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First clinical isolate in Europe of clindamycin-resistant group B *Streptococcus* mediated by the *lnu*(B) gene

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ABSTRACT

We characterize the mechanisms implicated in an unusual phenotype of resistance to macrolides-lincosamides (no halos of inhibition around clindamycin and lincomycin discs, and a 15 mm halo around erythromycin disc) in a *Streptococcus agalactiae* isolate recovered in Spain. The presence of macrolide or lincosamide resistance genes [*erm*(A), *erm*(B), *erm*(C), *erm*(T), *mef*(A), *mrs*(A), *lnu*(A), *lnu*(B), *lsa*(B), *lsa*(C) and *vga*(C)] was investigated by PCR and sequencing. The strain showed a resistant phenotype to erythromycin and clindamycin (MIC = 2 mg/L and MIC = 8 mg/L, respectively) and the presence of *lnu*(B) and *mef*(A) genes was demonstrated. Clinical microbiology laboratories should be aware of this unusual phenotype due to the association of two mechanisms mediated by *lnu*(B) and *mef*(A) genes. This constitute, to our knowledge, the first report of *lnu*(B) in *S. agalactiae* in human isolates in Europe.

Key words: nucleotidyltransferase, lincosamide resistance, macrolide resistance, clindamycin resistance, *Streptococcus agalactiae*.

Primer aislado clínico de *Streptococcus* grupo B con resistencia a clindamicina mediada por el gen *lnu*(B) en Europa

RESUMEN

Caracterizamos los mecanismos implicados en un fenotipo muy raro de resistencia a macrólidos-lincosamidas (sin halo de inhibición en los discos de clindamicina y lincomicina, y con 15 mm de halo en el disco de eritromicina; CMI de eritromicina = 2 mg/L y CMI de clindamicina = 8 mg/L) en una cepa clínica de *Streptococcus agalactiae* de España. Se investigaron los genes de resistencia a macrólidos y lincosamidas [*erm*(A), *erm*(B), *erm*(C), *erm*(T), *mef*(A), *mrs*(A), *lnu*(A), *lnu*(B), *lsa*(B), *lsa*(C) and *vga*(C)] mediante PCR y secuenciación y se demostró la presencia de los genes *lnu*(B) y *mef*(A). Los laboratorios de microbiología clínica deben ser conscientes de este fenotipo raro en el cual se asocian dos mecanismos mediados por los genes *lnu*(B) and *mef*(A). En nuestro conocimiento, este es el primer caso de una cepa de *S. agalactiae* de humanos con el gen *lnu*(B) en Europa.

Palabras clave: nucleotidiltransferasa, resistencia a lincosamidas, resistencia a macrólidos, resistencia a clindamicina, *Streptococcus agalactiae*.

INTRODUCTION

Group B *Streptococcus* (GBS, *Streptococcus agalactiae*) is a commensal microorganism of the gastrointestinal and genitourinary tracts. In some circumstances, it is able to cause invasive infections in neonates, pregnant women, and non-pregnant adults with underlying medical conditions, such as diabetes mellitus, or immunosuppression[1]. The vaginal and/or rectal colonisation in pregnant women represents an important risk factor to develop sepsis, pneumonia and meningitis in neonates. Penicillin G and ampicillin are the antibiotics of choice for intrapartum prophylaxis and treatment of invasive infections, while clindamycin is the recommended agent for patients who are allergic to beta-lactams[2]. However, although *S. agalactiae* remains almost always susceptible to penicillins, there is a significant and rising resistance to macrolides and lincosamides[3].

Two major resistance mechanisms to macrolides and lincosamides have been reported: alteration of the antibiotic target site and active drug efflux pump[4]. Ribosomal alteration is mediated by ribosomal methylases encoded by *erm*(B) and/or *erm*(A) genes. The drug efflux pump by a membrane-bound protein is encoded by the *mef*(A) gene. Expression of *erm*(B) and/or *erm*(A) confer cross-resistance to all macrolides, lincosamides, and streptogramins B (MLS_B phenotype), and this mechanism of resistance can be expressed constitutively (MLS_Bc) or inducibly (MLS_Bi), while expression of *mef*(A) confers resistance to 14- and 15-membered ring macrolides (M phenotype, erythromycin resistant but clindamycin susceptible).

