Chapter 4

Low rate of carriage of macrolide-resistant group B streptococci in pregnant women in the Netherlands

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Eur J Obstet Gynecol Reprod Biol. 2008 Mar;137(1):17-20

ABSTRACT

Objectives To describe prevalence of phenotypic and genotypic macrolide-resistance among GBS isolates in pregnant women and explore the possibility of clonal spread of resistant GBS isolates in a multicultural population.

Study design Antimicrobial resistance patterns of 107 GBS isolates obtained from asymptomatic pregnant women were determined using Etests.
Macrolide resistance genes *mef*(A), *erm*(TR) and *erm*(B) were determined with PCR and a subset of 39 isolates, including the 8 isolates harbouring macrolide resistance genes, was subjected to RAPD analysis to detect clonal spreading.

Results Resistance to erythromycin and clindamycin was found in 8% and 7%, respectively. Macrolide resistance genes *mef*(A), *erm*(TR) and *erm*(B) were found in 1, 2 and 5 isolates, respectively; only five of these eight isolates exhibited both genotypic as well as phenotypic resistance. One genotype occurred in 36% of the subset.

Conclusions Earlier reports on prevalence of phenotypic resistance were confirmed. Among the susceptible isolates one clonal type of GBS was clearly predominant; one of the resistant isolates shared its genotype. When such clonal types acquire resistance traits in the future, GBS disease may become harder to control.

INTRODUCTION

Neonatal infection with group B streptococci (GBS, *Streptococcus agalactiae)* is a universal cause of neonatal morbidity and mortality. To prevent GBS acquisition of the child during labor and delivery, intrapartum antibiotic prophylaxis is applied, usually with benzylpenicillin or, otherwise, with cefazolin, clindamycin, erythromycin or vancomycin. Emergence of resistance against these antimicrobials would decrease prophylactic efficacy. Resistance against erythromycin and clindamycin has been found in 0.7%-29% and 1.7%-21% of the strains, depending on geographical origin and temporal trends.(1-4) Fortunately, decreased susceptibility to benzylpenicillin has been scarcely reported(4;5), but clonal dissemination of such strains of decreased susceptibility would be worriesome.(6) Clonal diversity of erythromycin-resistant strains was documented in Portugal(3) and Spain.(7) Purpose of the present study was to assess phenotypic and genotypic antibiotic susceptibility patterns and putative epidemicity of GBS strains from a multicultural obstetric population in The Hague (the Netherlands).

MATERIALS AND METHODS

GBS isolates were obtained from rectovaginal cultures from asymptomatic women in the third trimester of pregnancy (January 2002 - February 2003). Isolates were kept in Amies transport medium. Incubation took place at 35-37°C for 18-24 hours in Todd-Hewitt broth with gentamycin (8 µg/ml) and nalidixic acid (15 µg/ml). Bacterial isolates were subcultured on 5% sheep blood agar in 5% CO_2. Suspect GBS colonies were subjected to Gram-staining. Catalase activity was assayed for Gram-positive cocci. GBS strains were identified by PathoDx group B® (DPC, Los Angeles, USA) and stored at -80°C in glycerol containing media.

Antibiotic susceptibility and presence of macrolide resistance genes were determined for 107 isolates from as many women. A subset of 39 strains was included in RAPD genotyping. This selection included all 8 isolates showing phenotypic macrolide resistance and 31 randomly selected isolates. Patient records revealed the countries of birth of the women.

Prior to susceptibility testing, GBS isolates were grown on 5% sheep blood agar plates for 18-24 hours in 5% CO_2. Dilutions of 0.5 McFarland were swabbed on Mueller-Hinton agar plates with 5% horse blood. E-tests (AB Biodisk, Solna, Sweden) for benzylpenicillin, cephalothin, erythromycin and clindamycin were performed according to the manufacturers' instructions. Cephalothin was tested as a representative first-generation cephalosporin. Cultures were incubated for 24 hours at 35-37°C in 5% CO_2. The MIC was recorded as indicated by the NCCLS guidelines for streptococci.

