

Portuguese piggeries as reservoir of antibiotic multi-resistant enterococci

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INTRODUCTION

Dissemination of antibiotic resistance in community and hospitals has been facilitated by the extensive use of different antibiotics in clinical practice as well as in veterinary, husbandry and agriculture. Evidence of spread of antibiotic resistant bacteria to humans via food chain or by contact with animals led to the ban of antibiotics as growth promoters in the Europe in 1997. However, different European countries, as UK and Denmark, have reported an increase use of these molecules as therapeutic agents in order to reduce of animal diseases (Phillips et al., 2004).

Beside food chain, piggeries have been reported to also contribute to environment contamination, such as soil, fresh vegetables and underground water, through land application of manure, sludge and wastewater and consequently by airborne dissemination of antibiotic resistant bacteria (Sarmah et al., 2006).

Antibiotic resistant strains may be co-selected by a diversity of biocides substances that include different antibiotics, antiseptics or metals which are given in feed or be present in the animal production environment. This occurs because genes coding for resistance to different compounds are often located in the same genetic platform (Hasman et al., 2006).

Recent Portuguese data revealed that, between 2004 and 2006, 632 tons of antibiotics were consumed by production animals being tetracyclines the most used (around 40 tons in 2006) (INFARMED, 2007).

PURPOSE

In order to evaluate the role of Portuguese piggeries as reservoir and contributor for the environment dissemination of antibiotic multi-resistant enterococci, our goal included the detection of multi-drug resistant phenotypes, the characterization of antibiotic resistance genes and their ability to be involved in transfer events.

METHODS

Bacterial strains and sample processing. Animal and environmental samples were collected in 5 intensive (A, B, C, E, F) and 1 extensive (D) production piggeries between April 2006 and December 2007 in the North, Centre and South of Portugal. Eighty-six samples were classified in 5 groups concerning their nature: (i) pigs (n=21; faeces, nostril/surface swabs); (ii) food/medicines (n=22, food, water, medicine, antiseptics) (iii) residues (n=17; waste lagoons, residual waters, manure, septic tank); (iv) piggeries facilities (n=23-air, water, walls/floors, dust, soil); (v) river receiving piggeries effluent (n=1).

Samples were enriched in peptone water (24h; 37°C) and plated on Slanetz-Bartley with (16 µg/ml tetracycline, 1000µg/ml streptomycin, 125µg/ml gentamicin, 8µg/ml erythromycin, 6µg/ml vancomycin, 16µg/ml ampicillin) and without antibiotics. One colony from each morphology and resistance phenotype was selected for further studies.

Antibiotic susceptibility. Susceptibility to 12 antibiotics of different families was determined by the disk diffusion methods following CLSI guidelines (CLSI, 2007). *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 as control strains.

Identification and characterization of antibiotic resistance genes. Genus identification were performed by Gram stain, catalase test, bilis-esculin hydrolysis and growth in NaCl 6.5%. Species identification (*E. faecium*, *E. faecalis*, *E. durans*, *E. hirae*) and detection of genes coding for antibiotic resistance (*vanA*, *vanB*, *vanC1*, *vanC2*, *tetM*, *tetL*, *tetO*, *tetS*, *tetK*, *ermA*, *ermB*, *ermC*, *aac(6)-Ie-aph(2)-Ia*, *aph(2)-Ib*, *aph(2)-Ic*, *aph(2)-Id*, *aph(3)-IIIa*) were searched by PCR as previously described (Aarestrup et al., 2000; Novais et al., 2005; Arias et al., 2006).

PFGE. Clonal relatedness was established by PFGE for 26 representative (16 *E. faecium* and 12 *E. faecalis*) strains with different antibiotic resistance phenotype from 3 intensive production farms. Clonal relationships were established following the criteria previously described (Novais et al., 2005).

Mating assays. Conjugation experiments were done in 67 tetracycline, 10 erythromycin and 6 gentamicin resistant isolates by filter mating methods at a 1:1 donor-recipient ratio, using the rifampicin and fusidic acid resistant *E. faecalis* JH2-2, *E. faecium* BM4105 or *E. faecium* GE-1 as recipients. Transconjugants were recovered in selection plates with 60mg/L of rifampicin plus 6mg/L of tetracycline, 8mg/L of erythromycin or 500 mg/L of gentamicin after 48h of incubation (37°C). Transconjugants were confirmed by resistance to rifampicin, fusidic acid and tetracycline, erythromycin or gentamicin (120µg) by the disc diffusion method.¹⁰⁰

RESULTS

For hundred and seventy-three enterococci were identified as *E. faecium* (n=171), *E. faecalis* (n=78), *E. hirae* (n=73), *E. gallinarum* (n=14), *E. casseliflavus* (n=5) and *Enterococcus* spp (n=132).

