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Short Communication

Detection and genetic characterisation of *van*A-containing *Enterococcus* strains in healthy Lusitano horses

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Summary

Lusitano horses were investigated in order to detect the presence of vancomycin-resistant *enterococci*. *van*A isolates showed high level vancomycin (Minimum inhibitory concentration; MIC ≥128 mg/l) and teicoplanin resistance (MIC 64 mg/l), as well as resistance to ciprofloxacin, erythromycin and tetracycline. The *tet*(L) and *erm*(B) genes, associated with tetracycline and erythromycin resistance, respectively, were found in all *van*A isolates. The intestinal tract of Lusitano horses can be a potential reservoir for *van*A-containing *enterococci*.

Introduction

Enterococci are Gram-positive bacteria that represent essential components of the natural intestinal microbiota in man and animals (Aarestrup *et al.* 2000). However, they are also considered as important causes of nosocomial and community-acquired infections (Franz *et al.* 1999). The emergence of vancomycin-resistant *enterococci* (VRE) has been associated with the use of glycopeptides, such as avoparcin, as growth promoters for livestock until its ban in 1997 in the European Union (Bager *et al.* 1997). Studies have indicated that the glycopeptide avoparcin selects for VRE and an association between the rates of vancomycin resistance in man and avoparcin usage in animals has been suggested (Aarestrup *et al.* 2000). Animals can be a source of this microorganism and the resistance determinants they contain can be transmitted to human bacteria representing a public health problem. The increase in *enterococci* resistance to commonly used antimicrobials both in the public health and veterinary sectors is one of the major threats to health care worldwide. The VRE that enter the intestinal tract might colonise the host or potentially transfer their resistance genes to the resident bacteria during their passage through the gut. Therefore, animal husbandries have become a large reservoir of resistance genes (*van*A gene cluster), which might also be transferred to other bacteria, including those potentially pathogenic (de Niederhäusern *et al.* 2007).

In Portugal, equestrian sport and horse riding have been recognised as significant leisure time activities. There are different varieties of horse that which are raised in the country and of them, the Lusitano breed is one of the foremost. The most commonly broad-spectrum therapies used for severe bacterial infections in mature horses include a combination of gentamicin for Gram-negative coverage and penicillin for Gram-positive and anaerobic coverage. Enrofloxacin can be used as a substitute of gentamicin in mature horses, whereas ampicillin or cephazolin can replace penicillin (Giguere 2007). Strains with vancomycin resistance were isolated from the intestines and faeces of several animal species (Poeta *et al.* 2006) including horses (Devriese *et al.* 1996; Rice *et al.* 2003), but there are no previous reports about detection of VRE on Lusitano horses. The objective of this work was to analyse the prevalence as well as the phenotypic and genotypic profiles of *van*A-containing *enterococci* in healthy Lusitano horses.

Materials and methods

Faecal samples and isolation of enterococci

Ninety faecal samples from mature Lusitano horses of Portugal were recovered from December 2007 to March 2008 and were studied for the presence of VRE isolates. All the faecal samples were collected individually from each animal rectum and obtained in collaboration with one of the oldest Equestrian Centre of Portugal. The Alter Real Foundation is a Public Utility Institution with the main objective of the improvement of native equine breeds. This foundation has about 400 Lusitano horses (150 mares, 60 stallions and 190 colts). All mature individuals were sampled; none had received antibiotic drugs in the previous 4 months (n = 90). Each sample was seeded on Slanetz-Bartley agar plates supplemented with 4 μg/ml of vancomycin and incubated 48 h at 37°C. Colonies with typical enterococcal morphology (2 per sample) were identified by biochemical tests and specific PCR for the different enterococcal species.

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Antibiotic susceptibility testing

Antibiotic susceptibility to 10 antibiotics (chloramphenicol, tetracycline, teicoplanin, quinupristin-dalfopristin, vancomycin, ciprofloxacin, erythromycin, streptomycin, gentamicin and kanamycin) was tested by the disc diffusion method in all of the selected VRE isolates. The agar dilution method (Anon 2007) was also used for susceptibility testing to vancomycin and teicoplanin.

Detection of genes encoding antibiotic resistance

Specific PCR reactions were used to detect the presence of vancomycin resistance genes (*van*A, *van*C-1, *van*C-2/3 and *van*B) in all isolates that showed resistance or reduced susceptibility to glycopeptides (Aarestrup *et al.* 2000; Torres *et al.* 2003; Poeta *et al.* 2006). Resistance genes for other antibiotics, including *tet*(M), *tet*(K), *tet*(L), *erm*(A), *erm*(B), *erm*(C) were also studied by PCR (Aarestrup *et al.* 2000; Torres *et al.* 2003; Poeta *et al.* 2006). Positive and negative controls were included in all assays and bacteria were part of the collection of the University of Trás-os-Montes and Alto Douro (Portugal).

