Macrolide and tetracycline resistance in clinical strains of \textit{Streptococcus agalactiae} isolated in Tunisia

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Between 2007 and 2009, 226 clinical strains of \textit{Streptococcus agalactiae}, recovered from female genital specimens and from gastric fluid or ear specimens from infected newborns, were isolated at the Laboratory of Microbiology of Charles Nicolle Hospital of Tunis. They were investigated to determine the prevalence of antibiotic resistance and to characterize the mechanisms of resistance to macrolide and tetracycline. All strains were susceptible to penicillin, ampicillin and quinupristin–dalfopristin. They were resistant to clindamycin (3.1\%), rifampicin (19.1\%), erythromycin (40\%) and tetracycline (97.3\%); 3.1\% were highly resistant to streptomycin and 1.3\% to gentamicin. Among the erythromycin-resistant isolates, 78.7\% showed a constitutive macrolide–lincosamide–streptogramin B (MLSB) phenotype with high-level resistance to macrolides and clindamycin (MIC\textsubscript{50} > 256 µg ml\textsuperscript{-1}), 10\% showed an inducible MLSB phenotype with high MICs of macrolides (MIC\textsubscript{50} > 256 µg ml\textsuperscript{-1}) and low MICs of clindamycin (MIC\textsubscript{50}=8 µg ml\textsuperscript{-1}) and 2.2\% showed an M phenotype with a low erythromycin-resistance level (MIC range=12–32 µg ml\textsuperscript{-1}) and low MICs of clindamycin (MIC range: 0.75–1 µg ml\textsuperscript{-1}). All strains were susceptible to quinupristin–dalfopristin and linezolid (MIC\textsubscript{90}=0.75 µg ml\textsuperscript{-1} for each). MLSB phenotypes were genotypically confirmed by the presence of the \textit{erm}(B) gene and the M phenotype by the \textit{mef}(A) gene. Resistance to tetracycline was mainly due to the \textit{tet}(M) gene (93.1\%) encoding a ribosome protection mechanism. This determinant is commonly associated with the conjugative transposon Tn916 (P≤0.0002). \textit{tet}(O) and \textit{tet}(T) existed in a minority (2.2\% and 0.4\%, respectively). The efflux mechanism presented by \textit{tet}(L) was less frequently present (4.5\%). No significant association was found between \textit{erm}(B) and \textit{tet}(M) genes.

\textbf{INTRODUCTION}

\textit{Streptococcus agalactiae} [also designated group B \textit{Streptococcus} (GBS)], although a normal commensal of the gastrointestinal and genitourinary tracts, is one of the leading causes of morbidity and mortality among newborns (Reid, 1975; Collins \textit{et al.}, 1998). Neonatal infection generally results from vertical transmission of GBS from colonized mothers to their infants (Schrag \textit{et al.}, 2000).

Penicillin and ampicillin are the drugs of choice for prevention or treatment of \textit{S. agalactiae} infections (Andrews \textit{et al.}, 2000), and macrolides and related drugs are the recommended alternatives for patients who are allergic to \textit{b}-lactam agents. GBS is considered to be susceptible to \textit{b}-lactam antimicrobial agents, but the emergence of strains resistant to macrolides and tetracycline has increased in recent years in several countries (Betriu \textit{et al.}, 2004; Bingen \textit{et al.}, 2004; Sahnoun \textit{et al.}, 2007; Lin \textit{et al.}, 2000; Morales \textit{et al.}, 1999). Erythromycin resistance in GBS is conferred either by methylases that modify the ribosomal target of macrolides, or by active drug efflux. Ribosomal modification encoded by \textit{erm} genes (\textit{erm}B or \textit{erm}A) results in cross-resistance to macrolide–lincosamide–streptogramin B (MLSB). The phenotypic expression of MLSB resistance can be inducible (iMLSB) or constitutive (cMLS B) (Roberts \textit{et al.}, 1999). Macrolide efflux is mediated by a membrane-bound protein encoded by the \textit{mef}(A) gene, which confers resistance only to 14- and 15-membered ring macrolides, resulting in the M phenotype of resistance (Clancy \textit{et al.}, 1996).

