SUSCEPIBILITY PROFILE TO PENICILLIN, ERYTHROMYCIN AND CLINDAMYCIN OF CLINICAL ISOLATES OF GROUP B STREPTOCOCCI RECENTLY ISOLATED IN BELGIUM AND DETECTION OF ERYTHROMYCIN RESISTANCE GENES

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P.Melin, C.Megali, M.P. Hayette and P. De Mol

Natl. Reference Lab. for GBS, Univ. Hosp. of Liège, Liège, Belgium

Medical Microbiology, CHU, B-23
Sart Tilman - B 4000 Liège, BELGIUM
Phone: +32-4 366 24 38
Fax: +32-4 366 24 40
Email: Pierrette.Melin@chu.ulg.ac.be

ABSTRACT

Background: Empiric therapy of severe group B streptococcal (GBS) infections, started before availability of susceptibility results, and intrapartum chemoprophylaxis to prevent early neonatal GBS disease are based on accurate susceptibility surveillance data. Increase of erythromycin (E) and clindamycin (C) resistance (R) is observed in GBS isolates in many countries. In Belgium, E-R increased up to 10 % through the 1990s and reached 19% overall with 30 % among adult isolates in 2001-2002.

Methods: 178 GBS clinical isolates from invasive GBS diseases consecutively received by the reference laboratory between January 2005 and June 2006, were from 32 neonates (22 early-onset and 10 late-onset diseases) and 146 adults. Penicillin (P), E and C MICs were determined by using Etest® strips (CLSI interpretive criteria). Furthermore, for the E-R isolates, the inducible (iMLS), constitutive (cMLS) and M phenotypes were assessed by a double-disk diffusion test and the distribution of genes encoding RNA methylases and efflux pumps was investigated by PCR.

Results: All strains were susceptible (S) to P, 32% of isolates were R to E, with a higher rate of R among serotype V (57%, p <0,001) and 19% were R to C.
Among the 58 E-R isolates, 79% exhibited the MLS_B phenotype (R to E and C), 26 were cMLS with E MIC_{50} >256 mg/L and 20 iMLS with E MIC_{50}/MIC_{90} 4/>256 mg/L. The M phenotype (R to E and S to C) was expressed by 12 (21%) of E-R isolates with E MIC_{50}/MIC_{90} 4/12 mg/L
The most common E-R genotypes were *erm*TR (36%) and *erm*B (34%), followed by *mef*A (14%), *erm*B+*erm*TR (12%) and *erm*TR+*mef*A (2%).

Conclusions: 1) P remains active against all isolates. 2) Prevalence of macrolides R has increased since the 1990s and is particularly high among serotype V isolates. 3) E-R is mainly caused by target-site modification (*erm*B, *erm*TR) mechanisms; efflux (*mef*A) R mechanism is also prevalent among these isolates. 4) R surveillance is mandatory to guide prophylaxis and treatment of serious GBS infections.

BACKGROUND

Group B streptococcus (GBS) or *Streptococcus agalactiae* is the leading cause of meningitis and sepsis in neonates and also a serious cause of mortality or morbidity in adults with underlying diseases. Penicillin is the first line antibiotic for the treatment of GBS infections or for intrapartum chemoprophylaxis, but erythromycin and clindamycin are effective recommended alternatives in the penicillin allergic patients. Empiric therapy of severe group B streptococcal (GBS) infections, starting before availability of susceptibility results, and intrapartum chemoprophylaxis to prevent early neonatal GBS disease are based on accurate susceptibility surveillance data.
Probably as a consequence of an important use of erythromycin, macrolides and related drugs, resistance among streptococcal isolates has been increasing in many countries. Through the 1990s, among clinically significant isolates of *S.agalactiae* collected in different areas of Belgium, erythromycin resistance increased from 3 to 10%. In 1999, erythromycin resistance among invasive and colonizing GBS isolates, fluctuated between 10 and 20 %. In 2001-2002, it reached 19% overall with 30 % among isolates from adults with invasive infection.
Different known mechanisms account for acquired resistance to macrolides in streptococci as the target site modification by 23S rRNA methylases, encoded by *erm* genes. The *Erm* enzymes confer cross-resistance to macrolides, lincosamides and the streptogramin B compounds, so-called MLS_B phenotype. MLS-resistance may be inducible or constitutive. Another mechanism involving an active efflux, only affects 14- and 15-membered macrolides but not 16-membered macrolides, neither lincosamides or streptogramins (M phenotype) and is encoded by *mef* genes.

OBJECTIVES

- **To monitor penicillin susceptibility among GBS recently isolated in Belgium from either neonates or adults with severe infections.**
- **To determine the rate of erythromycin (ERY) and clindamycin (CLI) resistance (R) among the same invasive isolates.**
- **To assess the distribution of macrolide resistance phenotypes and to identify the resistance genes, *erm*B, *erm*TR and *mef*A, among the erythromycin-resistant isolates.**

METHODS

Clinical isolates

Any Belgian laboratory is invited to send to the National Reference Laboratory (NRL) for GBS all their *S.agalactiae* isolated from severe infection (from blood, CSF or any deep normally sterile site) for further characterization and epidemiological purposes. A total of 178 clinical GBS strains isolated between January 2005 and June 2006 were included in this study: 22 isolates from early-onset neonatal disease, 10 from late-onset neonatal disease and 146 from adult invasive infections.

Serotypes were distributed as it follows: 17% Ia, 6% Ib, 10% II, 33% III, 6% IV, 21% V and 7% others (VI, VII and non typable).

All strains were stored in skimmed milk at -80°C.