Results

Eight of the 90 faecal samples tested contained VRE isolates (8.8%) and 2 VRE isolates/sample were identified to the species level and their antibiotic susceptibility profiles were determined. The 2 VRE isolates from each positive sample exhibited the same enterococcal species and the same antibiotic resistance profile and for this reason, only one VRE isolate per positive sample was maintained for further studies making a collection of 8 isolates (Table 1). Four of these isolates presented an acquired mechanism of vancomycin resistance and the remaining 4 an intrinsic mechanism of vancomycin resistance.

The 4 VRE isolates that exhibited an acquired mechanism of resistance were identified as *E. faecium* (one isolate) and *E. durans* (3 isolates). The characteristics of these isolates are shown in Table 1. The *van*A gene was identified by PCR in all these 4 isolates and they showed high level vancomycin (minimum inhibitory concentration; MIC $\geq$128 mg/l) and teicoplanin resistance (MIC 64 mg/l). The strains also exhibited resistance to ciprofloxacin, erythromycin and tetracycline. The *tet*(L) gene as well the *erm*(B) gene, related with tetracycline and erythromycin resistance, respectively, were found in all *van*A isolates.

The 4 VRE isolates with intrinsic mechanism of resistance contained the *van*C-1, specific of the *E. gallinarum* species (Table 1). They presented low level resistance to vancomycin (MIC 8 mg/l) and susceptibility to teicoplanin (MIC 1 mg/l). Three of these isolates exhibited resistance to ciprofloxacin, erythromycin and tetracycline [all with the *erm*(B) gene and one of them with the *tet*(L) gene], and the remaining one showed only ciprofloxacin resistance in addition to vancomycin.

Discussion

This work showed the presence of *van*A-containing enterococcal strains in the faecal samples of the studied horses. Other studies have found higher percentages of vancomycin resistant *enterococci* (37.2%) in horse farm isolates; however, the presence of *van* genes was not studied (Lauková *et al.* 2008). Isolates containing the *van*A gene were previously observed in 10% of faecal samples of horses, while avoparcin was still used as feed additive in European countries (Devriese *et al.* 1996). A more recent analysis of *enterococci* isolates from horses showed a lower prevalence of VanA phenotype (6.7%) (de Niederhäusern *et al.* 2007), compared to what was registered by Devriese *et al.* (1996) before avoparcin was banned in 1997, but still higher than that observed in the present study.

Enterococci showing an intrinsic mechanism of vancomycin resistance were also found in the study, although the frequency (4.4%) was lower (4.3%) to that found in horses by Rice *et al.* (2003), *E. cassilifavus* (*van*C2-3) being the most prevalent species detected. It is interesting to underline that despite the previously documented association between *tet*(L) and *tet*(M) genes on animal and human isolates and also in food products (Aarestrup *et al.* 2000; Huys *et al.* 2004; Poeta *et al.* 2006), the presence of both genes in the same strain was not observed in any of our horse isolates. On the other hand, *tet*(M) gene has been referred as the most frequent tetracycline resistance gene (Huys *et al.* 2004; Poeta *et al.* 2006); however, in our strains only *tet*(L) gene was found. This report of tetracycline resistance among our isolates is important, as it follows previous studies on horses, were no resistance to tetracycline was observed (Lauková *et al.* 2008).

All the *van*A isolates exhibited erythromycin resistance as previously reported by de Niederhäusern *et al.* (2007). It is known that *erm*B gene encoding for macrolide resistance can be carried by the same conjugative plasmid harbouring *van*A gene (Aarestrup *et al.* 2000), and in this sense, our results support the hypothesis that this linkage could be a causative factor for the ongoing persistence of VRE, even after the avoparcin ban. In fact, in this study the *van*A-enterococcal strains were erythromycin resistant and harboured the *erm*(B) gene. Moreover, some authors also demonstrated a genetic linkage in animal *enterococci* between the *erm*(B) gene and the tetracycline resistance gene *tet*. In fact, the use of one of these antimicrobial groups favours spread of resistance against all of these antimicrobial agents groups (de Graef *et al.* 2007).

