Invasive neonatal GBS infections from an area-based surveillance study in Italy

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Abstract

During an area-based study, 75 group B streptococcus (GBS) strains isolated both from early-onset disease (EOD, 37 strains) and from late-onset disease (LOD, 38 strains) were analysed for serotype, pulsed field gel electrophoresis (PFGE) and multilocus sequence typing profiles, protein markers and antibiotic resistance. Serotype III, possessing the *rib* gene, was the most frequent (54 strains, 72%) and responsible for 89.5% and 54% of LOD and EOD, respectively. Forty-six serotype III strains belonged to the same PFGE type and clonal complex 17, already described as an over-represented clone in neonatal invasive GBS infections. Other serotypes were Ia (9.3%), II (6.7%), Ib (5.3%), V (5.3%) and IV (1.3%). Seventeen PFGE groups were identified comprising strains with related sequence types; conversely, strains displaying the same sequence type could belong to different PFGE groups. When both neonate and maternal strains from vaginorectal swabs and/or milk were available (eight cases), they were indistinguishable. Resistance to erythromycin (12%) was associated with a constitutive resistance to clindamycin in five cases (four carrying the *erm*(B) gene and one both the *erm*(B) and *mef*(E) genes) and with an inducible clindamycin resistance in two cases (one possessing the *erm*(A) gene, the other the *erm*(T) gene). Two isolates displayed the M phenotype (*mef*(E) gene). All strains but five were resistant to tetracycline, mostly mediated by the *tet*(M) gene (97.1%). The study underlined the importance of an active surveillance system for the elucidation of a GBS population structure causing neonatal infections and allowed the detection of rare antibiotic resistance determinants [*erm*(T)].

Keywords: Alpha-like protein family, antibiotic resistance, group B streptococcus, MLST, molecular epidemiology, neonatal infection, PFGE, *S. agalactiae*, serotype
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Introduction

Group B streptococcus (GBS) is the leading cause of infectious diseases among newborns. Invasive neonatal GBS infections can present either as an early-onset disease (EOD) occurring within the first week (generally within the first day) and resulting in sepsis and/or pneumonia or as a late-onset disease (LOD) occurring between 1 week and 3 months of life and accounting for most meningitis cases and deaths [1].

Since 2002, CDC recommendations for the prevention of invasive GBS disease in newborns opted for the universal prenatal culture-based screening for vaginal and rectal GBS Centre for Disease Control colonization of pregnant women at 35–37 weeks of gestation, with intrapartum antibiotic prophylaxis (penicillin/ampicillin or macrolide in the case of serious penicillin allergy) for those with positive cultures [2]. Nevertheless, the observed decline in the incidence of GBS disease since the adoption of the prevention strategies related mostly to EOD, with only a slight reduction in LOD [3].

A total of ten different GBS capsular polysaccharide antigenes (Ia, Ib, II–IX) have been described so far; prevalence studies indicated that EOD is associated with Ia, Ib, II, III and V serotypes whereas LOD is associated primarily with type III [4–8]. Other serotypes have been reported mainly in colonization studies, with a predominance of serotypes VI and VIII in Japan and serotype IV in the United Arab Emirates [9,10].

Besides the capsule type, the Alpha-like protein (Alp) family, which comprises the surface-localized protein antigens Alpha-C, Alp1 to 3, and Rib/R4, is commonly used as a GBS protein marker in epidemiological studies [11].

The analysis of serotypes, protein markers, antibiotic resistance profiles and clonal relationships of invasive neonatal GBS strains from an area-based multicentre study is reported here. This is the first time this has been investigated in our country.

Materials and Methods

Bacterial strains and serotyping

From February 2005 to June 2008, 75 non-redundant GBS strains were isolated from blood and/or cerebrospinal fluid of infected newborns and infants. In one EOD and seven LOD cases, neonatal strains were accompanied by isolates from the maternal vaginorectal swabs collected at the onset of the neonatal disease symptoms and, in four LOD cases, from the maternal milk. In total, 87 GBS strains were analysed.

