Demonstration of a shift towards penicillin resistance in the *Streptococcus uberis* population

Penicillin resistance has been particularly well studied in *Streptococcus pneumoniae* (Denapate *et al.*, 2007; Hoban *et al.*, 2005). In this organism, resistance frequently occurs through modifications of the penicillin-binding proteins (PBPs) and/or the generation of mosaic PBPs, leading to a decreased affinity for the drug (Nagai *et al.*, 2002). Among clinically relevant streptococci, *S. pneumoniae* primarily infects humans, while the environmental *Streptococcus uberis* principally colonizes cattle, in which it is responsible for about one third of cases of clinical and subclinical mastitis (Botrel *et al.*, 2010; Bradley *et al.*, 2007), a type of disease causing major economic loss in the dairy industry worldwide. Currently, penicillin remains one of the first-line antibiotics for the treatment of such pathologies but, in contrast to pneumococci and despite several decades of widespread use, no field case of true penicillin-resistant *S. uberis* [MIC >16 mg l⁻¹, according to the CA-SFM (Comité de l'Antibiogramme de la Société Française de Microbiologie) – Antibigram Committee of the French Society for Microbiology] has been described yet. Furthermore, it is still commonly believed that *S. uberis* and other *Streptococcus* spp. implicated in animal intramammary infections are susceptible to this drug. However, isolates presenting decreased susceptibility (MIC of 0.25 to 0.5 mg l⁻¹) are regularly reported to our laboratory, and a recent study demonstrated the capacity of *S. uberis* to develop penicillin resistance through a PBP-mediated mechanism similar to *S. pneumoniae* and the other streptococci studied so far (Haenni *et al.*, 2010).

At present, the official criteria for the MIC determination of penicillin-resistant *S. pneumoniae* (sensitive, <0.06 mg l⁻¹; resistant, ≥2 mg l⁻¹) are coherent between the European Committee on Antimicrobial Susceptibility Testing (<http://www.eucast.org>), the American Clinical and Laboratory Standards

Institute and the French CA-SFM (CA-SFM, 2008; CLSI, 2008). On the contrary, criteria for non-*S. pneumoniae* streptococci are not harmonized and the classification of *S. uberis* itself is problematic. This bacterium was formerly associated to the viridans group because of its α-haemolysis, but is now considered as a member of the pyogenic group with a close phylogenetic link to *Streptococcus pyogenes*, which is apparently incapable of acquiring penicillin resistance *in vivo* (Perez-Trallero *et al.*, 2001). In France, the CA-SFM recommends that penicillin resistance is suspected for all non-pneumococcal streptococci isolated from animals when a decreased susceptibility to 5 µg oxacillin is detected by disc diffusion (resistant if <21 mm). Since it was recently shown that *S. uberis* can develop resistance to penicillin *in vitro* (Haenni *et al.*, 2010), and since isolates presenting decreased susceptibility have already been reported (Guerin-Faubleee *et al.*, 2002; Mevius *et al.*, 2007; Rossitto *et al.*, 2002), this work intended to further characterize field isolates classified as either oxacillin resistant or oxacillin sensitive, in order to get a

better insight of the potential population shift towards penicillin resistance in *S. uberis*.

A total of 185 *S. uberis* strains isolated from bovine mastitis and identified by species-specific PCRs (Riffon *et al.*, 2001) was included in this study. A total of 90 strains were considered as oxacillin resistant (oxa^R) with disc diffusion diameters ranging between 15 and 20 mm, whereas 95 were oxacillin sensitive (oxa^S) with diameters ranging from 21 to 40 mm. MICs to oxacillin, penicillin, amoxicillin and ampicillin were then determined by the agar dilution method on Muller–Hinton agar containing 5% sheep blood (Bio-Rad), by twofold dilutions ranging from 0.002 to 8 mg l⁻¹. All isolates were tested according to the recommendations of the CA-SFM (2008).

As presented in Fig. 1, the 90 oxa^S strains detected by disc diffusion were confirmed as negative by the agar dilution method. On the contrary, only 2 strains out of 95 oxa^R were considered as resistant, despite the fact that both populations differed significantly (*P*<0.01, Student's *t*-test).

Fig. 1. Distribution of the MIC values (mg l⁻¹) for oxacillin. Results of the population described as oxacillin sensitive by disc diffusion are indicated by white bars, and those described as oxacillin resistant by disc diffusion are indicated by black bars. The arrow indicates the breakpoint between sensitive and resistant isolates.

This discrepancy proves that the oxacillin disc diffusion test is a poor indicator of clinical resistance and should not have been used as a predictor of the therapeutic outcome.

Similar results were observed for penicillin and amoxicillin (Fig. 2a, b). Indeed, the bimodal distributions demonstrated a shift in the population, yet all isolates were classified as sensitive according to the CA-

SFM. For ampicillin (Fig. 2c), there was a step-by-step evolution towards decreased resistance, and 46 *oxa^R* strains (48 %) were considered as having intermediate resistance by the agar dilution method. Thus, for this particular antibiotic, the population shift towards resistance was congruent with a modification in the resistance pattern. Therefore, the oxacillin disc result can be used as a marker of

epidemiological evolution, independently of any clinical consideration.

The reality of this shift can also be inferred from our recent data on the molecular mechanisms of penicillin resistance in *S. uberis* (Haenni *et al.*, 2010). Indeed, in that study, the presence of penicillin-resistance-associated mutations (including the prototypic Q₅₅₄E substitution in the PBP2X) was systematically detected in *S. uberis* presenting an MIC above 0.12 mg l⁻¹ – in both laboratory-evolved mutants and field isolates – whereas these mutations were absent in the isolates presenting a MIC of 0.06 mg l⁻¹. Therefore, while still considered as sensitive according to the French clinical breakpoints, isolates presenting an MIC ≥ 0.12 mg l⁻¹ already display resistance mechanisms to β -lactams. Consequently, these isolates cannot be considered anymore as epidemiologically representative of a wild-type sensitive population and, moreover, contribute to an animal reservoir of potentially exchangeable PBP-resistant determinants.

In conclusion, this study confirms that a subpopulation of *S. uberis* field isolates shifted towards penicillin resistance, despite the fact that these isolates were still all considered as sensitive to this drug. These results raise the question of the pertinence of using penicillins to treat streptococcal bovine mastitis, a consideration in tight relation with the rapidity with which this population shift might evolve. However, only a combination of field and scientific observations might help tackle this difficult issue. Indeed, on the one hand, veterinary habits tend to indicate that penicillins are still widely and successfully used in the case of intramammary infections and, on the other hand, the slow development of resistance-associated mutations *in vitro*, as well as the apparent absence of mosaic PBPs in mastitis isolates (Haenni *et al.*, 2010), does not argue for a rapid and broad emergence of resistance, contrary to pneumococci in human medicine. Nevertheless, the reality of these phenotypic and molecular changes in *S. uberis* must not be underestimated, especially as similar trends were recently reported in *Streptococcus agalactiae*, a pathogen that is associated both with animal and severe human diseases

Fig. 2. Distribution of the MIC values (mg l⁻¹) to penicillin (a), amoxicillin (b) and ampicillin (c). Results of the population described as oxacillin sensitive by disc diffusion are indicated by white bars, and those of the oxacillin resistant population by disc diffusion are indicated by black bars. The arrows indicate the breakpoints between sensitive and intermediate isolates.

(Kimura *et al.*, 2008). Thus, taken together, these results underline the need to perform regular surveys on *S. uberis* field isolates, first to monitor the evolution of this population shift, and second to avoid a wider spread of penicillin-resistant streptococci in veterinary medicine.

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