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A third unusual mechanism of resistance has been previously reported, mediated by lincosamide nucleotidyltransferases encoded by *lnu* genes, responsible for the enzymatic inactivation of lincosamides by a mechanism of adenylation [5-7]. Inactivation of lincosamides mediated by *lnu* genes was firstly described in *Enterococcus faecium* HM1025 strain[8]. The L-phenotype (erythromycin susceptible but clindamycin resistant) mediated by *lnu*(B) expression has been recently described in *S. agalactiae* in specific areas from Korea[7], Latin-America[5] and Canada[6], while, to our knowledge, no cases have been previously reported in strains from humans in Europe.

The aim of this report is to describe the emergence in Europe of *S. agalactiae* harbouring the *lnu*(B) gene.

MATERIAL AND METHODS

S. agalactiae isolate 12/30482 was recovered from a vaginorectal exudate of a pregnant woman in a Spanish hospital (Madrid, Spain) and tested for clindamycin and erythromycin susceptibility by using disc-diffusion and epsilon test methods. Minimal inhibitory concentrations of penicillin, cefotaxime, vancomycin, erythromycin, clindamycin, tetracycline, and levofloxacin were determined by microdilution using WIDER system, being the results interpreted according to EUCAST[9].

DNA extraction of isolate 12/30482 was carried out by InstaGene Matrix (BioRad, Hercules, CA, USA). The presence of the genes *erm*(A), *erm*(B), *erm*(C), *erm*(T), *mef*(A), *mrs*(A), *lnu*(A), *lnu*(B), *lsa*(B), *lsa*(C), and *vga*(C) was analysed by PCR using primers and conditions previously described (table 1). Multilocus sequence typing (MLST) was performed by PCR/sequencing[10], and molecular capsular typing was developed by multiplex-PCR[11].

RESULTS

The *S. agalactiae* isolate 12/30482 was resistant to clindamycin and erythromycin by disc-diffusion. In order to study the resistant profile to macrolides and lincosamides, a triple disc diffusion assay with clindamycin-erythromycin-lincomycin was developed. No halos of inhibition around clindamycin and lincomycin discs were observed, while a 15 mm halo around erythromycin disc was visualized, indicating a phenotype different from the *erm*-mediated phenotypes (MLS_Bc or MLS_Bi) previously reported and suggesting that this strain had

Table 1 **Primers and conditions used for detection by PCR of the resistance genes in this study.**

Target	Primer 5'→3'	Annealing temperature (ºC)	Amplicon size (bp)	Reference
erm(A)	F: TCTAAAAAGCATGTAAAAGAA	52	645	14
	R: CTTCGATAGTTTATTAATATTAG			
erm(B)	F: GAAAAGTACTCAACCAAATA	52	639	14
	R: AGTAACGGTACTTAAATTGTTTA			
erm(C)	F: TCAAAACATAATATAGATAAA	52	642	14
	R: GCTAATATTGTTTAAATCGTCAAT			
erm(T)	F: ATTGGTTCAGGGAAAGGTCA	45	550	15
	R: GCTTGATAAAATTGGTTTTTGG			
mef(A)	F AGTATCATTAATCACTAGTGC	52	348	14
	R TTCTTCTGGTACTAAAAGTGG			
mrs(A)	F: GCAAATGGTGTAGGTAAGACAACT	55	399	16
	R: ATCATGTGATGTAAACAAAAT			
lnu(A)	F: GGTGGCTGGGGGGTAGATGTATTAACTGG	57	323	17
	R: GCTTCTTTTGAAATACATGGTATTTTTCGATC			
lnu(B)	F: CCTACCTATTGTTTGTGGAA	54	944	8
	R: ATAACGTTACTCTCCTATTC			
lsa(B)	F: TGCCGAAGCCATGTACCGTCC	55	396	18
	R: CGGTTAGACCAACCAGCCGAACG			
lsa(C)	F: GGCTATGTAAAACCTGTATTTG	55	429	19
	R: ACTGACAATTTTTCTTCCGT			
vga(C)	F : CCGTATGCCCAGAGTGAGAT	58	671	15
	R : TGCTTGGGAACAAGTCCTTC			

a L-phenotype. MICs by WIDER system showed that this isolate was susceptible to penicillin (0.06 mg/L), cefotaxime (≤ 0.03 mg/L), vancomycin (0.5 mg/L), but resistant to clindamycin (> 1 mg/L), erythromycin (> 1 mg/L), tetracycline (> 4 mg/L) and levofloxacin (> 8 mg/L). MICs by the epsilon test were 2 mg/L for erythromycin and 8 mg/L for clindamycin.