Bacterial strains were plated on defibrinated sheep blood agar plates and incubated at 37°C in 5% CO_2. Identification was confirmed by the Dryspot Streptococcal Grouping Kit (Oxoid). Serotyping was performed by using both a latex agglutination test (Statens Serum Institut) and a multiplex PCR assay [12].

Alpha-like protein (Alp) family

Surface protein markers were inferred by using a multiplex-PCR for the direct identification of the Alp protein genes [13].

Pulsed field gel electrophoresis (PFGE) analysis

Total DNA was extracted as previously described [14] and digested with 40 U of *Sma*I. Four isolates, all serotype III – *rib*, were not DNA-digested by the *Sma*I enzyme but they could be resolved by using the *Xma*I restriction enzyme, a *Sma*I isoschizomer. Interpretation of PFGE results and assignation to PFGE group were performed according to the previously reported criteria [14]. In particular, isolates with indistinguishable profiles were assigned to the same PFGE type and subtype; isolates with similar profiles (differing by up to five bands) were considered possibly related and assigned to different subtypes within the same PFGE type [15]. Isolates with more than five bands of difference were considered unrelated and were identified as different PFGE types.

Multilocus sequence typing (MLST) analysis

A subset of 32 isolates from each PFGE type and possessing different serotype/surface protein combinations were further genotyped by MLST (http://pubmlst.org/sagalactiae/). Sequence types (STs) were assigned to one of the previously described clonal complexes (CCs) included in the GBS MLST database if they shared five or more alleles from the most frequent ST in that CC.

Erythromycin, clindamycin and tetracycline resistance determinants

Resistance to erythromycin and clindamycin was assessed phenotypically by both Etest (Biomériela Italia, Milan, Italy) for determination of MIC values and the Kirby–Bauer double-disk diffusion method to assign the constitutive (CR), inducible (IR) and M resistant phenotype [16]. The presence of the macrolide resistance genes *erm*(A) (subclass *erm*(TR)), *erm*(B) and *mef* was investigated in a multiplex PCR, as already described [14]. The *mef* amplicon was sequenced for the identification of the *mef* class. PCR conditions and primer sequences used for amplification of the *erm*(T) gene were as described [17] and the amplicon was then sequenced to confirm its identity. Tetracycline resistance was determined both phenotypically by E-test and genotypically by studying the occurrence of the resistance genes *tet*(M) and *tet*(O) [18].

Statistical analysis

Fisher's exact test was used to evaluate the differences in distribution of isolates. Two-sided p values <0.05 were considered statistically significant (SPSS 17 for Windows).

Results

Clinical features

Among the 37 EOD cases, the most common manifestation was sepsis (59.4%), followed by septic shock (18.9%) and bacteraemia (13.5%). Sepsis was defined as GBS bacteraemia in the presence of clinical signs and symptoms consistent with a systemic inflammatory response. A septic shock was defined as a sepsis with tachycardia and signs of decreased perfusion. GBS bacteraemia was confirmed by a blood culture obtained from a peripheral or umbilical vessel (if <6 h after catheter insertion) [19].

TABLE 1. Classification of the GBS strains responsible for EOD and LOD by identification strings (serotype/surface protein/PFGE type/CC)

Serotype	*alp*	PFGE type	CC	EOD (no. isolates)	LOD (no. isolates)
III	*rib*	3	17	18	28
III	*rib*	6	19	2	1
III	*rib*	37	ST-449	–	1
III	*rib*	38	17	–	2
III	*rib*	39	17	–	1
III	*rib*	42	19	–	1
Ia	*alp*1	2	23	4	1
Ia	*alpha*	2	23	1	–
Ia	*alp*1	36	ST-4	1	–
II	*alpha*	12	12	1	1
II	*alpha*	8	ST-22	1	–
II	*rib*	4	19	1	–
II	*alp*2	1	1	1	–
V	*alp*3	1	1	3	–
V	*alp*1	41	19	1	–
Ib	*alpha*	10	12	1	1
Ib	*alpha*	40	12	–	1
Ib	*alpha*	43	7	1	–
IV	*alp*1	1	1	1	–
Total				37	38

Meningitis occurred only in 8.1% of cases of EOD but it was the prominent clinical condition, together with sepsis in LOD (42.1% of the cases for each). Other less frequent manifestations observed in LOD were bacteraemia and septic arthritis (10.5% and 5.3%, respectively).

