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ARTICLE

Phenotypes, genotypes, serotypes and molecular epidemiology of erythromycin-resistant *Streptococcus agalactiae* in Italy

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Abstract The purpose of this investigation was to analyse *Streptococcus agalactiae* (group B *Streptococcus*, GBS) isolates collected in Italy from vaginal and urine samples in respect to their clonality, distribution of virulence factors and antimicrobial resistance determinants. Three hundred and eighty-eight GBS were recovered from clinical samples. They were analysed for antibiotic resistance profiling. Erythromycin-resistant strains were further characterised by multilocus sequence typing (MLST), serotyping and the detection of *alp* genes of the alpha-like protein (Alp) family. GBS isolates represented 40 different sequence types (STs), grouped in five clonal complexes (CCs) and belonged to seven serotypes. Most serotype V strains (81%) possessed *alp2-3*; serotype Ia carried mainly *epsilon*, while the serotype III mainly *rib*. All isolates were susceptible to penicillin, whereas resistance to erythromycin was detected in 15% of isolates. Most erythromycin-resistant GBS strains were of serotype V (56.8%) and belonged to the CC-1 group (50%). Macrolide resistance phenotypes were the $cMLS_B$ (46.5%) and the M phenotypes (46.5%) due to the presence of *ermB* and *mefA/E* genes, respectively. These results provide data which establish a baseline for monitoring erythromycin resistance in this region and also provide an insight into the correlation among clonal types, serotypes, surface protein and resistance genes. The increased prevalence of strains that displayed the M phenotype strengthens the importance of the epidemiological surveillance of macrolide resistance in GBS, which may also represent an important reservoir of resistance genes for other species.

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Introduction

Streptococcus agalactiae (group B *Streptococcus*, GBS), a commensal of human gastrointestinal and genitourinary flora, is now the major cause of early-onset sepsis in newborns, as well as an important factor of post-partum maternal sepsis and late-onset invasive infections in infants. GBS is also increasingly isolated from bacteraemia, endocarditis, skin and soft tissue infections, pneumonia, and bone and joint infections in non-pregnant adults with underlying medical conditions [1]. β-lactam agents such as penicillin or ampicillin are the antibiotic agents of choice for the prophylaxis and treatment of GBS infections. Macrolides are the recommended second-line drugs and the first alternative in cases of β-lactam allergy. Resistance to the alternative drugs of choice for the treatment of GBS infections, including lincosamides such as clindamycin and macrolides such as erythromycin, has increased during the last decade in several countries, with some geographical variations [2–4]. The resistance is commonly caused by three genes: *ermB*, *ermA* (subclass *ermTR*) and *mefA/E*. Mechanisms which confer resistance to macrolide, lincosamide and streptogramin B (MLS_B) antibiotics include target site modification by methylation of the 23SrRNA binding site. This mechanism is mediated by the *ermB* and *ermTR* genes, which confer cross-resistance to all MLS_B antibiotics. Resistance can be either inducible ($iMLS_B$), where the methylase is produced in the presence of an inducer (such as erythromycin), or constitutive ($cMLS_B$), where the methylase is produced constitutively [5]. The *mefA* and *mefE* genes, which are 90% identical, encode 14- and 15-member macrolide efflux pumps, respectively, and lead to the macrolide-only (M) resistance phenotype [6].

This increasing resistance to macrolides observed worldwide underlines the need for detailed studies on

GBS resistance determinants and population structure. Among typing methods applied to GBS, multilocus sequence typing (MLST) possesses a high discriminatory power [7].

Capsular serotyping is the classical method used in epidemiological studies. Nine capsular types are recognised: Ia, Ib and II to VIII, along with a new provisional serotype IX, which has been recently proposed [8]. Comparison of the capsular locus genes suggested that the structural diversity of the capsular polysaccharide is associated with the genetic diversity of the capsular locus, possibly driven by horizontal gene transfer [9]. The capsular polysaccharide is a major GBS virulence factor and also the main target of antibody-mediated killing [7]. In the last decade, conjugated multivalent vaccines have been developed and proved to be highly immunogenic, raising the possibility of the prevention of perinatal GBS disease through maternal immunisation [10].

Proteins of the α-like protein (Alp) family play an important role in GBS pathogenesis and are also vaccine candidates [11]. The Alp family includes the alpha, Rib, Alp2, Alp3/R28, Alp4 and epsilon proteins, encoded by the allelic *bca*, *rib*, *alp2*, *alp3*, *alp4* and *alp1/alp5* genes, respectively, located on the pathogenicity island IV [12, 13].

