Detection of vancomycin-resistant *Enterococcus faecalis* ST6-*vanB2* and *E. faecium* ST915-*vanA* in faecal samples of wild *Rattus rattus* in Spain

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Abstract

The detection of vancomycin-resistant-enterococci (VRE) among wild animals represents a worrisome public health concern. The objectives of the study were to determine the possible presence of VRE in faecal samples of wild small mammals in Spain, to characterize the vancomycin resistance mechanisms and genetic lineages of recovered isolates and to know the diversity of enterococcal species in these animals. A total of 155 faecal samples from small mammals were inoculated in Slanetz-Bartley agar supplemented or not with vancomycin (Van-SB/SB plates). The antimicrobial susceptibility profile to 12 antimicrobials and the presence of 20 antimicrobial resistance genes was analyzed. The structure of Tn*1546* and the presence of *gelE*, *cylA*, *asa*, *esp* and *hyl* genes was studied. Multilocus-sequence-typing (MLST) technique was also performed. VRE isolates were recovered in Van-SB plates in 11 samples. Two samples contained *van*B2-positive *E. faecalis* isolates of lineage ST6, which showed a multiresistance phenotype and harboured the virulence genes *gelE* and *asa*. One sample contained a vancomycin-resistant *E. faecium* isolate of the new lineage ST915, with the *van*A gene included into Tn*1546* (truncated with IS*1542* and IS*1216* elements). The *vanB2* and *vanA* isolates were obtained from *Rattus rattus*. The remaining eight VRE-positive samples contained species with intrinsic vancomycin-resistance mechanisms: *E. casseliflavus* (n=5) and *E. gallinarum* (n=3). One-hundred-forty-seven vancomycin-susceptible-enterococcal isolates were obtained in SB plates, and *E. faecalis* and *E. faecium* were the most frequent detected species. This is the first report of *vanB2*-containing enterococci in wild animals.

Keywords

Enterococci, *vanB2*, *vanA*, wild small mammals, ST6, ST915, *Rattus rattus*

1. Introduction

Enterococcus spp. are commensal microorganisms that colonize the gastrointestinal tract in humans and animals. They can also produce opportunistic infections, highlighting the predominance of *E. faecalis* and *E. faecium* species. Clinical cases caused by other species, including *E. casseliflavus*, *E. gallinarum*, *E. hirae* or *E. mundtii*, have been identified sporadically (Brulé et al., 2013; Higashide et al., 2005; Sambhav et al., 2012; Swampillai et al., 2011). These microorganisms have some intrinsic antimicrobial resistance mechanisms and are able to acquire other mechanisms, which limits severely the therapeutic options.

Among the existing antimicrobial agents, vancomycin is one of the most clinically important and is one of the last lines of defense against many Gram positive bacteria. For this, the emergence and spread of vancomycin resistant enterococci (VRE) in hospital settings has been, and continue being, a highly worrisome problem. Remarkably, VRE isolates containing acquired vancomycin resistance mechanisms (especially those encoded by the genes *vanA* and *vanB2*) represent a major public health problem, due to their demonstrated capacity of transference (Orsi et al., 2013). Some enterococcal species, as *E. gallinarum* and *E. casseliflavus*, present an intrinsic mechanism of vancomycin resistance, mediated by the *van*C mechanism (variants *van*C-1 and *van*C-2/3), that confers low level vancomycin resistance and is not transferable (Sambhav et al., 2011; Swampillai et al., 2012).

In the last two decades it has been reported the detection of VRE of the *vanA* genotype in farm animals and in derived food and it was associated with the use of the glycopeptide avoparcin as animal growth promoter in many countries. For this reason, this specific use was banned in the EU since 1997 (Hammerum et al., 2012). A decrease in the prevalence of *vanA*-enterococci was observed after the ban of avoparcin as

growth promoter (Bager et al., 1999; van den Bogaard et al., 2000). However, the problem persists and several hypotheses point out that the co-selection phenomenon could be one of the causes (Aarestrup 2000; Hammerum et al., 2012). Moreover, *vanA* containing enterococci have been also described in different wild animal species such as woodmice, badgers, crows, wild boars, seagulls, and song thrush (Mallon et al., 2002; Oravcova et al., 2014; Poeta et al., 2007; Radhouani et al., 2010, 2011; Silva et al., 2012), but never before in Spain.

