

## **HIGH PREVALENCE OF *TCRB*, A COPPER RESISTANCE GENE, IN ANTIBIOTIC-RESISTANT ENTEROCOCCI FROM SWINES AND PIGGERIES ENVIRONMENT**

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**ABSTRACT**

Copper is largely used as an animal growth-promoter in intensive animal production. Different Portuguese piggeries are reservoir of *tcxB* gene (coding for copper resistance) which is disseminated in different enterococcal species, samples and piggeries. Resistance to erythromycin seems to be more common among enterococci harbouring *tcxB*. This gene is frequently transferred using tetracycline or erythromycin as selective agents. The selection of antibiotic resistance bacteria in the animal setting might be associated with the use of antibiotics and/or biocides.

**KEYWORDS**

Copper, biocides, antibiotic resistance, *Enterococcus*, pigs.

## RESUMO

Suplementos de cobre são largamente utilizados como promotores de crescimento na produção animal intensiva. Diferentes suiniculturas Portuguesas são reservatório do gene *tcrB* (resistência ao cobre), disseminado em várias espécies de *Enterococcus* provenientes de amostras animais e ambientais. A resistência à eritromicina parece ser mais comum entre bactérias *tcrB*<sup>+</sup>. Este gene é frequentemente transferido usando quer a tetraciclina quer a eritromicina como elementos de pressão selectiva. A selecção de bactérias resistentes aos antibióticos no ambiente de produção animal parece estar relacionada quer com o uso destas moléculas quer com produtos biocidas.

## PALAVRAS-CHAVE

Cobre, biocidas, resistência a antibióticos, *Enterococcus*, suínos.

## 1. INTRODUCTION

In the last decades, the menace imposed by multidrug resistant microorganisms has been attributed to the selective pressure exercised by the consumption of large amount of antimicrobial agents used for different purposes in different ecological niches as hospitals and the intensive animal production (Hasman et al. "Copper Resistance"; Schwarz et al.; WHO/FAO/OIE). However, despite the efforts to decrease the use of antibiotics (e.g. ban of antibiotics as animal growth promotion, control hospital policies) antibiotic resistant microorganisms are increasingly recovered from farm animals, pets and aquacultures (Phillips et al.). Recently, the European Commission suggested that the use of non-antibiotic substances classified as biocides (e.g. metals, quaternarium ammonium compounds, bisguanidines, etc.) in disinfectants, antiseptics, cosmetics and other widely distributed products, might contribute to the selection and maintenance of antibiotic resistant bacteria in some ecological niches (SCENIHR). This hypothesis has been suggested since the activity of antibiotics and biocides is often diminished by the same bacterial mechanism or by different mechanisms encoded by genes located in common mobile genetic elements (Hasman et al. "*tcrB*, a gene"; SCENIHR). Hence, the knowledge improvement concerning microorganism's resistance/tolerance to biocides and the identification of particular clones or genetic elements that contribute to the dissemination of biocide and antibiotic resistance genes is of utmost importance.

Copper has been used in different settings due to its cytotoxic properties (as fungicide/bactericide in agriculture activities, disinfectant in human and animal settings, or as preservative in different industrial processes) and also as animal growth promoter (Berg et al.; EFSA; SCAN). It plays a vital role in the physiology of all living organisms being largely used in Europe and United States in swine, poultry and fish farming's (Amachawadi et al.; SCAN). The emergence of bacteria exhibiting copper resistance/tolerance mechanisms in the presence of high levels of this metal was predictable and we now recognize that many microorganisms use homeostasis proteins to maintain a strict cellular copper quota to avoid toxic concentrations (Stoyanov et al.). However, an association between copper and antibiotic resistance has scarcely been investigated.

Enterococci, which have been used in the last years as good indicators of antibiotic resistance gene flux in particular niches, have developed resistance to copper by the acquisition of the *tcrYAZB* gene cluster (Hasman et al. "The *tcrB* gene"). This operon was initially described on pA17sv1, a plasmid also harbouring genes encoding resistance to different antibiotics as macrolides (*ermB*) or glycopeptides (*vanA*), showing an association between *tcrYAZB* ope-

ron and antibiotic resistance (Hasman et al. "The *tcrB* gene"). To date the number of isolates carrying this plasmid is scarce although its real distribution has not been evaluated outside a few geographical locations (Hasman et al. "tcrB, a gene"). Our goal was to analyze the occurrence of *tcrB* gene (coding for a CPx-type copper efflux ATPase) among enterococci from swine and the environment of six Portuguese piggeries and to evaluate the frequency of its co-transference with tetracycline and erythromycin, antibiotics largely used as veterinary agents in intensive animal production.

