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Research Paper

Conjugative transfer of resistance determinants among human and bovine *Streptococcus agalactiae*

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Abstract

Streptococcus agalactiae (GBS) is a major source of human perinatal diseases and bovine mastitis. Erythromycin (Ery) and tetracycline (Tet) are usually employed for preventing human and bovine infections although resistance to such agents has become common among GBS strains. Ery and Tet resistance genes are usually carried by conjugative transposons (CTns) belonging to the Tn*916* family, but their presence and transferability among GBS strains have not been totally explored. Here we evaluated the presence of Tet resistance genes (*tet*M and *tet*O) and CTns among Ery-resistant (Ery-R) and Ery-susceptible (Ery-S) GBS strains isolated from human and bovine sources; and analyzed the ability for transferring resistance determinants between strains from both origins. Tet resistance and *int*-Tn genes were more common among Ery-R when compared to Ery-S isolates. Conjugative transfer of all resistance genes detected among the GBS strains included in this study (*erm*A, *erm*B, *mef*, *tet*M and *tet*O), in frequencies between 1.10^{-7} and 9.10^{-7}, was possible from bovine donor strains to human recipient strain, but not the other way around. This is, to our knowledge, the first report of *in vitro* conjugation of Ery and Tet resistance genes among GBS strains recovered from different hosts.

Key words: *Streptococcus agalactiae* (GBS), conjugative transposons, erythromycin resistance, tetracycline resistance.

Introduction

Streptococcus agalactiae (group B *Streptococcus*; GBS) emerged as a major source of perinatal diseases in the 60's (Eickhoff *et al.*, 1964), after a long history of contributing mostly to mastitis in cows. In Brazil, GBS prevalence among herds has been reported to be around 60% (Duarte *et al.*, 2004; Keefe, 2012). Bovine mastitis control is easily achieved using intramammary infusions containing high levels of one or more antibiotics, including mainly penicillins, cephalosporins, tetracyclines, aminoglycosides and macrolides (Keefe, 2012). On the other hand, rates of gastrointestinal and urogenital colonization among humans range between 10 and 30% (Simões *et al.*, 2007; Linhares *et al.*, 2011). Most of this number is constituted by pregnant women, and penicillin or macrolides are the recommended drugs for intrapartum chemoprophylaxis (CDC, 2010).

GBS remains susceptible to penicillin, with rare exceptions (Kimura *et al.*, 2011). Resistance to erythromycin (Ery), however, has emerged in the USA and Europe, with rates between 20 and 50% (Dipersio and Dipersio, 2006; Sadowy *et al.*, 2010). In Brazil, Ery-resistant (Ery-R) GBS strains usually don't exceed 10% of prevalence (D'Oliveira *et al.*, 2003; Duarte *et al.*, 2005; Palmeiro *et al.*, 2010; Corrêa *et al.*, 2011). Tetracycline (Tet) resistance rates are known to be as high as 70-80% among strains from both human and bovine origins (D'Oliveira *et al.*, 2003; Duarte *et al.*, 2004, 2005; Dogan *et al.*, 2005; Gao *et al.*, 2012). Resistance to tetracycline in GBS is typically mediated by the *tet*M gene, while Ery resistance is usually due to the pres-

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ence of the *erm*B gene (Poyart *et al.*, 2003). These resistance genes are usually present in conjugative transposons (CTns), which can be horizontally transferred; most of them belong to the Tn*916*-Tn*1545* family (Roberts and Mullany, 2011).

Recently, we reported a considerably higher Ery resistance level among bovine GBS strains (27.6%) when compared to human isolates (4.0%; p = 0.0277) in Brazil (Pinto *et al.*, 2013). Moreover, in the same study, around 90% of both human and bovine isolates were Tet-resistant (Tet-R). The presence and transferability of CTns among GBS isolates, however, have not been totally explored. Therefore, in this study, we evaluated the presence of Tet resistance genes and CTns among Ery-R and Ery-S GBS strains isolated from human and bovine sources, and analyzed the ability for transferring erythromycin and tetracycline resistance determinants between strains from different origins.