Tetracycline resistance is due to acquisition of two mechanisms: an efflux-mediated mechanism encoded by \textit{tet}(K) or \textit{tet}(L) genes; and ribosomal protection proteins, which protect the ribosome from the action of tetracycline, mediated by \textit{tet}(M), \textit{tet}(O), \textit{tet}(S), \textit{tet}(P), \textit{tet}(Q) and \textit{tet}(U) genes (Chopra \& Roberts, 2001). Tetracycline-resistance genes are often found on mobile genetic elements that carry macrolide-resistance genes, so co-occurrence of resistance to both classes of drugs can be observed.

The aim of this study was to assess the prevalence of antibiotic resistance in GBS isolated at Charles Nicolle

\textbf{Abbreviations}: GBS, group B \textit{Streptococcus}; MLSB, macrolide–lincosamide–streptogramin B.
hospital of Tunis (a 1000-bed university hospital) from January 2007 to December 2009 and to characterize the molecular mechanisms of macrolide and tetracycline resistance.

**METHODS**

**Bacterial strains.** Between 2007 and 2009, a total of 226 clinical strains of GBS isolated at the Laboratory of Microbiology of Charles Nicolle Hospital of Tunis were investigated. They were recovered from vaginal swabs (n=120) and from gastric fluid or ear specimens from infected newborns (n=106).

All GBS strains were grown on blood agar plates incubated in 5% CO₂ at 37 °C overnight. They were identified by their colony morphology, β-haemolysis, absence of catalase, group B latex agglutination (bioMérieux) and the API Rapid ID32 STREP system (bioMérieux).

**Antimicrobial susceptibility testing.** Antimicrobial susceptibility testing was done by the disc diffusion method on Mueller–Hinton agar supplemented with 5% defibrinated horse blood according to the Clinical and Laboratory Standards Institute criteria (CLSI, 2006).

**MIC determination.** MICs of macrolides (erythromycin, clarithromycin and azithromycin), lincosamides (clindamycin), streptogramins (quinupristin–dalfopristin) and oxazolidinone (linezolid) were assessed by Etest (AB Biodisk) according to the manufacturer’s instructions.

**Detection of macrolide- and tetracycline-resistance genes.** The erm(B), erm(A) and mef(A) erythromycin-resistance genes were detected by multiplex PCR (Lopardo et al., 2003). DNA was extracted using Instagene Matrix (Bio-Rad Laboratories) and 10 μl DNA was added to 40 μl PCR mixture containing 2.5 U GoTaq DNA polymerase (Promega), 1.5 mM MgCl₂, 0.2 mM of each deoxynucleoside triphosphate and primers. Amplification was carried out in a Perkin-Elmer 2400 GeneAmp thermal cycler. The cycling conditions consisted of an initial denaturation at 95 °C for 1 min, followed by 35 cycles of denaturation at 95 °C for 1 min, primer annealing at 57 °C for 1 min and extension at 72 °C for 1 min, with a final elongation step at 72 °C for 5 min.

The tet(M), tet(O), tet(S), tet(T), tet(L) and tet(K) tetracycline-resistance determinants and the int-Tn gene, encoding the integrase of Tn916, were determined by single PCRs (Poyart et al., 2003). Each PCR was performed by adding 5 μl chromosomal template DNA to a 45 μl PCR mixture that included 2.5 U GoTaq DNA polymerase (Promega), 1.5 mM MgCl₂, 0.2 mM of each deoxynucleoside triphosphate and 0.4 μM of each primer. Amplification was performed in a Perkin-Elmer 2400 GeneAmp thermal cycler. The cycling conditions consisted of an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 1 min and 72 °C for 45 s. A final elongation step was performed at 72 °C for 7 min.