## 2. MATERIALS AND METHODS

### 2.1. STUDY DESIGN

Between April 2006 and December 2007 we conducted a large study to determine the incidence of antibiotic resistant enterococci present in the animal production setting (Novais et al., unpublished). It included isolates recovered from five intensive (A, B, C, E, F) and one extensive (D) production piggeries located in North, Centre and South of Portugal. From the 473 enterococci obtained, we selected for this specific study 197 isolates representative of different antibiotic resistance profiles, species and piggeries. They were originated from animals (31 from feces, 2-nostril and 8-skin), residues (36-waste lagoons, 16-manure), animal feed (18-food, 14-drinking water) and piggeries environment (6-disinfectant-Mystral®, 8-air, 16-residual waters, 12-soils, 12-dust, 18-piggeries facilities as walls and floors from disinfected rooms and equipment). Previously, samples were enriched in Buffered Peptone Water for 16h (37 °C) and a 0,1 ml aliquot were plated onto Slanetz-Bartley agar plates without antibiotics and supplemented with 16 mg/L of tetracycline, 1000 mg/L of streptomycin, 125 mg/L of gentamicin, 8 mg/L of erythromycin, 6 mg/L of vancomycin or 16 mg/L of ampicillin.

### 2.2. BACTERIAL IDENTIFICATION

Genus identification was based on the results of Gram stain, catalase test, bilis-esculin hydrolysis and growth in NaCl 6,5%. Identification at species level (*Enterococcus faecium*, *Enterococcus faecalis*, *Enterococcus gallinarum*, *Enterococcus casseliflavus*, *Enterococcus durans*, *Enterococcus hirae*) was performed by amplification of species specific genes by PCR as previously described (Arias et al.; Novais et al.).

### 2.3. STUDY OF ANTIMICROBIAL SUSCEPTIBILITY

All isolates were tested for susceptibility to tetracycline and erythromycin using the disk diffusion method and following *Clinical Laboratory Standards Institute* guidelines (CLSI).

### 2.4. SEARCH OF THE GENE ENCODING COPPER RESISTANCE

Search of the gene encoding to copper (*tcrB*) was performed by PCR as previously described (Hasman et al. "Copper"). Positive and negative controls were included in all assays.

### 2.5. MATING ASSAYS

Conjugation experiments were done for representative *tcrB*<sup>+</sup> enterococci (n=42) of different species and antibiotic resistance profiles. They were performed by filter mating method at

a 1:1 donor-recipient ratio using the rifampicin and fusidic acid resistant strains *E. faecalis* strain JH2-2, *E. faecium* strain BM4105RF or *E. faecium* GE1 as recipients. Transconjugants were recovered from selection plates containing 30mg/L of rifampicin, 25mg/L of fusidic acid plus 8mg/L of tetracycline or 8mg/L of erythromycin. Transconjugants were recovered after 24-48h of incubation (37 °C) and they were verified by confirming the resistance to rifampicin, fusidic acid and tetracycline or erythromycin by the disc diffusion method (CLSI).

### 3. RESULTS

#### 3.1. BACTERIAL IDENTIFICATION

We identified 79 *E. faecium*, 41 *E. faecalis*, 33 *E. hirae*, 6 *E. gallinarum*, 3 *E. casseliflavus* and 35 *Enterococcus* spp.

#### 3.2. STUDY OF ANTIMICROBIAL SUSCEPTIBILITY

Most enterococci isolates included in this study showed resistance to tetracycline (92%, n=192/197) or erythromycin (86%, n=170/197). Resistance was scattered by different species, samples and piggeries.

#### 3.3. SEARCH OF GENES ENCODING FOR ANTIBIOTIC OR COPPER RESISTANCE

The *tcrB* gene was detected in 29% (n=57/197) of enterococci from all piggeries and samples (except those from a sample of drinking water and nostril swab). Most of these isolates were identified as *E. faecium* (60%, n=34/57), followed by *E. faecalis* (14%; n=8/57), *E. hirae* (10%; n=6/57) and other species (*E. gallinarum*-2%, n=1/57; *E. casseliflavus*-2%, n=1/57; *Enterococcus* spp.-12%, n=7/57) (Figure 1).

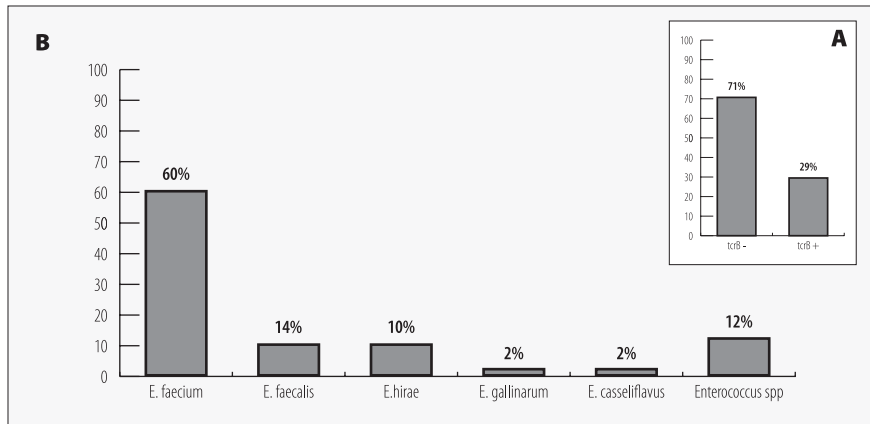


FIGURE 1 - Incidence of *tcrB* gene among the isolates studied (A) and its distribution by species (B).