Invasive GBS infection among preterm infants (gestational age between 22 and 36 weeks) occurred in 32% of cases (14 EOD and 10 LOD); mortality occurred only in this category (five EOD cases, one of which involved a twin birth). The male:female ratio was 0.88% and 69.3% of infants were Caucasian (Table S1).

Serotyping and alpha-like surface protein genes profile

Serotype III strains were the most frequent (54 strains, 72%) and possessed only the *rib* gene. Other serotypes were Ia (seven strains, 9.3%), II (five strains, 6.7%), V (four strains, 5.3%), Ib (four strains, 5.3%) and IV (one strain, 1.3%). A different distribution of serotypes among EOD and LOD cases could be noted but only serotype III was significantly associated with LOD ($p < 0.05$) (Table 1 and Table S1).

Early-onset infections were caused by serotype III (20 cases, 54%), serotype Ia (six cases, 16.2%), serotype II (four cases, 10.8%), serotype V (four cases, 10.8%), serotype Ib (two cases, 5.4%) and serotype IV (one case, 2.7%). The spectrum of serotypes responsible for the late-onset disease was restricted to serotypes III (34 cases, 89.5%), Ib (two cases, 5.3%), Ia (one case, 2.6%) and II (one case, 2.6%) (Table 1 and Table S1). The *rib* gene was the most common (55 strains, 73.3%) because of its association with serotype III strains. Only in one case was it present also in serotype II. The *alp1* gene was present in eight strains (10.7%; six serotypes Ia, one serotype IV and one serotype Ib), the *alpha* gene was present in seven strains (9.3%; five serotype Ib, two serotype II, one serotype Ia), the *alp3* gene was present only in the three serotype V strains (4%) and the *alp2* gene was detected once in a serotype II strain (1.33%) (Table 1 and Table S1).

PFGE, ST profiles and clonal complexes

Analysis of clonal relatedness of the neonatal strains by PFGE distinguished 17 groups. PFGE patterns that were found to be superimposable on those already identified in our previous study [14] received the same PFGE type designation. In particular, nine PFGE types (1, 2, 3, 4, 6, 8, 10, 12 and 19) were previously identified and eight types (from 36 to 43) were newly identified (Table S1). The PFGE groups, encompassing at least five strains, defined group 1 (five strains of which three were serotype V, one serotype II and one serotype IV), group 2 (six strains of serotype Ia) and group 3 (a very large cluster comprising 46 strains of serotype III – *rib*) (Table S1). The MLST analysis identified 17 STs, three of which were newly assigned by the database curator: ST-467 (a single-locus variant of ST-17), ST-420 (a double-locus variant of ST-17) and ST-449. The sequence types could be grouped into six clonal complexes, CC17 (49 isolates, 65.3%), CC19 (seven isolates, 9.3%), CC23 (six isolates, 8%), CC1 (five isolates, 6.7%), CC12 (five isolates, 6.7%) and CC7 (one isolate, 1.3%), plus three singletons (ST4, ST22 and ST449). The PFGE groups included strains displaying the same ST or belonging to the same clonal complex, while the same sequence type as for ST-17 and ST-19 or the same clonal complex as for CC17, CC19 and CC12 could be shared by different PFGE groups (Table 1 and Table S1).