The purpose of this paper was to characterise a collection of GBS isolated both from urine and vaginal samples by antimicrobial susceptibility testing, the presence of antimicrobial resistance genes, serotyping, MLST and analysis of the *alp* gene distribution.

Materials and methods

Strain collection

A total of 388 GBS isolates were collected between June 2009 and January 2010 from patients aged between 23 and 70 years (median 40.5 years) who visited Brescia's main hospital (Spedali Civili); 265 originated from vaginal samples and 123 originated from urine samples. All strains were isolated from consecutive outpatients who for gynaecologic healthcare control, for normal routine screening during pregnancy or for the presence of symptoms of genital infections had attended Brescia's hospital. GBS strains were isolated by streak plating 1 to 10 μl of transport medium onto chromID Strepto B agar plates (bioMérieux, St. Louis, MO, USA). The plates were incubated at 37°C for 18 to 24 h in aerobic conditions. GBS was selected by the production of a pink pigment when grown aerobically on chromID Strepto B agar. GBS identification was performed by means of the VITEK™ system (bioMérieux).

Alpha-like protein (Alp) genes and serotyping

Surface protein markers were detected by using a multiplex polymerase chain reaction (PCR) for the direct identification of the Alp protein genes [12]. Primer nucleotide sequences used as the reverse primers and the nucleotide sequence, common to all of the surface protein genes, used as the forward primer are reported in the Table 1. Capsular serotyping was carried out by latex agglutination method with anti-type Ia, Ib, II, III, IV, V, VI, VII and VIII sera (Statens Serum Institute, Copenhagen, Denmark; distributed in Italy by Biogenetics). Isolates that failed to type using the serotyping kit were deemed non-typable (NT) by latex agglutination.

MLST

DNA was extracted from each strain using a DNeasy kit (Qiagen). Internal fragments (size~400–500 bp) of seven housekeeping genes, chosen for GBS characterisation by the use of MLST, were amplified from DNA extract by PCR. The seven loci were **pheS** (phenylalanyl transfer RNA synthetase), **atr** (amino-acid transporter protein), **tkt** (transketolase), **glcK** (glucose kinase), **sdhA** (L-serine dehydratase), **glnA** (glutamine synthetase) and **adhP** (alcohol dehydrogenase).

The MLST analysis of macrolide-resistant strains was performed as described elsewhere [7]. Alleles for the seven loci were analysed on the MLST website (http://pubmlst.org/sagalactiae), and its combination provided an allelic profile or sequence type (ST). The STs were grouped with the eBURST program [14] into clonal complexes (CCs)

Table 1 Nucleotide primer sequence and amplicon size expected for each *Streptococcus agalactiae* surface protein gene

Primer	Sequence (5′–3′)	Amplicon size (bp)
Universal forward	TGATACTTCACAGACGAAACAACG	
Alpha-C reverse	TACATGTGGTAGTCCATCTTCACC	398
Rib reverse	CATACTGAGCTTTTAAATCAGGTGA	295
Epsilon reverse	CCAGATACATTTTTTACTAAAGCGG	200
Alp2-3 reverse	CACTCGGATTACTATAATATTTAGCAC	334
Alp4 reverse	TTAATTGCACCGGATTAACACCAC	110

whose members shared at least five of the seven MLST loci [15]; otherwise, an ST was considered to be a singleton.

Antimicrobial resistance phenotype and determinants

Antimicrobial susceptibility was tested using VITEK2 (bioMérieux). VITEK2 susceptibility testing was performed according to the manufacturer's instructions by using the AST-P586 card. The results obtained after a maximum of 15 h of incubation were analysed and interpreted by AES 4.02 software. The minimum inhibitory concentrations (MICs) determined by the system identified the class of microbial susceptibility as susceptible, intermediate or resistant according to the interpretative criteria published by Clinical and Laboratory Standards Institute (CLSI) guidelines [16]. The phenotypic characterisation of macrolide-resistant strains was performed by double-disc diffusion testing as described previously [17]. Erythromycin (15 μg) and clindamycin (2 μg) discs were placed 20 mm apart. Isolates resistant to erythromycin with blunting of the clindamycin inhibition were of the $iMLS_B$ phenotype, isolates that demonstrated resistance to both erythromycin and clindamycin were of the $cMLS_B$ phenotype, isolates showing resistance to erythromycin without blunting of the clindamycin inhibition zone were of the M phenotype and isolates resistant to clindamycin yet susceptible to erythromycin belonged to the L phenotype. Interpretative criteria were in accordance to the CLSI guidelines [16]. A multiplex PCR was used to identify the *ermB*, *ermTR* and *mefA/E* genes from the GBS strains, using primers (Table 2) and conditions previously reported [17, 18], and a separate PCR was used to amplify the *linB* gene [19, 20].