Materials and Methods

Bacterial strains

Eighty-nine GBS strains were selected according to their susceptibility profile, among a collection previously evaluated by our group (Pinto *et al.*, 2013), and clustered into three main groups as follows:

The first group comprised twenty-three Ery-R GBS strains, including fifteen of human and eight of bovine origin, which were also Tet-R and represented different Ery resistance genotypes. The second group included 23 erythromycin-susceptible (Ery-S)/Tet-R GBS strains, comprising twelve of human and eleven of bovine origin. In the third group, 43 Ery-S and Tet-susceptible (Tet-S) GBS strains were included.

Detection of tetracycline resistance genes and *int*-Tn gene

The presence of *tet*M and *tet*O genes was evaluated by PCR as previously described (Trzcinski *et al.*, 2000). Likewise, the presence of the gene coding for the integrase of the Tn*916*-Tn*1545* family of CTns was evaluated by PCR as recommended elsewhere (Poyart *et al.*, 2003). DNA extraction was performed according to Sambrook *et al.* (1989).

Statistical analysis

The Student's *t* test was employed and differences were considered statistically significant when $p < 0.05$.

Filter mating experiments

Seven Ery-R GBS strains were selected as donors according to their Ery resistance profile, while two Ery-S strains were selected as the recipients. Donors were susceptible to chloramphenicol while recipients were resistant to this drug. Their characteristics are illustrated in Table 1. Minimal inhibitory concentrations (MIC) of erythromycin, tetracycline and chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA) were determined by the broth dilution method (CLSI, 2011) for the nine GBS strains.

Filter mating experiments were done using previous recommendations (Horaud *et al.*, 1996), with the following modifications: strains were grown in Todd-Hewitt broth (THB; BD Difco, BD Diagnostics, Franklin Lakes, NJ, USA), and the initial inoculum was adjusted to OD_{540} of 0.4. Chloramphenicol, erythromycin and tetracycline were used as the selective drugs for transconjugants, with concentrations varying according to the MIC levels of each strain.

Frequency of conjugation was expressed as the number of transconjugants per colony forming unit (CFU) of re-

Table 1 - Characteristics of the nine *Streptococcus agalactiae* strains included in the filter mating conjugation experiments performed in this study.

Strain	Clinical origin	Erythromycin genotype	Tetracycline genotype	*int*-Tn	Frequency[a]	Added to the medium[b]
Donor strains						
87169	Bovine	*erm*A/*mef*	None	+	1.10^{-7}	None
90003	Human	*mef*	*tet*M	+	9.10^{-7}	Erythromycin
96008	Human	*erm*B	*tet*O	+	9.10^{-7}	Tetracycline
96009	Human	*erm*B	*tet*O	-	ND	NA
02031	Human	*erm*A	*tet*O	-	ND	NA
06003	Bovine	*erm*B/*mef*	*tet*M/*tet*O	-	ND	NA
06005	Bovine	*erm*B/*mef*	*tet*M/*tet*O	+	1.10^{-7}	Erythromycin/ tetracycline
Recipient strains						
88596	Human	None	None	-	NA	NA
B66	Bovine	None	None	-	NA	NA

[a]Frequency of conjugation expressed as the number of transconjugants per cfu of recipient; ND, not determined since transconjugants were not detected; NA, not applicable.

[b]Antimicrobials added to the conjugation medium in some replicas: erythromycin at 0.5 μg/mL and tetracycline at 4 μg/mL; NA, not applicable.

cipient. Donor and recipient control plates were included to discard the possibility of spontaneous mutation, and all the experiments were done in duplicates, in three independent assays. DNase (1 g/L) (Sigma-Aldrich) was added to the conjugation medium to exclude the possibility of transformation. Moreover, subinhibitory concentrations of erythromycin (0.5 μg/mL) and tetracycline (4 μg/mL) were also added in some replicas to verify their influence on conjugation frequency.