Antibiotic resistance

Resistance to erythromycin was possessed by nine strains (12%) and was phenotypically associated with a constitutive resistance to clindamycin in five cases (four carrying the *erm*(B) gene and one carrying both the *erm*(B) and *mef*(E) genes) and with an inducible phenotype in two cases (one possessing the *erm*(A) gene and one the *erm*(T) gene). Notably, this is the first report of the presence of the *erm*(T) gene in GBS in our country. Two isolates displayed the M phenotype carried by the *mef*(E) gene. All strains but five were resistant to tetracycline (93.3%), mediated mainly by the *tet*(M) gene (97.1%). Three strains possessed both the *tet*(M) and *tet*(O) gene (Table S1).

Strain identification strings and disease manifestation

On the basis of serotype, *alp* gene, PFGE group and clonal complex, an identification string was assigned to each strain

(Table 1). The clone identified by the string 'III, *rib*, PFGE type 3, CC-17' was the most common and responsible for 18 out of the 20 EOD cases (90%) and for 28 out of the 34 LOD cases (82.3%) caused by serotype III.

The string that identified the single case of LOD caused by serotype Ia (Ia, *alp*1, PFGE type 2, CC-23) was prominent within EOD infections caused by the same serotype. Serotype Ib and II strains displayed individual strings, of which only one, for each serotype, was shared by EOD and LOD ('II, alpha, PFGE type 12, CC-12' and 'Ib, alpha, PFGE type 10, CC-12'). Serotype V strains were isolated only from EOD cases and constituted a more homogeneous clone identified mainly by the string 'V, *alp*3, PFGE type 1, ST-1'. One EOD case caused by a serotype IV isolate was also identified, which is extremely rare in infections in newborns. The identification string 'IV, *alp*1, PFGE type 1, ST-196' indicated that it belonged to the same PFGE group that included the serotype V strains, all sharing the CC1 (Table S1).

When the neonatal-maternal coupled GBS strains were available, the typing analysis demonstrated them to be indistinguishable in terms of serotype, surface proteins, PFGE subtype and MLST typing (Table S1).

Discussion

From February 2005 to June 2008 we received and analysed 75 GBS strains from neonatal invasive infections occurring in an area where an active surveillance was set up [19–21]. By combining PFGE and MLST analyses we observed a correspondence between PFGE and clonal complex. A total of 17 different PFGE types were identified among 75 GBS strains. A total of five CCs (CC-1, CC-12, CC-17, CC-19 and CC-23) plus one CC-7 strain and three singletons (ST-4, ST-22 and ST-449) were identified, with CC-17 accounting for 65.3% of all strains. In a previous survey from Italy including invasive, non-invasive and colonizing GBS strains mainly from adult patients, four major clonal groups accounted for 52.7% of all isolates: PFGE type 1/CC1 comprising mainly serotype V isolates carrying the *alp3* gene, PFGE type 2/CC23 encompassing serotype Ia isolates with the *alp1* or *alpha* gene, PFGE type 3/CC17 comprising serotype III isolates carrying the *rib* gene, and PFGE type 4/CC19 consisting of serotype II and serotype III isolates possessing the *rib* gene [14]. The same 'string associations' were observed in the present study, indicating a highly clonal GBS population structure. CC-1, CC-12, CC-17, CC-19 and CC-23 are common GBS genetic lineages encountered in studies dealing with both colonization and disease that, presumably, resulted from the emergence of host-specific clones that successfully spread globally [22]. Nevertheless, peculiar clone enrichments are represented within adult and neonatal categories [4,23,24]. It has been hypothesized that CC-1, along with CC-23, is part of those genotypes more commonly found in pregnant women, well adapted to the female genitourinary tract and easily acquired and transmitted to susceptible neonates [23]. Indeed, the serotype distribution in EOD cases was superimposable on that found in pregnant women, corroborating the hypothesis of a vertical transmission (R Creti *et al.*, unpublished data). CC-19 seems likely to cause invasive diseases among both neonates and adults [4,23,25].

