatal infections.

Streptococcus agalactiae, a Group B *Streptococcus*, remains the leading cause of neonatal sepsis and meningitis in many countries, while colonizing frequently the genital track of women (Phares et al., 2008). The estimated rate of transmission from colonized mothers to newborns during childbirth varies from country to country and may result in invasive infection in the newborn during the first week of life, known as early-onset group B streptococcal infection (Phares et al., 2008). Molecular typing methods and population genetics of *S. agalactiae* have shown that the majority of neonatal infections including meningitis are caused by a limited number of "highly virulent clones", such as ST17 belonging mainly to serotype III (Kong et al., 2008; Lin et al., 2006; Manning et al., 2009; Martins et al., 2007; Van der Mee-Marquet et al., 2009).

A previous study in Greece has demonstrated that the incidence of *S. agalactiae* neonatal invasive diseases is very low (0.5%), while the maternal and neonatal colonization rates reach approximately to 6.6% and 2.4%, respectively (Tsolia et al., 2003). Until now, there are no data about the prevalence of "highly virulent clones" among *S. agalactiae* strains isolated from neonatal invasive diseases and colonized pregnant women in our country. In an effort to determine the predominant clones among *S. agalactiae* isolates in Greece, molecular characterization of 171 vaginal and 46 invasive neonatal isolates were assessed by multilocus sequence typing (MLST) and serotyping.

The cervical isolates were recovered from 171 individual colonized pregnant women from four tertiary hospitals located in different areas of Greece (Northern Greece [n = 39], Southern Greece [n = 30], Central Greece [n = 46], and Eastern Greece [n = 56]), during the period 2007–2010. After identification, the isolates of each participating hospital were sent to the Department of Microbiology of the University Hospital of Larissa (UHL) for further investigation. A collection of 46 invasive *S. agalactiae* recovered from blood cultures and cerebrospinal fluids, all consecutively isolated between 1995 and 2010 from equal number of neonatal invasive infections (sepsis and meningitis) in the Paediatric Hospital of Athens "Aghia Sofia", were also included in this study. Ten out of 46 isolates from neonatal infections were consecutively isolated between 2007 and 2010. We should note that the collection of the *S. agalactiae* isolates recovered from the colonized pregnant women did not include samples from the mothers of the neonates, for which no data were available.

The identification of the isolates to species level was confirmed in the Department of Microbiology of UHL based on Gram staining,

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colony morphology, haemolysis, and latex agglutination test with specific antisera, using the Slidex Strepto Plus (BioMerieux, Marcy l'Etoile, France). Antimicrobial susceptibility testing to various antimicrobial agents (penicillin, ampicillin, cefepime, ceftriaxone, chloramphenicol, erythromycin, clindamycin, levofloxacin, and tetracycline) was performed by the disk diffusion method according to the guidelines and interpretive criteria of the CLSI, (2011). The Double Disk Synergy Test and Gots' test were performed in all erythromycin- and/or clindamycin-resistant isolates in order to distinguish $MLS_{B\text{-inducible}}$, M and L phenotypes, as previously recommended (Gots, 1945; Hamilton-Miller and Saroj, 2000).

DNA was extracted from all isolates using the Quick-gDNA TM MiniPrep kit (Zymo Research, Irvine, CA, USA), and the presence of the commonly found antimicrobial resistance genes in streptococci for tetracycline [*tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(S), *tet*(T)], erythromycin [*mef*(A), *erm*(A), *erm*(B), erm(TR)] and clindamycin [*vga*(A), *vga*(B), *vga*(C), *vga*(D), *vga*(E), *lsa*(A), *lsa*(B), *lsa*(C), *lsa*(E), *lnu*(A), *lnu*(B), *lnu*(C), *lnu*(D)] was determined by PCR with primers and conditions as described previously (Culebras et al., 2002; Dogan et al., 2005; Leclercq, 2002; Poyart et al., 2003; Roberts, 2008).

Genetic characterization of the isolates was performed by MLST, as previously recommended (Jones et al., 2003). Sequences were obtained on both DNA strands; the allelic profiles were determined and assigned to sequence types (STs), and novel alleles and STs were submitted to the *S. agalactiae* MLST website (http://pubmlst.org/sagalactiae/). For each ST, the sequences of the 7 housekeeping gene fragments were concatenated maintaining the +1 reading frame aligned, and a neighbor-joining tree was constructed from the aligned sequences using the MEGA software (Tamura et al., 2007). Non overlapping groups of related STs were identified using eBURST, with the default setting for the definition of groups (Feil et al., 2004). The presence of the ST17 specific *gbs2018* allele, encoding for the surface protein HvgA was assessed by PCR with primers and conditions as described previously (Lamy et al., 2006). In addition, serotyping was performed with a coagglutination method using antisera against types I to VIII (Essum, Umeå, Sweden) according to the manufacturer's instructions.

Although *S. agalactiae* clinical strains with reduced sensitivity for penicillin G have been recorded elsewhere, all 217 isolates were sensitive to all beta-lactams tested as well as chloramphenicol and levofloxacin (Nagano et al., 2012). The various patterns of antimicrobial resistance are described in Table 1; 35 isolates (16.3%) were co-resistance to tetracycline, macrolides, and lincosamides.

Interesting finding of the present study was the high rates of resistance to tetracycline (97.2%). In more details, 169 out 171 isolates derived from pregnant women (98.83%) exhibited resistance to tetracycline and carried the *tet* (M) gene (162 isolates), the *tet* (O) gene (6 isolates), and the *tet* (L) gene (1 isolate). Among the 46 isolates from infected neonates, 42 (91.3%) were resistant to tetracycline and carried the *tet*(M) gene (41 isolates) and the *tet*(O) gene (1 isolate).