The *S. agalactiae* isolate 12/30482 harboured the *lnu*(B) and *mef*(A) genes, being negative for the other resistance genes tested. In the case of *lnu*(B), 95% of the gene was sequenced and compared to GenBank accession no. AJ238249. Besides, this isolate was ascribed to the sequence type 19 (ST19) and belonged to capsular type III.

DISCUSSION

Our study shows a new antibiotic resistance phenotype in GBS mediated by *lnu*(B) and *mef*(A) genes, which confers resistance to erythromycin (with halo of inhibition around the disc) and a clear resistance to lincosamides (clindamycin and lincomycin). The *lnu*(B) phenotype is identified for the first time in a human clinical isolate in Europe, since other cases have been recently published in other parts of the world[5-7].

A recent study in Spain showed that in GBS (n=689) the rate of erythromycin and clindamycin resistance were 15.7 % and 15.8%, respectively, and that the MLS_Bc was the predominant phenotype (75%)[12]. Interestingly, the uncommon erythromycin-susceptible and clindamycin-resistant phenotype was found in four GBS, but *lnu*(B) gene was not detected by PCR suggesting that other genes could be involved in this resistance phenotype.

In this case, we describe two mechanisms (*lnu*(B)- and *mef*(A)-mediated) in the same isolate. The presence of *lnu*(B) gene confers resistance to clindamycin, different from the constitutive and inducible *erm*-mediated phenotypes (MLS_Bc or MLS_Bi). Additionally, resistance to erythromycin was also observed, explained by the *mef*(A) gene expression which was also detected by PCR and sequencing. No other gene conferring macrolide or lincosamide resistance was detected. The association of *lnu*(B) and *mef*(A) suggest that these genes could be linked either physically or functionally in circulating *S. agalactiae* strains. Previous reports have described *mef*(A) gene associated with the carrying element mega in several strains of *S. agalactiae*, while the single *mef*(A) subclass gene has been found to be associated with the genetic element Tn1207[6] suggesting that transformation is the main mechanism through which this resistance gene is acquired[13]. To date, it is not known if the *lnu*(B) gene in *S. agalactiae* is also located on mobile genetic elements[7].

On the other hand, it is important to take into account that this mechanism occurs in an isolate which belongs to a capsular type III (serotype III), consistent with a previous report in which the GBS strains harbouring *lnu*(B) gene belonged to this serotype[7]. This is an important conclusion due to the possible consequences regarding changes in molecular epidemiology and transmission.

In conclusion, the emergence of *S. agalactiae* harbouring the *lnu*(B) gene in Europe is described for the first time. Nevertheless, more clinical isolates need to be detected and more studies need to be done to know how this mechanism is acquired, because *S. agalactiae* is a common commensal microorganism and could exchange genetic material with other bacteria of the human microbiota.