Among all species, more than 50% of isolates were nonsusceptible to tetracycline, minocycline, erythromycin or quinupristin-dalfopristin (Fig1). Antibiotic resistance to gentamicin, ampicillin, chloramphenicol was only detected in piggeries with intensive production.

tetM (63%; including 9 susceptible isolates), *tetL* (53%; including 4 susceptible isolates) and *ermB* (50%; including 4 susceptible isolates) were observed in a high number of strains (Fig.2). Absence of *tet* or *erm* genes tested were observed in 13% and 19% of tetracycline or erythromycin nonsusceptible enterococci, respectively. *aac(6)-Ie-aph(2)-Ia* was detected in most of gentamicin nonsusceptible isolates tested and 79% of them presented simultaneously *aph(3)-IIIa*. *vanA* genes were observed in 5 *E. faecium* (5 piggery facility samples from 2 farms of intensive and 1 of extensive production) and 1 *E. faecalis* (1 faeces sample from 1 farm with intensive production).

Forty-one resistance genotypes were observed and the ten more predominant were harbored by 75% of the isolates. They were scattered in different piggeries, species and sample types that included those such air, manure to be used in agriculture, animal food and water disinfected with UV and *vanA*, *vanB*. Representative strains of most genotypes could transfer by conjugation tetracycline, macrolide and/or aminoglycoside resistance (Table 1).

A polyclonal population was present (11 PFGE types for *E. faecium* and 9 for *E. faecalis*), although common clones were detected in different samples of the same farm (Manure and waste lagoons of B farm: 1 *E. faecalis* and 1 *E. faecium* clones; Faeces, animal drinking water, dust of maternity of C farm: 1 *E. faecalis* clone; Two different faeces samples of distinct animals from C farm: 1 *E. faecalis* clone).

Table 1. Percentage, species distribution, samples, piggeries and mating assays of predominant antibiotic resistance genotypes

Predominant genotype	Percentage of isolates	Species	Samples type (nº piggeries)	Tetracycline	Mating Assays	Aminoglycosides
<i>ermB</i> , <i>tetM</i> , <i>tetL</i>	17% (78/473)	<i>E. faecium</i> (n=38); <i>E. faecalis</i> (n=18); <i>E. hirae</i> (n=11); <i>Enterococcus</i> spp (n=13)	Pigs (n=14; C, D, E, F); Residues (n=14; A,B,C,D,E,F); Piggeries facilities (n=10; B,C,D,E,F); Food/medicines (n=9; B,C,F)	+/-	ND	ND
<i>tetM</i> , <i>tetL</i>	12% (57/473)	<i>E. faecium</i> (n=27); <i>E. faecalis</i> (n=3); <i>E. hirae</i> (n=4); <i>Enterococcus</i> spp (n=13)	Pigs (n=8;C); Residues (n=11;A,B,C,E); Piggeries facilities (n=14;B,C,D,E,F); Food/medicines (n=2; B,D)	+/-	ND	ND
<i>tetM</i>	10% (48/473)	<i>E. faecium</i> (n=22); <i>E. faecalis</i> (n=7); <i>Enterococcus</i> spp (n=12)	Pigs (n=7;C,E,F); Residues (n=7;A,B,D,E); Piggeries facilities (n=13;B,C,D,E,F); Food/medicines (n=4; E)	+/-	ND	ND
<i>ermB</i> , <i>tetL</i>	7% (33/473)	<i>E. faecium</i> (n=10); <i>E. faecalis</i> (n=9); <i>Enterococcus</i> spp (n=14)	Pigs (n=13;C,E,F); Residues (n=11;A,B,C,D,E,F); Piggeries facilities (n=10;B,C,D,E); Food/medicines (n=12; B,C,F)	+/-	+/-	ND
<i>ermB</i>	6% (30/473)	<i>E. faecium</i> (n=13); <i>E. faecalis</i> (n=2); <i>E. hirae</i> (n=4); <i>Enterococcus</i> spp (n=11)	Pigs (n=5;C,D,E,F); Residues (n=8;A,B,C,E); Piggeries facilities (n=8;B,C,D,E,F); Food/medicines (n=3;C,E)	+/-	-	ND
<i>tetL</i>	6% (29/473)	<i>E. faecium</i> (n=15); <i>E. faecalis</i> (n=1); <i>E. hirae</i> (n=6); <i>Enterococcus</i> spp (n=15)	Pigs (n=3;C); Residues (n=8;B,C); Piggeries facilities (n=3;B,C,D); Food/medicines (n=3;C)	ND	ND	ND
<i>ermB</i> , <i>tetM</i>	6% (27/473)	<i>E. faecium</i> (n=17); <i>E. faecalis</i> (n=2); <i>E. hirae</i> (n=3); <i>Enterococcus</i> spp (n=9)	Pigs (n=5;D,E); Residues (n=5;A,B,C,E); Piggeries facilities (n=7;B,D,E); Food/medicines (n=3; D,E,F)	-	ND	ND
<i>ermB</i> , <i>tetM</i> , <i>aac(6)-Ie-aph(2)-Ia</i> , <i>aph(3)-IIIa</i>	4% (20/473)	<i>E. faecium</i> (n=3); <i>E. faecalis</i> (n=2); <i>Enterococcus</i> spp (n=6)	Pigs (n=4;C,E,F); Residues (n=9;A,B,C,E); Piggeries facilities (n=3;B,C,E); Food/medicines (n=3; C,E)	+/-	-	-
<i>tetM</i> , <i>aac(6)-Ie-aph(2)-Ia</i> , <i>aph(3)-IIIa</i>	4% (17/473)	<i>E. faecium</i> (n=1); <i>E. faecalis</i> (n=2); <i>E. hirae</i> (n=1); <i>Enterococcus</i> spp (n=6)	Pigs (n=4;C,E); Residues (n=8;A,B,C,E); Piggeries facilities (n=1;F); Food/medicines (n=1; E)	+	ND	-
<i>ermB</i> , <i>tetM</i> , <i>tetL</i> , <i>aac(6)-Ie-aph(2)-Ia</i> , <i>aph(3)-IIIa</i>	3% (15/473)	<i>E. faecium</i> (n=3); <i>E. faecalis</i> (n=1); <i>Enterococcus</i> spp (n=2)	Residues (n=6;B,C,F); Food/medicines (n=6;B,C,F)	+/-	+	+/-
Total	75% (338/473)					