The transconjugants were isolated, identified as GBS using a Streptococcal Grouping Kit (Oxoid, Basingstoke, Hampshire, UK), and characterized regarding antimicrobial susceptibility profile (disk-diffusion and MIC), presence of resistance genes and of *int*-Tn gene, as described above.

Results

Distribution of *tet* and *int*-Tn genes among Ery-R GBS strains

The *tet*M gene was detected in fourteen of the twenty-three Ery-R strains (60.9%), while *tet*O was found in seventeen (73.9%). Ten strains harbored both genes simultaneously, and two had none of the genes. Combining Ery and Tet determinants, the most common genotype among Ery-R GBS strains was *erm*B/*mef*/*tet*M/*tet*O.

A total of 87.0% (20/23) of the Ery-R GBS strains had the *int*-Tn gene, including thirteen from human origin and seven from bovine origin. Only two *int*-Tn-positive strains had no *tet* genes, and only one strain harboring a *tet* gene (*tet*O) did not present *int*-Tn.

Distribution of *tet* and *int*-Tn genes among Ery-S/Tet-R GBS strains

Among the twenty-three Ery-S/Tet-R GBS strains, *tet*M was detected in seven isolates (30.4%), while *tet*O was observed in ten (43.5%). Five strains had both genes, and eleven did not present any of them. A total of 65.2% (15/23) harbored the *int*-Tn gene, including five from human origin and ten from bovine origin. Four *int*-Tn-positive strains had no *tet* genes, and only one *tet*O-positive strain did not present *int*-Tn.

Distribution of *tet* and *int*-Tn genes among Ery-S/Tet-S GBS strains

Among the forty-three Tet-S GBS strains, forty-two did not amplify any of the *tet* genes tested. One single Tet-S strain, isolated from human oropharynx and belonging to serotype Ia, had the *tet*M gene. A total of 11.6% (5/43) harbored the *int*-Tn gene, comprising three of human origin (*tet*M-positive isolate included) and two of bovine origin.

Conjugative transfer of resistance-associated genes

Matings with the human GBS isolate as the recipient were successful when using four of the seven selected donors, in frequencies between 1.10^{-7} and 9.10^{-7} (Table 1). Addition of subinhibitory concentrations of erythromycin and/or tetracycline to the conjugation medium was a crucial factor for three of them. No transconjugants were observed when the bovine GBS isolate was used as the recipient strain.

All the resistance determinants observed among the selected GBS strains (*erm*A, *erm*B, *mef*, *tet*M and *tet*O) could be transferred *in vitro*, always in combination with *int*-Tn gene (Table 1). The three donor strains that did not generate transconjugants lacked the *int*-Tn gene.

Discussion

Contrasting with data from the literature (Poyart *et al.*, 2003; Dipersio and Dipersio, 2006; Cochetti *et al.*, 2007), *tet*O gene was the prevalent Tet determinant among both human and bovine GBS strains in this study. While only one Ery-R strain did not amplify any of the *tet* genes tested, 11 Ery-S did not present any *tet* gene. This statistically significant difference ($p = 0.0282$) suggests a linkage between *tet* genes and erythromycin-resistance determinants among our strains.

There are multiple descriptions of association between *tet*M and *erm*B genes on the same mobile genetic element in *Streptococcus pyogenes* and *Streptococcus pneumoniae* (Varaldo *et al.*, 2009). Such association, however, has been rarely evaluated in *S. agalactiae* (Varaldo *et al.*, 2009; Haenni *et al.*, 2010), and might be underestimated.

The *int*-Tn gene was detected in 11.6% of the Ery-S/Tet-S GBS strains, in 65.2% of the Ery-S/Tet-R strains, and in 87.0% of the Ery-R/Tet-R isolates, suggesting an association between resistance determinants and CTns ($p = 0.0334$).