PCR products were resolved by electrophoresis on 1% agarose gels.

**Statistical analysis.** Pearson’s χ² test was used to assess inter-group significance. Statistical significance was set at P-value ≤ 0.05.

**RESULTS**

**Antibiotic susceptibility**

The susceptibility analysis showed that all isolates were fully susceptible to penicillin, ampicillin and pristinamycin; 3.1% and 19.1% of isolates were resistant to chloramphenicol and rifampicin, respectively; and 3.1% were highly resistant to streptomycin and 1.3% to gentamicin.

Forty per cent (n=90) of isolates were resistant to erythromycin and 38.4% (n=87) to clindamycin. Among the 90 erythromycin-resistant isolates, 79, 9 and 2 strains expressed the cMLSₐ, iMLSₜ and M phenotypes, respectively. All the erythromycin-resistant strains were concomitantly resistant to tetracycline.

MIC ranges, MIC₅₀ and MIC₉₀ of erythromycin, clari-thromycin, azithromycin, clindamycin, quinupristin-dalfopristin and linezolid are summarized in Table 1. The cMLSₜ and iMLSₜ phenotypes were genotypically confirmed by the presence of the erm(B) gene and the M phenotype by the mef(A) gene.

*S. agalactiae* showed a high rate of resistance to tetracycline (97.3%; n=220).

Among the 220 tetracycline-resistant isolates, tet(M) was the most frequently found determinant (n=205; 93.1%), followed by tet(L) (n=10; 4.5%), tet(O) (n=5; 2.2%) and tet(T) (1 strain; 0.4%). Four isolates harbouring the tet(O) gene also presented tet(M), while all tet(L)-positive isolates also possessed the tet(M) gene simultaneously. Neither the tet(K) nor tet(S) determinant was detected in the present study. Fourteen isolates did not harbour any of the tetracycline-resistance genes tested.

The statistical analysis revealed that there was no significant association between erm(B) and tet(M) genes.

Eighty-five per cent of tetracycline-resistant isolates harboured the int-Tn gene, encoding the integrase, and a significant correlation was found between the presence of the tet(M) gene and the int-Tn gene (P≤0.0002).

**DISCUSSION**

Penicillin is the first-line agent recommended for prophylaxis and treatment of GBS diseases. Our study confirms the uniform susceptibility to penicillin, to which resistance is rarely described (Hsueh et al., 2001). Macrolides are the recommended alternatives for penicillin hypersensitive patients, while resistance to macrolides has increased during the last decade in several countries with some geographical variations. In our hospital, 40% of GBS were resistant to erythromycin. This rate of resistance can be compared to data from Taiwan (43–46%) and the USA (54%) (Hsueh et al., 2001; DiPersio & DiPersio, 2006), but it is higher than rates reported in recent Arabian studies [0.7% in Kuwait and 13.15% in Egypt (Al-Sweih et al., 2008)].
In the present study, target site modification mediated by the presence of the \( \text{erm}(B) \) gene was the main mechanism (97.7 %) associated with cMLSB or iMLSB, as reported in Spain (Betriu et al., 2003).

The \( \text{mef}(A) \) gene, encoding an active efflux pump, was represented by only 2.2 % of isolates bearing the M phenotype. This low prevalence of the \( \text{mef}(A) \) gene among our isolates agrees with that described in France, Spain and Canada (Fitoussi et al., 2001; Betriu et al., 2003; de Azavedo et al., 2001). An overall concordance was noted between the MLS phenotype and resistance genotype.

As previously reported (de Azavedo et al., 2001), all the cMLSB\(_9\) phenotype isolates showed high-level resistance to macrolides and clindamycin (MIC\(_{90} > 256 \mu g \text{ ml}^{-1}\)); however, strains with the M phenotype showed low macrolide-resistance levels with MICs not exceeding 32 \( \mu g \text{ ml}^{-1} \).