For molecular typing, bacterial DNA was extracted using lysostaphin treatment, the Bacterial DNA kit III and the MagnaPure Robot (Roche Diagnostics, Almere, The Netherlands). Macrolide resistance genes *mef*(A), *erm*(B) and *erm*(TR) were amplified from 50 ng DNA

using primers and protocols as described by Sutcliffe et al.(8) and Seppala et al.(9) PCR results were scored on the basis of the absence or presence of the correctly sized amplicon after agarose gel electrophoresis. No positive control isolates were included, allthough all of the individual PCR runs showed positive results. Negative controls involved water samples. Genotyping by RAPD was performed according to Ahmed et al.(10) using primers 12/13 (AAGTAAGTG-ACTGGGGTGAGCG), 46 (GGTTGGGTGAGAA-TTGCACG). 48 (GGCCATAGAGTG-TTGCAGACAAACTGC), 50 (GCGATCCCA) and 52 (GTGGATGCGA) DNA fingerprints were scored by two independent individuals and any change in banding pattern led to definition of a novel genotype. In case of discrepant interpretations consensus was sought through intervention of a third examiner. This resolved all discrepancies. Genotypes were defined for all separate RAPD tests, the combination of all five types led to the composite, overall RAPD genotype.(11) Finally, PCR ribotyping was performed according to Martirosian et al.(12)

RESULTS AND DISCUSSION

Samples were obtained from 1702 pregnant women between 35 and 37 weeks of pregnancy. In 365 (21%) samples GBS was cultured. From this isolate collection, we randomly selected 107 isolates. Of all 107 isolates, over 84% were susceptible to all antimicrobials tested (Table 1). Of the 9 isolates not susceptible to erythromycin, resistance or intermediate susceptibility to clindamycin was present in seven cases. Macrolide resistance genes were detected in 8 of 107 strains. Phenotypic and genotypic data for the subset of 39 strains are summarized in Table 2. No strain contained more than one macrolide resistance gene. Out of these 8 strains, 5 showed phenotypic resistance towards macrolides on lincosamides. In two phenotypically macrolide-resistant or -intermediate strains resistance genes were not detected. PCR ribotyping produced a single PCR fragment of which the nucleotide sequence precisely matched the sequence of the reference strain 2603 V/R (results not shown), confirming GBS identity. Per GBS-isolate 5 separate RAPD reactions were performed. When

Table 1 Susceptibility patterns of 107 tested GBS strains

Antibiotic*	**Susceptible (S)**		**Intermediate (I)**		**Resistant (R)**	
Benzylpenicillin	107	(100%)	0	(0%)	0	(0%)
Cephalothin	107	(100%)	0	(0%)	0	(0%)
Erythromycin	98	(92%)	2	(2%)	7	(6%)
Clindamycin	100	(93%)		(1%)	6	(6%)

*MIC breakpoints (µg/mL) used in our study are as recommended by NCCLS(8) for benzylpenicillin: S =< 0.12, I = 0.25-2, R >= 4, cephalothin: S =< 8, I = 16, R >= 32, erythromycin: S=< 0.25, I = 0.38-0.75, R >= 1, clindamycin: S =< 0.25, I = 0.38-0.75, R >= 1.

Table 2 Summary of typing data obtained from a subset of GBS isolates from The Hague

E tests (MIC values in µg/ml)					Resistance Genes			RAPD Types					Overall RAPD genotype
	penicillin G	Cephalothin	Erythromycin	Clindamycin	*mef*(A)	*erm*(B)	*erm*(TR)	12/13	46	48	50	52	
1	0.032	0.094	0.064	0.064				A	A	A	A	B	*
2	0.047	0.064	0.047	0.094				B	B	B	B	G	*
3	0.032	0.094	0.047	0.047				A	A	C	A	H	*
4	0.032	0.094	0.047	0.047				A	A	C	A	A	*
5	0.047	0.094	0.047	0.094				A	A	D	A	A	I
6	0.047	0.125	0.094	0.19				A	A	E	A	A	*
7	0.032	0.125	0.094	0.064				A	A	F	A	A	II
8	0.064	0.125	0.125	0.125				A	A	G	A	A	*
9	0.032	0.094	0.125	0.19				A	A	F	A	A	II
10	0.047	0.094	0.125	0.094				A	C	*	A	A	*
11	0.047	0.094	**2**	0.064	+			A	C	H	A	A	*
13	0.047	0.064	0.016	0.047				A	A	F	A	A	II
14	0.032	0.094	0.125	0.094				A	A	D	A	B	III
15	0.047	0.064	0.047	0.064				A	A	D	C	A	*
16	0.047	0.094	0.064	0.094				A	A	D	C	B	*
23	0.047	0.094	0.064	0.094		+		A	A	D	A	B	III
36	0.064	0.094	**>256**	**>256**			+	A	D	F	A	A	*
44	0.064	0.094	***0.50***	***12***				A	A	D	A	A	I
45	0.047	0.064	**4**	**>256**				A	E	D	A	A	IV
59	0.032	0.094	**1.5**	0.19			+	B	F	*	B	C	*
68	0.023	0.047	**>256**	*R*[a]		+		A	A	D	A	D	*
72	0.047	0.094	**>256**	**>256**		+		A	G	D	A	E	*
76	0.064	0.064	*0.50*	*0.50*				C	D	F	A	F	*
78	0.064	0.064	0.19	0.094		+		C	H	F	A	C	*
91	0.047	0.094	0.016	0.064		+		D	H	*	D	G	*
92	0.094	0.094	0.094	0.094				E	A	F	A	A	*
96	0.064	0.064	0.094	0.19				F	A	D	E	A	*
97	0.047	0.125	0.016	0.032				G	F	*	E	C	*
98	0.032	0.094	0.064	0.064				C	A	D	A	A	V
99	0.032	0.094	0.047	0.047				A	A	D	A	A	I
100	0.047	0.125	0.094	0.094				A	A	D	A	A	I