Only one strain showing phenotypic susceptibility to Tet harbored the *tet*M gene, in combination with the *int*-Tn gene. This strain presented an inhibition zone indicative of Tet susceptibility in the disk-diffusion test, which was confirmed by MIC levels (2 μg/mL). Although reported for other streptococcal species, such as *S. pneumoniae* (Cochetti *et al.*, 2007) and *S. pyogenes* (Brenciani *et al.*, 2007), and for some bovine GBS isolates (Gao *et al.*, 2012), the reasons behind the lack of expression of such resistance gene still have to be evaluated. Possible explanations include distant, weak or absent promoter, or presence of mutations (Gao *et al.*, 2012). However, although in a silent form, they can still be carried by Tn*916*-related genetic elements (Cochetti *et al.*, 2007).

Although *tet*M is the typical Tet resistance determinant shared among the members of Tn*916*-Tn*1545* family, mobile elements carrying other less common tetracycline resistance genes, such as *tet*O (Varaldo *et al.*, 2009; Brenciani *et al.*, 2010) and *tet*S (Haenni *et al.*, 2010), have been reported. Likewise, a variety of Ery resistance genes, such

as *mef*A and *erm*A, besides the most commonly found *erm*B, have also been associated with *tet* genes and CTns, mainly in *S. pyogenes* isolates (Brenciani *et al.*, 2007; Varaldo *et al.*, 2009). Indeed, all the five resistance-associated genes detected among the GBS strains included in this study (*erm*A, *erm*B, *mef*, *tet*M and *tet*O) could be transferred *in vitro*, in different combinations. Moreover, no transconjugants were observed when using donor strains presenting identical genotypes but lacking the *int*-Tn gene (Table 1), suggesting the association of lateral dissemination of resistance genes with CTns.

In this study, conjugation was evident from bovine donor strains to human recipient strain, but not from human donor strains to bovine recipient strain. Additional experiments are required to check whether this limitation was strain-specific, since only one bovine strain was tested as the recipient, or if it is universally observed for bovine isolates. Despite this fact, the higher resistance levels observed among bovine isolates and their probable transmission to humans uncover possibilities of a bovine origin for erythromycin and tetracycline resistance among human GBS isolates.

Conjugation frequencies ranged between 1.10^{-7} and 9.10^{-7}, which are similar to indexes observed by other authors for other streptococcal species (Giovanetti *et al.*, 2002; Martel *et al.*, 2005), but represent numbers 2 to 90 times higher than those previously observed for bovine GBS isolates (Haenni *et al.*, 2010).

The addition of Ery and/or Tet in the conjugation medium was a decisive step for three of the four successful conjugation experiments, as it was previously suggested (Bahl *et al.*, 2004), indicating the important role of selective pressure in the dissemination of resistance determinants. In the veterinary field, this selective pressure is represented by the intramammary infusions, which are slowly released for therapeutic purposes. As consequences, active levels of antimicrobials are maintained for an extended period of time and drug residues can be found in the milk of treated cows (Keefe, 2012).

Genomic studies have demonstrated the presence of CTns and the importance of horizontal gene transfer to the evolutionary process of GBS (Brochet *et al.*, 2008). These elements usually carry resistance determinants and their dissemination is probably also driven by the abusive usage of antimicrobials. High Tet resistance rates currently observed among GBS isolates, and possibly driven by mobile genetic elements such as those from the Tn*916* family, indicate the danger of a similar scenario for macrolides in the future. Although some CTns carrying Ery and Tet resistance determinants had been described among GBS strains (Marimón *et al.*, 2005; Achard and Leclercq, 2007), the transferability of such elements between human and bovine isolates had never been demonstrated. Therefore, this is, to our knowledge, the first report of *in vitro* conjugation of Ery and Tet resistance genes, in combination with *int*-Tn gene, among GBS strains recovered from different hosts.

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