### 3.4. ASSOCIATION OF ANTIBIOTIC RESISTANCE PROFILE AND TCRB GENE

A higher percentage of resistance to erythromycin was detected among *tcrB*<sup>+</sup> isolates (98%) than among *tcrB*<sup>-</sup> (81%). No significant difference was observed for tetracycline resistance among these two groups (*tcrB*<sup>+</sup>-95% and *tcrB*<sup>-</sup> -91%).

### 3.5. MATTING ASSAYS

We obtained 57% of positive matting assays for tetracycline or erythromycin resistance (n=24 out of 42 enterococci included). Among them, a high number of transconjugants (92%, n=22/24) also carried *tcrB* gene (Table 1). *tcrB*<sup>+</sup> transconjugants were resistant to erythromycin (n=7), to tetracycline (n=1) or both (n=14) antibiotics (Table 1).

Antibiotic used as selective agent	Isolates tested	Positive mating assays; Species	<i>tcrB</i> co-transferred to transconjugants	Antibiotic resistance patterns in transconjugants harboring <i>tcrB</i>		
				Ery	Tet	Ery+Tet
Erythromycin (8mg/L)	26	n=15/26; <i>E. faecium</i> (n=12), <i>Enterococcus</i> spp (n=3)	100% (n=15/15)	47% (n=7/15)	0% (n=0/15)	53% (n=8/15)
Tetracycline (8mg/L)	16	n=9/16; <i>E. faecium</i> (n=6), <i>E. faecalis</i> (n=2), <i>Enterococcus</i> spp (n=1)	78% (n=7/9)	0% (n=0/7)	14% (n=1/7)	86% (n=6/7)

**TABLE 1** - Features of *tcrB*<sup>+</sup> transconjugants obtained from different species of enterococci (Footnote: Tet=tetracycline; Ery=erythromycin).

## 4. DISCUSSION

This study shows that *tcrB* is widely distributed among enterococci from different species, samples and piggeries located in several Portuguese geographical regions. Incidence rates of *tcrB* are based on reported studies performed in different European countries which have described higher (Denmark, Spain) and lower (Sweden) percentages than those observed in this study (Aarestrup et al.; Hasman et al. "*tcrB*, a gene"). It was not a surprise to detect this gene in our samples, since pigs are the farm animals that consume the highest concentrations of CuSO<sub>4</sub> as growth promoter in comparison with animal from other production types (SCAN). In fact, absence or lower percentages of this gene were previously observed in enterococci from poultry, calves or sheep (Hasman et al. "*tcrB*, a gene"; SCAN).

The European Commission has been worried about the selection of antibiotic resistant bacteria by the use on nonantibiotic molecules (SCENIHR). In this study, erythromycin resistance was more frequent among *tcrB*<sup>+</sup> than among *tcrB*<sup>-</sup> isolates. Moreover, the success of mating assays show that horizontal transfer plays an important role in the co-dispersion of antibiotic and copper resistance genes. These data are corroborated by previous studies which described *tcrB* in different Gram positive genus located either in ICESde3396, a conjugative integrative element or at the transferable multiresistance plasmid pA17sv1, harbouring *ermB* and *vanA* (Davies et al.; GenBank accession numbers EU142041.1, AE009948.1, CP001849.1, CP001708.1; Hasman et al. "*tcrB*, a gene"). Although we did not characterize the genetic platform in which *tcrB* was located, we demonstrated its transferability with erythromycin and/or tetracycline resistance. The large spread of particular capture genetic units associated with these two widely used antibiotics in piggeries environments (Aarestrup et al.; Hasman et al. "*tcrB*, a gene") might

contribute to the frequent co-transfer of the resistances mentioned above, either within common genetic platforms or in different elements transferred during the same genetic event. The predominance of *tcrB* among *E. faecium* suggests that this particular species might be more prone to acquire and/or to maintain the genetic elements carrying this gene. Similar results for *E. faecium* were observed in previous studies (Aarestrup et al.). Although horizontal transfer seems to have an important role in *tcrB* dissemination, a clonal expansion cannot be ruled out, and thus further molecular studies should be performed to test this hypothesis.

In summary, Portuguese pigs and piggeries environment are reservoirs of *tcrB* which is frequently co-transferred with antibiotic resistance genes by conjugation. The selective pressures associated with the use of antibiotics and/or copper compounds in the animal production might favor the maintenance of particular clones or the horizontal transfer of mobile genetic elements carrying genes coding for antibiotic resistance and/or biocides.

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