Statistical analysis

Fisher's exact test was used to evaluate the differences in the distributions of isolates. A p-value of <0.05 was considered to be significant.

Results

Serotypes and the *alp* family genes

All 58 GBS isolates resistant to macrolides were serotyped by the latex agglutination method. Four strains were non-typable (NT). Overall, the most represented serotypes isolated were types V (33 isolates), Ia (7 isolates) and III (6 isolates). Serotype Ib was represented by three isolates, while serotypes II and IV were represented by two isolates each. One strain belonged to serotype VI. No strains of serotypes VII and VIII were found. The presence of a particular Alp gene in relation to the serotype was also noted (Table 3). Of the 27 *alp2-3*-positive strains isolated, 22 were of serotype V; 9 of 17 *epsilon*-positive strains corresponded to serotype V and five corresponded to serotype Ia; *rib*-positive strains were present in almost all of the serotypes isolated. Conversely, a certain serotype commonly corresponded to a particular Alp gene: serotype Ib and II presented *rib*, serotype IV carried either *rib* and *epsilon*, most serotype V strains (81%) possessed *alp2-3*, serotype Ia carried mainly *epsilon*, while serotype III carried mainly *rib*. Different associations of *alp* genes were presented in a single strain.

MLST of GBS

The MLST analysis of 58 macrolide-resistant strains demonstrated the existence of different genetic lineages, inclusively among strains expressing the same serotype (Table 4). In particular, 40 STs were observed. Despite the high number, all STs were grouped into five CCs; 11 singleton STs were identified that were not a part of a cluster. The eBURST groups were: CC-1 (including STs: 1, 2, 6, 86, 153, 170, 186, 196, 242, 297, 318, 333, 364, 433, 436, 455, 466; $n=29$, 50%); CC-46 (including STs: 46, 243, 268, 273; $n=5$, 8.6%); CC-24 (including STs: 24, 33, 163, 444; $n=4$, 6.8%); CC-27 (including STs: 106, 181; $n=3$, 5.1%); CC-352 (including ST-352 and ST-460; $n=2$, 3.4%) and singletons (STs: 55, 129,

Table 2 Primers and products

Primer name	Sequence	Gene target(s)	Product size (bp)
ermB1 ermB2	5′-GAA AAG GTA CTC AAC CAA ATA-3′ (forward) 5′-AGT AAC GGT ACT TAA ATT GTT TAC-3′ (reverse)	*ermB*	639
ermTR1 ermTR2	5′-GAA GTT TAG CTT TCC TAA-3′ (forward) 5′-GCT TCA GCA CCT GTC TTA ATT GAT-3′ (reverse)	*ermTR*	395
mefA1 mefA2	5′-AGT ATC ATT AAT CAC TAG TGC-3′ (forward) 5′-TTC TTC TGG TAC TAA AAG TGG-3′ (reverse)	*mefA* and *mefE*	346
linB1 linB2	5′-CCT ACC TAT TGT TTG TGG AA-3′ (forward) 5′-ATA ACG TTA CTC TCC TAT TC-3′ (reverse)	*linB*	944

Table 3 Distribution of the *alp* genes among the observed group B *Streptococcus* (GBS) serotypes

Surface alpha-like protein genes (no. of isolates)	No of isolates (%) of serotypes							
	Ia	Ib	II	III	IV	V	VI	NT
Alp2-3 (27)	1 (3.7)			2 (7.4)		22 (81)*	1 (3.7)	1 (3.7)
Rib (24)	2 (8.3)	2 (8.3)	2 (8.3)	5 (20.8)*	1 (4.1)	9 (37.5)		3 (12.5)
Epsilon (17)	5 (29.4)*				2 (11.7)	9 (52.9)		1 (5.8)
Alpha c (8)	1 (12.5)			1 (12.5)		5 (62.5)		1 (12.5)
Alp 4 (13)	1 (7.6)					9 (69.2)		3 (23)

*$p<0.05$

**$p<0.01$

168, 171, 185, 198, 231, 247, 413, 434, 457, 461, 480; n =15 (25.8%). There was a statistically significant association ($p<0.05$) between CC-1 and the surface protein *alp2-3* and *epsilon* genes, and between CC-1 and serotype V. Furthermore, a statistically significant correlation was observed between CC-27 and the surface protein *rib* gene.