TABLE 1: Characteristics of vancomycin-resistant enterococcal strains recovered from Lusitano horses

Enterococcus strain	Minimum inhibitory concentration (μg/ml)		Vancomycin resistance gene detected	Resistance phenotype for other antibiotics[a]	Genes detected by PCR
	Vancomycin	Teicoplanin			
E. faecium LH32	>128	64	*van*A	ERY-TET- CIP	*erm*(B)+ *tet*(L)
E. durans LH37	>128	64	*van*A	ERY-TET- CIP	*erm*(B)+ *tet*(L)
E. durans LH46	>128	64	*van*A	ERY-TET- CIP	*erm*(B)+ *tet*(L)
E. durans LH51	>128	64	*van*A	ERY-TET- CIP	*erm*(B)+ *tet*(L)
E. gallinarum LH58	8	1	*van*C-1	CIP	–
E. gallinarum LH63	8	1	*van*C-1	ERY-TET- CIP	*erm*(B)
E. gallinarum LH76	8	1	*van*C-1	ERY-TET- CIP	*erm*(B)+ *tet*(L)
E. gallinarum LH82	8	1	*van*C-1	ERY-TET-CIP	*erm*(B)

[a]ERY; erythromycin; TET; tetracycline; CIP; ciprofloxacin.

Although the percentages and diversity of genotypes and phenotypes of resistance were inferior to that observed in other animal species and human isolates (Aarestrup *et al.* 2000; Poeta *et al.* 2006), the impact of the detection of *van*A genes together with resistance to some other antibiotics, show these animals as potential reservoirs of antibiotic-resistance genes. VRE have been found in horses, for which avoparcin or related glycopeptides are not used either as growth promoter or in equine medicine (de Niederhäusern *et al.* 2007). The possibility that these animals might have acquired these isolates from the environment or from focally contaminated foods cannot be excluded. Vancomycin is a glycopeptide antibiotic that is very important in human medicine because of its activity against multi-drug resistant organisms, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *enterococci*. However, recently, at the Veterinary Microbiological Diagnostic Centre of The Netherlands, the percentage of MRSA isolates found in equine clinical samples increased from 0% in 2002 to 37% in 2008 (van Duijkeren *et al.* 2009). Additionally, in horses, diarrhoea associated with *Clostridium difficile* responds to metronidazol or to vancomycin. However, horses may be affected with metronidazol resistant strains (Viveca *et al.* 2004). Although vancomycin is generally not used in equine medicine, it can at times represent unique effectiveness as a therapeutic alterative. In this way, the presence of *van*A-*enterococci* can represent a clinical problem.

Enterococci that harbour antibiotic resistance genes are common in the digestive tract of animals and it has been suggested that these bacteria might serve as a reservoir of resistance genes for human digestive microflora. Previous investigations have revealed the occurrence of horizontal gene transfer between bacteria that colonise livestock and those that colonise man. In our study the presence of *van*A enterococcal strains in horses has been detected, indicating that these animals can excrete VRE in their faeces and may be a reservoir for such resistant bacteria that can be transmitted to other animals. On the other hand, the genes detected in the *enterococci* of these animals are the same already found in enterococcal strains of man and other animals indicating that the genes can circulate between different sources and can be transferred from animals to humans. Enhancing the emergence of resistance is the ease by which resistance determinants and resistant bacteria can spread locally and globally, selected by widespread use of the same antibiotics in human medicine, animal husbandry and agriculture. Although antibiotics are certainly important elements in the resistance imbalance, the transfer of antibiotic resistance genes and selection for resistant bacteria can occur through a variety of mechanisms, which may not always be linked to specific antibiotic use.

On the other hand, although *Enterococcus faecalis* is responsible for the majority of registered cases of human enterococcal infections, followed by *E. faecium;* an increasing incidence was reported for infections with *E. durans* and *E. hirae* as causative agents (Franz *et al.* 1999). Six different glycopeptide resistance phenotypes (*Van*A to *Van*E and *Van*G) have been described in *enterococci* but *Van*A and *Van*B are of greatest clinical relevance (Werner *et al.* 2008). Although *E. gallinarum* is not a relevant problem because the *van*C gene is not transferable, the presence of one *E. faecium* and 3 *E. durans* that harboured the *van*A gene detected in the present study represents a clinical problem, of the location in mobile genetic elements transferable to other microorganisms.

Monitoring the level of antimicrobial resistance in commensal bacteria allows a comparison between the prevalence and evolution of resistance patterns over time. Future studies should be carried out to analyse the evolution of VRE colonisation in different ecosystems, including wild animals of different species and countries.

Therefore, a surveillance programme to detect the VRE prevalence is necessary to increase the knowledge about resistance patterns and its way of transmission and spreading in different ecosystems.

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