Reference strains for specific genes,

GBS previously characterized, belonging to the collection of the NRL for GBS:

- Two strains for *erm*B gene (Ref.1546 and 1628), one for *erm*TR gene (Ref. 97123) and one for *mef*A gene (ref.167).
- Five strains as negative control (Ref. 1732, 1734, 1741, 1745 and 1747)

Determination of susceptibility and MICs

- Disks diffusion : erythromycin (15 µg) and clindamycin (2 µg) (Becton Dickinson, USA) for all isolates
- Etest® method (AB Biodisk, Sweden): benzylpenicilline strips for all isolates, erythromycin & clindamycin strips for all isolates identified ERY-R by the disk diffusion test
- Inoculum 0.5 McFarland on Mueller-Hinton agar + 5% sheep blood
- Incubation 18-24h at 35°C
- MIC breakpoints and interpretive standards (CLSI January 2006)

Determination of macrolide resistance phenotypes

- Erythromycin (15 µg) and clindamycin (2 µg) double-disk diffusion assay
- Disks placed 18 mm apart (edge to edge) on inoculated Mueller Hinton agar + 5% sheep blood ; 18-24 h incubation at 35°C.
- ***MLS_B phenotypes:***
  - ***Inducible resistance (IR):*** blunting of the clindamycin zone of inhibition proximal to the erythromycin disk or "D shaped zone"
  - ***Constitutive resistance (CR):*** resistance to both erythromycin and clindamycin
- ***M phenotype:*** susceptibility to clindamycin with no blunting of the clindamycin zone of inhibition.

Detection of resistance genes

- DNA extraction using QIAmp DNA Mini Kit (Qiagen)
- PCR amplifications with specific primers enabling detection of target genes

Protocol of PCR using gene specific primers for known macrolide resistance markers

Targets	Initial denaturation	Number of cycles	Denaturation	Annealing	Extension	Final extension	Product sizes (bp)
erm B	94°C - 10 min	30	94°C - 60 s	54°C - 60 s	72°C - 1 min	72°C - 5 min	640
erm TR		35	94°C - 30 s	45°C - 90 s			530
mef A		30	94°C - 60 s	52°C - 60 s			348

Thermocycler : PxE1,2 (Hybaid, Canada)
Following amplification and further separation by electrophoresis on 2% agarose gel combined with ethidium bromide staining, the product of PCR was visualized under UV light.

RESULTS

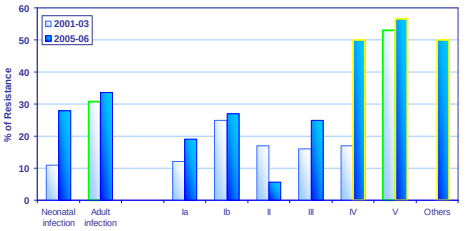
Antimicrobial susceptibility profile

Antimicrobial susceptibility profile of 178 clinical isolates of GBS (January 2005 - June 2006)

	% of Resistance	MIC_{90} (mg/L)	MIC Range (mg/L)
Penicillin	0	0.094	0.016-0.094
Erythromycin	32		
Clindamycin	19		

Erythromycin Resistance & evolution

GBS Resistance to erythromycin among different groups of patients and per serotype (January 2001-March 2003 and January 2005-June 2006)



Within the first period, isolates from adults and serotype V isolates were significantly more resistant to erythromycin ($p<0.01$ and $p< 0.001$).
More recently, neonatal isolates have demonstrated the same level of resistance to erythromycin as adult isolates. And, serotypes IV, V, VI and VII are more resistant to erythromycin ($p<0.001$).

Macrolide resistance phenotypes

Distribution of macrolide resistance phenotypes among 58 isolates of GBS resistant to erythromycin

Phenotype		%	Ery MIC_{50} / MIC_{90} (mg/L)
MLS	*Constitutive*	45	>256 / >256
	Inducible	34	4 / >256
M		21	4 / 12

Distribution of macrolide resistance genes within the different phenotypes

Distribution of the ermTR, ermB and mefA genes within the different macrolide resistance phenotypes of 58 GBS isolates

Resistance phenotype	Resistance genotype	Number of isolates (% / phenotype)
MLS constitutive (26 isolates)	***erm*B**	**19 (73)**
	*erm*TR	2 (8)
	*erm*B & *erm*TR	5 (19)
MLS inducible (20 isolates)	*erm*B	1 (5)
	***erm*TR**	**16 (80)**
	*erm*B & *erm*TR	1 (5)
	*erm*TR & *mef*A	1 (5)
	unknown	1 (5)
M (12 isolates)	*erm*TR	3 (25)
	***mef*A**	**8 (67)**
	*erm*B & *erm*TR	1 (8)

GBS strains containing the *erm*B gene resulted in a variety of phenotypes as well as for isolates containing the *erm*TR genes. The mechanisms of the phenotypic variation or expression of these genes were not investigated.

DISCUSSION & CONCLUSION

- **As in the different surveillance studies of GBS antimicrobial susceptibilities, all isolates remain fully susceptible to penicillin.**
- **The increase of resistance to macrolides becomes a relevant problem. In the 1990s, ERY-R increased in Belgium from 3 to 10% and the increase is still ongoing. In 2005-2006, with 32% of ERY-R, GBS isolated in Belgium are still quite susceptible by comparison to the 73% of resistance recently observed in France, a neighbor country.**
  - **High rates of resistance are particularly associated with serotypes IV, V, VI and VII.**
  - **In this study, by comparison to a Belgian previous surveillance (2001-2003), no more difference was observed in the rate of ERY-R among isolates from either adults or neonates.**
  - **As previously reported in Belgium or in other European countries, most of ERY-R isolates had a MLS phenotype.**
  - **The detection of MLS-IR is important: the simple and reliable double-disk diffusion test is strongly recommended.**
  - **Owing to this increasing prevalence of MLS resistance in GBS, neither macrolides no lincosamides should no longer be used without susceptibility testing.**

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