Macrolide resistance phenotypes and genotypes

Erythromycin resistance was possessed by 15% of the strains (58 out of 388). Most of the resistant isolates (33 isolates, 56.8%) belonged to serotype V. The majority of resistant isolates belonged to the cluster CC-1 (29/58, 50%). Fifty-four of 58 erythromycin-resistant isolates

Table 4 Correlation between clonal clusters (CCs) and *alp* genes, serotypes, phenotypes and genotypes

Alp gene (no.)	CC-1 (%)	CC-24 (%)	CC-27 (%)	CC-46 (%)	CC-352 (%)	Singletons (%)
Alp2-3 (27)	66**	1	0	2	6	25
Alp4 (13)	53	7.6	4.5	0	0	34.8
Rib (24)	54	12	17*	0	0	17
Alpha c (8)	25	12.5	0	25	0	37.5
Epsilon (17)	67**	3	0	11	0	19
Serotype (no.)						
Ia (7)	14	28	0	28	0	28
Ib (3)	33.3	33.3	33.3	0	0	0
II (2)	100	0	0	0	0	0
III (6)	25	0	25	0	0	25
IV (2)	100	0	0	0	0	0
V (33)	51*	3	0	6	6	34
VI (1)	100	0	0	0	0	0
NT (4)	100	0	0	0	0	0
Phenotype (no)						
M (27)	55	7	4	4	4	26
$cMLS_B$ (27)	37	7	7	14	4	30
$iMLS_B$ (4)	75	0	0	0	0	25
Genotype (no)						
ermB (22)	41	4.5	4.5	2.9	4.5	36
ermTR+ermB (1)	0	100	0	0	0	0
mefA/E (27)	53	7.1	3.5	3.5	3.5	28
mefA/E+ermB (4)	75	0	25	0	0	0
Negative (4)	50	25	0	25	0	0

*$p<0.05$

**$p<0.01$

displayed half the $cMLS_B$ resistance phenotype and the other half the M resistance phenotype, while the $iMLS_B$ phenotype was found in four isolates (Table 5).

We isolated four strains with unidentified antibiotic resistance genotypes. These unknown resistant strains were found to have the $cMLS_B$ resistance phenotypes. Most of the screened strains presented solely a single resistance gene. An exception occurred for five strains that exhibited a combination of *ermB* and *mefA/E* genes (four isolates) and of *ermB* and *ermA* genes (subclass *ermTR*) (one isolate). Twenty-two GBS strains carried the *ermB* gene and twenty-seven possessed the *mefA/E* gene (Table 4). All *ermB* gene-positive isolates expressed the $cMLS_B$ resistance phenotype, while the M resistance phenotype was expressed by all of the isolates found to carry the *mefA/E* gene.

Discussion

One of the properties specific for GBS seems to be a remarkably clonal population structure, with strong differences in the degree of divergence within particular clonal groups. In this study, 58 isolates belonged to 40 STs. Other studies showed the presence of various STs among the collection of isolates [7, 15, 21]. Despite this high genetic diversity, the STs belonged to relatively few CCs, five in this study. The degree of variability of pathogenic factors, such as serotype and surface protein genes, differed for various CCs. Some CCs such as CC27 are more homogeneous, while others, e.g. CC1 and CC23, are much more internally variable [7, 15, 22]. These differences among CCs may be due to different capabilities for horizontal gene transfer, whose mechanism remains unclear in GBS.

It has been hypothesised previously that the spread of strains of particular surface protein profiles and serotypes reflects the selection of the best evolutionary lineages by the immune system [13]. In this study, we found that the five major CCs comprised isolates presenting serotype–surface protein gene combinations (serotype V-*alp2-3*; serotype III-*rib*) already reported [13, 23].

Nonetheless, a different combination (serotype Ia–*epsilon*) was observed in this study, suggesting that new successfully selected clones may be emerging.