+ Positive mating assays, - Negative mating assays, +/- Different isolates presented distinct results in mating assays, ND- Not determined.

CONCLUSION

Antibiotic multi-resistant enterococci were commonly detected among Portuguese piggeries probably reflecting the antibiotic use in veterinary medicine. The presence of such strains in samples such manure used in agriculture and in air, is of concern since they can be spread outside piggeries frontiers to environment and consequently to other animals and humans.

The presence of a polyclonal population associated to the ability of isolates to transfer different genes by conjugation supports that horizontal transfer events might have an important role in antibiotic resistant dissemination in Portuguese animal setting.

The absence of tetracycline or erythromycin resistance genes in nonsusceptible phenotypes suggests that other resistant mechanism less common in enterococci might be present.

Whether persistence of these antibiotic resistant isolates is only due to selection by antibiotics or also by other biocide agents used in piggeries environment deserves further studies in order to better understand the epidemiology of antibiotic resistance in the animal setting of our country and to decrease the presence of such strains/genes.

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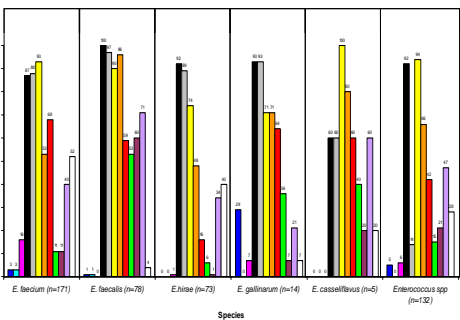


Fig.1 Percentage of resistance of different enterococci species to 12 antibiotics.

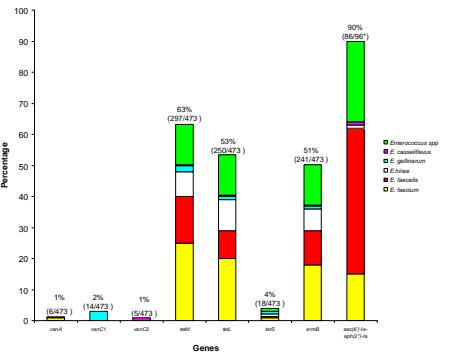


Fig.2 Distribution of antibiotic resistance genes by Enterococcus spp isolates. * *aac(6)-Ie-aph(2)-Ia* was only searched in gentamicin nonsusceptible isolates.

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