Among the 171 *S. agalactiae* isolates recovered from colonized pregnant women, 33 (19.3%) exhibited cross-resistance to erythromycin and clindamycin (MLS_B phenotype), while 3 (1.75%) and 2 (1.17%) isolates were only resistant to clindamycin (L phenotype) and erythromycin (M phenotype), respectively. On the other hand, among the 46 isolates recovered from neonatal invasive diseases, 2 (4.35%) isolates exhibited MLS_B phenotype, and 1 (2.17%) exhibited M phenotype. Of the 35 isolates exhibiting cross-resistance to erythromycin and clindamycin (MLS_B phenotype), 27 (77.14 %) showed the constitutive MLS_B ($cMLS_B$) phenotype and carried the *erm*(B) gene, whereas only 8 (22.86%) isolates showed the inducible MLS_B ($iMLS_B$) phenotype and carried the *erm*(TR) gene. The 3 erythromycin-resistant isolates exhibiting M phenotype carried the *mef*(A) gene, but none of the resistant genes tested was found in the clindamycin-resistant isolates exhibiting L phenotype.

Table 1
CCs, MLST STs, serotypes, and antimicrobial resistance patterns of 217 *S. agalactiae* isolates from neonates and colonized pregnant women.

CC	MLST ST[a]	Serotypes (No.)	Antimicrobial resistance patterns (No.)	Neonatal isolates, No. (%)	Colonizing isolates, No. (%)	Total of isolates, No. (%)
CC-1	**1**	IV(15), V(28)	**TC (23)TC, EM, CM (20)**		**43 (25.15)**	**43 (19.82)**
	2	V (2)	TC (2)		2 (1.17)	2 (0.92)
	196	IV (2)	TC (2)		2 (1.17)	2 (0.92)
CC-10	**8**	Ib (21)	**TC (17)CC (2) TC, EM, CM (2)**	**3 (6.52)**	**18 (10.53)**	**21(9.68)**
	10	Ib (2)	TC (2)		2 (1.17)	2 (0.92)
	12	Ib (15)	**TC (14)TC, EM, CM (1)**		**15 (8.77)**	**15 (6.91)**
CC-17	**17**	III (35)Ia (3)	**TC (35)TC, EM, CM (3)**	**31 (67.39)**	**7 (4.09)**	**38 (17.51)**
	146	III (3)	TC (3)	3 (6.52)		3 (1.38)
CC-19	**19**	III (27)	**TC (21)EM (4) TC, EM, CM (2)**	**6 (13.05)**	**21 (12.28)**	**27 (12.44)**
	28	III (6)	TC (6)		6 (3.51)	6 (2.76)
	106	III (2)	TC, EM, CM (2)		2 (1.17)	2 (0.92)
	197	III (3)	TC (3)		3 (1.75)	3 (1.38)
	219	III (2)	TC (2)		2 (1.17)	2 (0.92)
	335	III (2)	TC (2)		2 (1.17)	2 (0.92)
CC-22	22	III (3)	TC (3)		3 (1.75)	3 (1.38)
CC-23	**23**	III (34)	**TC (28)TC, EM, CM (6)**	**3 (6.52)**	**31 (18.13)**	**34 (15.67)**
	24	III (3)	TC (3)		3 (1.75)	3 (1.38)
	220	III (2)	TC (2)		2 (1.17)	2 (0.92)
	464	III (2)	TC (2)		2 (1.17)	2 (0.92)
	498	III (3)	TC (3)		3 (1.75)	3 (1.38)
Singleton	314	III (2)	TC (2)		2 (1.17)	2 (0.92)
Subtotal				**46 (100)**	**171 (100)**	**217 (100)**

TC = tetracycline; EM = erythromycin; CM = clindamycin.
[a] The most prevalent STs are indicated in bold.

The distribution into STs and clonal complexes (CCs) of the *S. agalactiae* isolates recovered from neonatal infections and colonized women genotyped by MLST is shown in Table 1. We have identified 21 STs among all isolates tested, which were clustered into 6 CCs; 1 ST was singleton (Table 1). The genetic relatedness of the 21 STs found in the present study was assessed by the Neighbour-Joining algorithm and is shown in Fig. 1.

STs 1, 8, 12, 17, 19, and 23 were the most prevalent ones, each comprised of 15–43 isolates, whereas the remaining STs were comprised of 2–6 isolates (Table 1). The distribution of the STs did not appear any annually variability, while no differences in the distribution of STs among the different areas of Greece were observed. STs 8, 17, 19, and 23 were shared between colonized women and infected neonates (Table 1). The most prevalent clone among pregnant women was ST1 (25.15%), while among neonatal isolates, ST17 predominated (67.39%). We note that 8 out of 10 neonatal isolates, recovered between 2007 and 2010, belonged to ST17; the remaining 2 isolates belonged to ST19. All ST17 *S. agalactiae* isolates, either from neonates or from pregnant women, were positive for the ST17-specific allele of the *hvgA* gene that confers hypervirulence and meningeal tropism in neonates, as reported previously (Lamy et al., 2006; Tazi et al., 2010). All erythromycin-resistant strains belonged to several STs, mainly to STs 1 and 23, while all ST17 strains were sensitive to macrolides and lincosamides (Table 1).

Serotyping results revealed that isolates belonging to ST8 and ST12 were classified as Ib type, ST1 as either IV or V type, ST23 and ST19 as III type, whereas ST17 as either Ia or III type (Table 1). In our study, a capsular switching of some STs was observed, resulting in the appearance of new serotype-genotype combinations. This finding emphasizes the need for the combined application of MLST and serotyping for surveillance purposes.

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