REFERENCES

1. Schuchat A. Epidemiology of group B streptococcal disease in the United States: shifting paradigms. Clin Microbiol Rev 1998; 11:497-513.
2. Matteson KA, Lievense SP, Catanzaro B, Phipps MG. Intrapartum group B streptococci prophylaxis in patients reporting a penicillin allergy. Obstet Gynecol 2008; 111:356-64.
3. Gygax SE, Schuyler JA, Kimmel LE, Trama JP, Mordechai E, Adelson ME. Erythromycin and clindamycin resistance in group B streptococcal clinical isolates. Antimicrob Agents Chemother 2006; 50:1875-7.
4. Domelier AS, van dM-M, Arnault L, Mereghetti L, Lanotte P, Rosenau A, et al. Molecular characterization of erythromycin-resistant *Streptococcus agalactiae* strains. J Antimicrob Chemother 2008; 62:1227-33.
5. Faccone D, Lalonardi F, Abel S, Machain M, Errecalde L, Littvik A, et al. Multiple-Clones of *Streptococcus agalactiae* harbouring lnuB gene. J Infect Dev Ctries 2010; 4:580-2.
6. de Azavedo JC, McGavin M, Duncan C, Low DE, McGeer A. Prevalence and mechanisms of macrolide resistance in invasive and noninvasive group B *Streptococcus* isolates from Ontario, Canada. Antimicrob Agents Chemother 2001; 45:3504-8.
7. Seo YS, Srinivasan U, Oh KY, Shin JH, Chae JD, Kim MY, et al. Changing molecular epidemiology of group B *Streptococcus* in Korea. J Korean Med Sci 2010; 25:817-23.
8. Bozdogan B, Berrezouga L, Kuo MS, Yurek DA, Farley KA, Stockman BJ, et al. A new resistance gene, linB, conferring resistance to lincosamides by nucleotidylation in *Enterococcus faecium* HM1025. Antimicrob Agents Chemother 1999; 43:925-9.
9. The European Committee on antimicrobial susceptibility testing. Break point tables for interpretation of MICs and zone diameters. Version 3.0. European Society of Clinical Microbiology and Infectious Diseases. 2013;Available from: URL: http://www.eucast.org
10. Jones N, Bohnsack JF, Takahashi S, Oliver KA, Chan MS, Kunst F, et al. Multilocus sequence typing system for group B *Streptococcus*. J Clin Microbiol. 2003; 41:2530-6.
11. Poyart C, Tazi A, Reglier-Poupet H, Billoet A, Tavares N, Raymond J, et al. Multiplex PCR assay for rapid and accurate capsular typing of group B streptococci. J Clin Microbiol 2007; 45:1985-8.
12. Merino DL, Torres Sanchez MJ, Aznar MJ. Prevalence and mechanisms of erythromycin and clindamycin resistance in clinical isolates of beta-haemolytic streptococci of Lancefield groups A, B, C and G in Seville, Spain. Clin Microbiol Infect 2008; 14:85-7.

13. Marimon JM, Valiente A, Ercibengoa M, Garcia-Arenzana JM, Perez-Trallero E. Erythromycin resistance and genetic elements carrying macrolide efflux genes in *Streptococcus agalactiae*. Antimicrob Agents Chemother. 2005; 49:5069-74.
14. Sutcliffe J, Grebe T, Tait-Kamradt A, Wondrack L. Detection of erythromycin-resistant determinants by PCR. Antimicrob Agents Chemother 1996; 40:2562-6.
15. Fessler A, Scott C, Kadlec K, Ehricht R, Monecke S, Schwarz S. Characterization of methicillin-resistant *Staphylococcus aureus* ST398 from cases of bovine mastitis. J Antimicrob Chemother 2010; 65:619-25.
16. Wondrack L, Massa M, Yang BV, Sutcliffe J. Clinical strain of *Staphylococcus aureus* inactivates and causes efflux of macrolides. Antimicrob Agents Chemother 1996; 40:992-8.
17. Lina G, Quaglia A, Reverdy ME, Leclercq R, Vandenesch F, Etienne J. Distribution of genes encoding resistance to macrolides, lincosamides, and streptogramins among staphylococci. Antimicrob Agents Chemother 1999; 43:1062-6.
18. Gomez-Sanz E, Torres C, Lozano C, Fernandez-Perez R, Aspiroz C, Ruiz-Larrea F, et al. Detection, molecular characterization, and clonal diversity of methicillin-resistant *Staphylococcus aureus* CC398 and CC97 in Spanish slaughter pigs of different age groups. Foodborne Pathog Dis 2010; 7:1269-77.
19. Malbruny B, Werno AM, Murdoch DR, Leclercq R, Cattoir V. Cross-resistance to lincosamides, streptogramins A, and pleuromutilins due to the *lsa*(C) gene in *Streptococcus agalactiae* UCN70. Antimicrob Agents Chemother 2011; 55:1470-4.