Vancomycin Resistance Genes in *Enterococcus* spp. Strains Isolated from Alpine Accentor and Chamois

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Abstract

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Enterococcus strains from highland animals the alpine accentor *Prunella collaris* (n = 19) and chamois *Rupicapra rupicapra* (n = 18) were isolated. One third of strains belonged to the species *E. casseliflavus*, while other were identified as *E. faecalis* and *E. gallinarum, E. faecium* and *E. mundtii* or *E. flavescens*. Intrinsic low level resistance to vancomycin (MIC 0.5 - 4 $\mu g \cdot ml^{-1}$), but not teicoplanin were found in 13 strains *E. casseliflavus* and *E. faecalis*. The *vanC2/C3* genes (4 strains) and *van C1* genes (2 strains) were detected by PCR in *E. casseliflavus* strains. The genes *van A* or *van B* were no detected. The results of our study have shown that in high altitude mountains where no antibiotic selection pressure exists only *vanC* genes coding the intrinsic resistance to vancomycin were recorded in enterococcal strains.

vanC1, vanC2/C3 , Enterococcus casseliflavus, PCR, alpine accentor, chamois

Enterococci are widespread bacteria which can be isolated from different ecological sources (Klare et al. 1993). These bacteria are part of normal intestinal flora of humans and animals.

In Europe, antimicrobial agents are widely used as feed additives for growth promotion in animal husbandry (Van den Bogaard and Stobberingh 1996). Avoparcin is a glycopeptide antibiotic used for this purpose in poultry, and it appears to be associated with the emergence of resistance to glycopeptides in general (Bager et al. 1997; Bates et al. 1994; Klare et al. 1995).

A possible source of vancomycin-resistant enterococci (VRE) is the food chain since VRE have been isolated from farm animals and animal products in several Europen countries (Bager et al. 1997; Van den Bogaard et al. 1997). It has been suggested that the use of antibiotic avoparcin as a feed additive in animal husbandry in numerous European countries has resulted in the selection of vancomycine resistance in strains from farm animals (Aarestrup 1995; Bager et al. 1997; Lukášová and Šustáčková 2003). Van den Braak et al. (1998) published that direct horizontal transmission of VRE from poultry to humans through the food chain is important transmission route. There are five recognized phenotypes of vancomycin resistance, VanA, VanB, VanC, VanD and VanE, from which only *vanC* locus is non-transferable. Transmission of resistance genes *(vanA, vanB, vanD* and *vanE*), rather than clonal dissemination of resistant microorganisms, could be the determining factor driving the extension of vancomycin resistance from poultry to humans. Enterococci are infamous for their ability to transfer their resistance genes to other enterococci rapidly (Moellering 1992) as well as to bacteria belonging to other genera (Leclerq et al. 1989; Noble et al. 1992). It has been proposed that strains are spread from animals to humans and the use of growth promoters and antimicrobial agents in animal husbandry often selects resistant strains (Kaukas et al. 1987; Kaukas et al. 1988).

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Another important issue with antibiotic resistance is the fact that the wide use of antibiotics not only selects for drug-resistant pathogenic bacteria but also exerts selective pressure on the normal commensal microbiota (Aminov et al. 2001).

The aim of our investigations was to estimate the occurrence of vancomycin-resistance genes in enterococci from highland animals (alpine accentor *Prunella collaris* and chamois *Rupicapra rupicapra*), which are without direct antibiotic pressure.

Materials and Methods

Origin of bacterial strains

The samples of fresh faeces came from the different areas of two National Parks (High Tatras from six localities and Low Tatras from four localities). One collecting place for alpine accentor was near Tery cottage in High Tatras. Samples were collected in sterile glass tubes and sent to the laboratory, within one day, where they were inoculated on non selective medium (Columbia blood agar, Oxoid) and selective medium (Slanetz Barley agar, Oxoid). Isolates recovered from faeces of wild animals (*Rupicapra rupicapra* subsp. *tatrica* - 18 strains from 18 samples, *Prunella collaris* - 19 strains from 19 samples). It was not possible precisely to distinguish the number of animals.

Microbial identification

Isolates were presumptively identified as enterococci by colonial morphology, Gram's stain, the absence of catalase production and growth on bile-esculine agar with esculin hydrolysis. Species identification was performed by computer program TNW Lite 6.0 with using Lachema Brno (Czech Republic) En-Coccus identification test.

Antibiotic susceptibility testing

Antibiotic susceptibility testing to vancomycin was performed by an agar dilution method and by following the current guidelines of the National Committee for Clinical Laboratory Standards (USA). We used Mueller-Hinton agar (Oxoid, England) with vancomycin and teicoplanin concentrations 0.5; 1; 2; 4; 8; 16; 32 $\mu g.ml^{-1}$. Minimal inhibitory concentrations (MIC) were determined after incubation during 24 hours at 37 °C.

DNA isolation

Chromosomal DNA was isolated from overnight Nutrient Broth (Oxoid, England) cultures of *Enterococcus* spp. by lysosyme-sodium dodecyl sulfate lysis at 60 °C, 1 hour incubation.