Among the 58 GBS strains studied, all capsular serotypes except VII and VIII were found. Globally, serotype V (56.8%) was the most common, followed by serotypes Ia (12%) and III (10%), contrasting with the low prevalence of serotypes Ib (5%), II and IV (3.4%). However, other studies showed a predominance of other

Table 5 Antibiotic resistance and molecular typing of group B streptococcal clinical strains

Serotype	No. of isolates	Phenotype	Genotype	ST
Ib	3	$cMLS_B$ (1)	ermB	1
		$cMLS_B$ (1)	ermB+mefA/E	181
		$cMLS_B$ (1)	ermB+ermTR	33
Ia	7	$cMLS_B$ (1)	–	163
		M (4)	mefA/E (4)	297, 243, 444, 198
		$cMLS_B$ (2)	ermB (2)	273, 461
II	2	$iMLS_B$ (2)	ermB (2)	436, 297
III	6	$cMLS_B$	–	1
		$cMLS_B$	ermB+mefA/E	466
		$cMLS_B$ (2)	ermB (2)	106, 247
		M (2)	mefA/E (2)	106, 55
IV	2	M	mefA/E	433
		$cMLS_B$	ermB	196
V	33	$cMLS_B$ (14)	ermB (11)	413 (3), 129, 268, 1, 86, 364, 460, 168, 273
			– (2)	46, 297
			ermB+mefA/E	1
		M (17)	mefA/E (17)	434, 1, 242, 352, 413, 170, 333, 185, 24, 153, 231, 455, 318 (3), 186, 2, 171
		$iMLS_B$ (2)	ermB (2)	247, 297
VI	1	$cMLS_B$	ermB	455
NT	4	$cMLS_B$	ermB+mefA/E	433
		M	mefA/E	433
		M (2)	mefA/E (2)	2, 6

serotypes (IV in the United Arab Emirates, and VI to VIII in Japan) [24, 25], which could reflect the specificities of immune responses that may vary according to the studied population.

In our study, the majority of resistant isolates belonged to serotype V and the CC-1 clonal group, and such an association was already found [4].

Other studies have also identified serotype V GBS strains associated with erythromycin resistance [26–28].

For a long time, GBS remained fully susceptible to penicillin [3, 4, 29], the first-line drug used in the treatment and prophylaxis of infections caused by this pathogen. The current alternatives for patients allergic to penicillin include macrolides and lincosamides. However, 19% of the *S. agalactiae* isolates were resistant to erythromycin and 53% of these presented resistance to clindamycin [30]. Regarding the erythromycin resistance among strains of *S. agalactiae*, our results (15%) were similar to what has already been observed in Italy, Spain, Portugal, Germany, France and Canada [4, 29, 31–34], but differ considerably from the 3.8% reported in the Czech Republic [35] and 38–41.9% reported in the United States [3, 17]. Together with social determinants and differences of healthcare structures, the factors most frequently associated with these large discrepancies in antimicrobial resistance are the antibiotic consumption and its inappropriate use [36]. The erythromycin resistance level observed, i.e. 15%, indicates the need for careful surveillance in the future, especially as the first penicillin-non-susceptible GBS were reported in USA and Japan [37, 38]; this dangerous phenomenon may lead to the further limitation of treatment options.

In our study, there was a predominance of $cMLS_B$ and M phenotypes, indicating that erythromycin resistance was mediated by the two principal mechanisms: methylation of 23S ribosomal RNA, determined by *erm* genes, and active drug efflux by pumps encoded by *mef* genes. To our knowledge, this is the first time that this high proportion of M phenotypes has been reported in Italy (46.5%). It has important implications. Firstly, on the laboratory reporting of clindamycin susceptibility results, as clindamycin can be reported as susceptible, and, secondly, it may influence the antibiotic choice, as clindamycin may still be a therapeutic option, even if the organism is resistant to erythromycin.

There were four isolates in the collection for which no resistance mechanism was found. These isolates may harbour mutations in genes coding for 23S rRNA or ribosomal proteins L4 and L22. Interestingly, there was one isolate of the $cMLS_B$ phenotype that had both *ermA* subclass *ermTR* and *ermB* genes; the coexistence of both genes has been documented previously [39]. Also, four isolates that displayed M phenotypes harboured both *mefA/E*, genes as well as *ermB* genes. This finding implies differential gene expression, as only the *mefA/E* gene was expressed.

In summary, GBS strains isolated represent a complex population where the detection of a correlation among clonal clusters, serotypes, surface-associated proteins and resistance genes show a clonal spread.

Finally, as erythromycin resistance rates in GBS have increased, continued surveillance is advisable and local statistics will be of crucial value in guiding empirical antibiotic therapy. It also highlights the role of efflux on macrolide resistance in GBS, which may have an impact on antibiotic choice in clinical practice. The current study provides a baseline from which future trends in macrolide resistance can be monitored.

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