In the present study, the clone identified by the string 'III, rib, PFGE type 3, CC-17' was the most prevalent and responsible for 18 out of the 20 cases of EOD (90%, $p < 0.05$) and for 28 out of the 34 cases of LOD (82.3%, $p < 0.05$). In our previous study, which comprised only 13% of perinatal infections, strains characterized by the string 'III, rib, PFGE type 3, CC17' were represented to a much lesser extent (10.6%).

The over-representation of ST-17 among invasive neonatal strains is recognized worldwide and it is a typical example of a homogeneous 'pandemic clone' with rapid dissemination and successful adaptation to human neonates [22]. It was responsible for 15 out of 19 meningitis cases. A very recent report demonstrated that the propensity of this clone to cause neonatal meningitis relies on a novel ST-17-specific surface-anchored protein (HvgA) that enhances GBS adherence and translocation across the intestinal epithelium and the blood-brain barrier [26].

One EOD case was caused by a serotype IV GBS. Both of the patient's parents were of Caucasian origin. The same indistinguishable strain was isolated from the mother's vaginorectal swab. This serotype has seldom been found as a cause of neonatal infections [4,8,27,28] but it predominates in colonized pregnant women in the United Arab Emirates and its incidence of carriage is increasing in the US, suggesting that this serotype has the potential to emerge as a neonatal pathogen [24].

A total of nine macrolide-resistant GBS strains (12%) were found. Even if not alarmingly high, this rate stresses the importance of testing GBS isolates from prenatal screening for susceptibility to clindamycin and erythromycin, used as a second choice for intrapartum antibiotic prophylaxis in the case of patients at high risk for anaphylaxis. All erythromycin-resistant isolates but one exhibited one of the resistance genotypes studied, with four isolates harbouring the *erm*(B) gene, two isolates carrying the *mef*(E) gene, one isolate possessing the *erm*(A) gene, and one isolate carrying both *erm*(B) and *mef*(E) genes. The erythromycin resistance

prevalence was slightly lower than that observed in our previous study on GBS strains from different sources of isolation (16.5%), where it was mostly due to serotype V strains belonging to CC-1 and possessing the *erm*(B) gene. Serotype V has emerged recently as a cause of human disease with a high propensity to acquire macrolide resistance and to spread throughout the population [8,14,25]. Also, a novel association between erythromycin resistance and serotype III/ST-19 has been recently reported [29]. Herein we found that macrolide resistance was not limited to a specific serotype but was present in four different serotypes, Ia (one isolate), Ib (three isolates), III (four isolates) and V (one isolate).

One GBS strain possessed the unusual macrolide resistance determinant *erm*(T). To date, inducibly clindamycin-resistant GBS *erm*(T) gene positive, have been reported only once [17]. The plasmid-borne *erm*(T) gene has been previously detected in erythromycin-resistant animal isolates of *Lactobacillus* spp. and human isolates of *Streptococcus bovis*, with which GBS shares the same ecological niche, thus suggesting that a lateral gene transfer might occur. Almost all GBS isolates (93.3%) were resistant to tetracycline, mediated by the *tet*(M) gene as already reported [14,25].

While the vertical transmission of GBS from mother to newborn is clearly involved in the development of EOD, the mode of LOD transmission is still poorly understood. In the present study we could observe that when the diseased newborn's mother was colonized at the vaginorectal level and/or her breast milk was positive for GBS, the neonate and maternal strains were identical; however, no hypothesis on the routes of transmission could be inferred.

The introduction of a GBS vaccine could have benefits in preventing GBS-related stillbirths or prematurity (32% of cases in our study) by providing protection earlier in gestation than any intrapartum prophylaxis strategy. It would also provide a longer, more effective, immunity, thus preventing late-onset GBS infections as well. Finally, an effective vaccine would also protect the mother against invasive GBS infection [30].

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Transparency Declaration

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Clinical and microbiological characteristics of the neonatal disease isolates.

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