The association of quinupristin–dalfopristin and linezolid showed good activity against all GBS isolates with an MIC\(_{90}\) of 0.75 \( \mu g \text{ ml}^{-1} \) for each. These data are similar to those reported in other studies (Gonzalez et al., 2005; Malbruny et al., 2002).

Tetracycline is a broad-spectrum antibiotic, not widely used as treatment for streptococcal infections; however, resistance to these drugs has become widespread among GBS. A high rate of tetracycline resistance was noted in our study, as was described in other studies performed in different countries: USA (96 %), Germany (74.5 %) and France (88.1 %) (Lin et al., 2000; Traub & Leonhard, 1997; De Mouy et al., 2001).

As previously reported, ribosome protection was the major mechanism detected among our isolates, and it is mainly conferred by the \( \text{tet}(M) \) resistance determinant (93.1 %), \( \text{tet}(O) \) existed in a minority (2.2 %), and \( \text{tet}(T) \), which was found for the first time in \( S. \ agalactiae \) by Poyart et al. (2003), was detected alone in one strain. Four isolates carried both \( \text{tet}(M) \) and \( \text{tet}(O) \) genes as described by Poyart et al. (2003). The efflux mechanism presented by \( \text{tet}(L) \) was less frequently present (4.5 %).

Fourteen isolates did not contain any of the tested tetracycline-resistance genes. It is possible that these isolates carried another \( \text{tet} \) determinant.

Our study confirms a significant association between the \( \text{tet}(M) \) gene and the conjugative transposon Tn916. Resistance to tetracyclines in \( S. \ agalactiae \) is assumed to be mainly due to the presence of conjugal transposons. \( \text{tet}(M) \) can be transferred via Tn916-related transposons (Flannagan et al., 1994), which may account for its spread under selective pressure.

### Table 1. MIC values (\( \mu g \text{ ml}^{-1} \)) according to macrolides-resistance phenotypes

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Erythromycin</th>
<th>Clarithromycin</th>
<th>Clindamycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC range</td>
<td>MIC(_{50})</td>
<td>MIC(_{90})</td>
<td>MIC(_{50})</td>
</tr>
<tr>
<td>cMLS(_9) (( n = 79 ))</td>
<td>4–256</td>
<td>&gt; 256</td>
<td>0.25–3.125</td>
</tr>
<tr>
<td>iMLS(_9) (( n = 9 ))</td>
<td>16–256</td>
<td>&gt; 256</td>
<td>0.125–1.5</td>
</tr>
<tr>
<td>M (( n = 2 ))</td>
<td>12–32</td>
<td>&gt; 256</td>
<td>0.25–0.5</td>
</tr>
</tbody>
</table>

[Shabayek et al., 2009], in many European countries [Germany (12 %), Belgium (16.7 %), Spain (8–18 %) and France (18–21.4 %) (Ruess et al., 2000; Decoster et al., 2005; Portillo et al., 2001; Fitoussi et al., 2001)], in Japan (3.0 %) and in Canada (18 %) (Matsubara et al., 2001; de Azavedo et al., 2001). The high rate of macrolide resistance appeared to be related to the high usage of these antibiotics.
Conclusions

To our knowledge, this is the first report to describe the distribution of phenotypic and genotypic characteristics of macrolide and tetracycline resistance among S. agalactiae strains in Tunisia.

Erythromycin resistance is relatively high in our country, and target site modification encoded by the *erm*(B) gene was the most predominant mechanism. Thus, antibiotic treatment for patients allergic to penicillin must be guided by macrolide susceptibility testing.

The high level of tetracycline resistance observed in this study was mainly due to the presence of the *tet*(M) gene encoding ribosomal protection proteins. *tet*(M) was commonly associated with the presence of the conjugative transposon Tn916.

Continued surveillance of the genetic backgrounds of antimicrobial-resistant GBS strains and more controlled use of these antibiotics in Tunisia are needed.

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**REFERENCES**