PCR

A PCR assay was used to detect *van A* and *van B* genes coding for vancomycine resistance in enterococci. Each 50 µl of PCR amplification mixture contained deionized sterile water, 0.5 $\mu mol.l^{-1}$ of each appropriate pair of oligonucleotide primers (Gibco BRL, UK) vanA forward GGGAAAACGACAATTGC and *vanA* reverse GTACAATGCGGCCGTTA or *vanB* forward ATGGGAAGCCGATAGTC and *vanB* reverse GATTTCGTTCCTCGACC, or *vanC1* forward GGTATCAAGGAAACCTC and *vanC1* reverse CTTCCGCCATCATAGCT or *vanC2/C3* forward CTCCTACGATTCTCTTG and *vanC2/C3* reverse CGAGCAAGACCTTTAAG; 1 U of the enzyme Platinum *Taq* DNA polymerase (Gibco BRL, UK); dNTPs (Promega, USA) 200 µM each; 1.5 mM $MgCl_2$; 10 × PCR buffer (Gibco BRL, UK) and 10 ng *Enterococcus* DNA. The thermal cycler protocol was as follows: denaturation at 94 °C for 5 min; then 32 cycles for denaturation (94 °C, 1 min), annealing (54 °C, 1 min) and extension (72 °C, 1 min), then a final extension (72 °C, 10 min). Amplification was carried out in a Progene thermocycler (Techne, Cambridge, UK). PCR products (*vanA* 732 bp, *vanB* 635 bp, *vanC1* 822bp and *vanC2/C3* 439 bp) were resolved by electrophoresis on a 1% agarose gel (Sigma, Germany) containing 0.5 µg ethidium bromide per ml. As the size marker, a 100 bp ladder (Promega) was used.

Results and Discussion

Enterococcus strains from highland animals the alpine accentor *Prunella collaris* (n = 19) and chamois *Rupicapra rupicapra* (n =18) were isolated. One third of strains belonged to the species *Enterococcus casseliflavus*, while other were identified as *Enterococcus faecalis* and *E. gallinarum, Enterococcus faecium* and *E. mundtii* or *E. flavescens*. Intrinsic low level resistance to vancomycin (MIC 0.5 - 4 $\mu g.ml^{-1}$) and teicoplanin sensitivity were found in 13 strains *E. casseliflavus* and *E. faecalis* (Table 1). The *vanC2/C3* genes (3 strains from chamois-10K,11K, 32K and one strain 43 from alpine accentor) and *van C1* genes (2 strains from chamois 35K, 36K) were detected by PCR in *E. casseliflavus* strains.

There is absence of data with vancomycin resistant enterococci isolated from highland animals, without antibiotic pressure. We can compare our results with few other current

Table 1
PCR detection of vancomycin resistance genes

Strain number	Vancomycin sensitivity		Species identification
	MIC vancomycin (μg/ml)	genotype	
28K	1	—-	*E. casseliflavus*
31K	2	—-	*E. casseliflavus*
32K	2	van C2 – van C3	*E. casseliflavus*
35K	4	van C1	*E. casseliflavus*
36K	4	van C1	*E. casseliflavus*
43	2	van C2 – van C3	*E. casseliflavus*
10K	2	van C2 – van C3	*E. casseliflavus*
9K	1	-	*E. casseliflavus*
11K	2	van C2 – van C3	*E. casseliflavus*
24K	0.5	-	*E. faecalis*
33K	2	-	*E. faecalis*
34K	1	-	*E. faecalis*
40	1	-	*E. faecalis*

K-chamois

studies. According to Iversen et al. (2002) from Sweden, they ceased using avoparcin in 1986. In spite of it they isolated VRE from untreated sewage samples, hospital sewage samples, treated sewage samples and also from surface water samples. Most isolates carried van A gene, and the majority of the isolates were *Enterococcus faecium.* Lemcke and Buelte (2000) described that out of 1643 *Enterococcus* isolates from 115 poultry and 50 pork samples, 420 isolates could be identified as vancomycin resistant, 202 isolates of which carry the vanA, one isolate both the vanA and the vanC1, 38 isolates the vanC1, 14 isolates the vanC2, nine isolates both the vanC1 and the vanC3 gene and 156 isolates carry no gene.The vanB gene was not found in these isolates

We did not detected *van A* or *van B* genes among tested enterococci from *Prunella collaris* and chamois, which are considered as transferable genes potentially dangerous for human population. Only vanC1 and vanC2/C3 genes, which are chromosomally located and nontransferabile, were detected in *E. casseliflavus*. These results suggest that comensal faecal microflora of the high land animals contains only naturally occurring vancomycine resistant enterococci. Similar observation of the occurrence only intrinsic antibiotic resistance of *Enterobacteriaceae* in faecal samples of alpine accentor was described by Timko and Kmeť (2003).

The summer study regions lie above the timber and dwarf pine line usually between 1 800 and 2 500 m above sea level. The habitats were dominated by alpine meadows and by rocky parts (Drgonová and Janiga 1989). Only few interactions (especially alpine skiing) exist between the alpine accentor or chamois and humans.

Výskyt génov rezistencie na vankomycín u enterokokov izolovaných z vysokohorského spevavca a kamzíka

Z 37 kmeňov enterokokov izolovaných z trusu vrchárky červenkastej *Prunella collaris* (n = 19) a kamzíka vrchovského *R. rupicapra* (n = 18) jedna tretina patrila do druhu *E. casseliflavus,* zatiaľčo ostatné boli identifikované ako *E. faecalis* a *E. gallinarum* (po 8 kmeňov), *E. faecium* a *E. mundtii* (po 4 kmene) a *E. flavescens* (dva kmene). Nízka hladina prirodzenej rezistencie na vankomycín (MIC 0.5 - 4 $\mu g.ml^{-1}$) a citlivosť na teikoplanín bola detekovaná u 13 kmeňov *E. casseliflavus* a *E. faecalis*. Gény *vanC1* a *vanC2/C3* boli pomocou PCR detekované u dvoch resp. štyroch kmeňov E. casseliflavus. Prítomnosť *vanA* a *vanB* génov rezistencie nebola zaznamenaná. Výsledky ukázali, že vo vysokohorskom

prostredí kde neexistuje selekčný tlak antibiotík bola dokázaná prítomnosť *vanC* génov kódujúcich iba prirodzenú rezistenciu u enterokokov.

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