E tests (MIC values in µg/ml)					Resistance Genes			RAPD Types					Overall RAPD genotype
	penicillin G	Cephalothin	Erythromycin	Clindamycin	*mef*(A)	*erm*(B)	*erm*(TR)	12/13	46	48	50	52	
101	0.064	0.125	0.047	0.094				C	A	D	A	A	V
102	0.094	0.19	0.047	0.094				A	C	D	A	E	*
103	0.094	0.125	0.047	0.094				A	A	D	A	A	I
104	0.064	0.125	0.032	0.094				A	A	D	A	A	I
105	0.094	0.125	0.047	0.064				A	E	D	A	A	IV
106	0.094	0.125	0.047	0.064				A	A	D	A	A	I
107	0.094	0.125	0.064	0.094				A	A	D	A	A	I
108	0.064	0.125	0.047	0.094				H	A	D	A	A	*

The overall RAPD genotype combines the outcome of the five assays as defined by the different primers listed in Methods. This led to the identification of 27 genotypes. Note that strains sharing the AA*AA genotype (* any other type) may also form a clonal cluster. Only types occurring more than once have been given a serial Roman cluster code, all others are unique. Resistant isolates are marked in bold and intermediate susceptible isolates in italic.
a Resistance to clindamycin was inducible

the amplimers were analysed by gel electrophoresis on avarage 5 DNA fragments were visible per fingerprint. This amounts to an approximate number of 25 scorable fragments per isolate. Per RAPD assay a type was assigned and a single band difference led to another type designation.

Overall, 27 RAPD genotypes were identified (Table 2), 5 of which occurred more than once (I: 8/39 (21%) ; II: 3/39 (8%) ; III: 2/39 (5%) ; IV: 2/39 (5%) ; V: 2/39 (5%)). The AA*AA type occurs 14 times (36%), showing that certain (sub)clones may spread more efficiently than others. These subclonal types, sharing 4 out of 5 individual RAPD test results, shared >80% band identity. Data on the countries of birth of the patients in the subset were obtained: 18 were born in the Netherlands and the others were from 10 different countries, showing the heterogeneity of the population. As expected in this small study group, no association between country of birth and phenotypic resistance or RAPD type was found.

All phenotypically macrolide-resistant isolates were RAPD-unique, but isolate 44 shared the epidemic genotype AA*AA with antibiotic susceptible GBS isolates.

CONCLUSIONS

The prevalence of macrolide resistance among our isolates agrees with recent reports.(2;4;5) Several discrepancies between the phenotypic resistance and the presence of resistance genes are identified. Phenotypic resistance without *erm* or *mef* genes may be due to the fact that also other genes are involved in macrolide resistance.(9;13) In addition, *erm*(B) expression, often needs to be induced.(14) Furthermore, genetic variation in the *erm* and *mef* genes may also lead to false-negative PCR results. All phenotypically macrolide resistant strains were RAPD typed as unique genotypes. But among the susceptible isolates, we found a predominant GBS clone. One of the resistant isolates shared the genotype with this predominant clone, but the presence of resistance within the predominant clone is still limited to a single GBS isolate. The existence of a major antibiotic-susceptible GBS clone indicates that epidemic expansion of resistant variants could easily happen even in a heterogenous population. When resistance would expand in such epidemic isolates, efficient spread of antimicrobial resistance could take place. Continuous surveillance for resistance and its clonal spread is therefore needed, especially in the light of the increased use of antibiotics for prophylactic indications. In addition, the spread of this clone beyond the limited geographic area we analyzed in this study should be monitored by modern genotyping methods including multi locus sequence typing to better estimate its possible clinical impact.

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