Regarding vancomycin resistance gene *vanB2* in enterococci, there is only one previous report in animals, in one *E. hirae* isolate recovered in 2003 from a healthy pig in Spain (Torres et al., 2003). This gene has been also found in food samples of animal origin (López et al., 2009). Moreover, the *vanB2* mechanism of resistance has been detected in the last years in *E. faecalis* or *E. faecium* isolates implicated in hospital outbreaks or in sporadic clinical cases in Spain (López et al., 2012; Nebreda et al., 2007). Recently, this gene has been identified in one *Staphylococcus succinus* isolate from a wild songbird (*Turdus migratorius*) (Ishihara et al., 2013). However, so far, it is not clear if it could be a potential animal reservoir for the *vanB2* resistance mechanism.

The objective of this study was to determine the presence of VRE in faecal samples of wild small mammals in Spain and to characterize the mechanisms of vancomycin resistance and the genetic lineages of recovered isolates. Moreover, the study was also focused to know the diversity of enterococcal species in these animals as well as their antimicrobial resistance phenotypes and genotypes.

2. Material and methods

2.1. Samples and bacterial isolates

One hundred and fifty-five faecal samples collected from free-ranging wild small mammals [54 common voles (*Microtus arvalis*), 41 wood mice (*Apodemus sylvaticus*), 6 Algerian mice (*Mus spretus*), 46 black rats (*Rattus rattus*), 6 greater white-toothed shrews (*Crocidura russula*), 1 garden dormouse (*Eliomys quercinus*) and 1 red squirrel (*Sciurus vulgaris*)] were analyzed in this study. Wild small mammals included in this study were captured in the framework of projects headed by researchers at the Spanish Wildlife Research Institute (IREC) from 2008 to 2013. The projects at which wild small mammals were captured had been subjected to the examination by ethical committees. These animals came from two different Spanish regions: i) North-Centre (South of the province of Palencia; N=75); and ii) South (Province of Cádiz; N=80). The North-Centre region of study is an agriculture-devoted landscape mainly composed of cereal plains within a continental Mediterranean climate. Small mammals coexist with extensively produced livestock, humans and agricultural landscape-associated wildlife. The South region is a hunting estate in a thermo-Mediterranean climate in which small mammals coexist with a high diversity of wildlife and with extensively farmed red deer. Faecal samples were collected from animals at necropsy into sterile vials, sealed, frozen at -20° C and transported frozen to the laboratory. Faecal samples were thawed at room temperature for 2 hours, suspended in 3 mL of saline solution, and 100 µL was seeded on Slanetz-Bartley agar plate (Scharlau Chemie S.A., Barcelona, Spain) both supplemented (Van-SB) and non-supplemented (SB) with 4 µg/ml of vancomycin. Plates were incubated 48 h at 37 °C. Two colonies from each positive plate with a typical enterococcal morphology were selected and initially identified by biochemical conventional methods (Gram staining, catalase, and bile esculin test). PCR experiments with specific primers for different enterococcal species (*E. faecalis, E. faecium, E. hirae, E. durans, E. casseliflavus,* and *E. gallinarum*) were carried out (Torres et al.,

2003). Moreover, identification of the remaining enterococcal species was performed by amplification and sequencing of the *sodA* gene (Poyart et al., 2000).

2.2. Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MICs) of vancomycin and teicoplanin were determined by the agar dilution method (EUCAST, 2015). Susceptibility testing to other 10 antimicrobial agents was performed by the disk diffusion method and the antimicrobials tested were (mug/disk): ampicillin (10), streptomycin (300), gentamicin (120), kanamycin (120), chloramphenicol (30), tetracycline (30), erythromycin (15), ciprofloxacin (5), trimethoprim–sulfamethoxazole (1.25+23.75), and linezolid (30) (CLSI, 2014). *E. faecalis* ATCC 29212 and *S. aureus* ATCC 29213 were used as quality control strains.

2.3. Study of vancomycin resistance mechanisms

The presence of the vancomycin resistance genes *vanA*, *vanB*, *vanC*-1, *vanC*-2/3, *vanD*, *vanE*, and *vanG* was studied by PCR in enterococcal isolates which showed resistance or reduced susceptibility to glycopeptides (Torres et al., 2003; Domingo et al., 2005). When *vanA* gene was detected, the whole structure of Tn*1546* was analyzed by PCR overlapping and sequencing, using a wide diversity of primers as previously described (López et al., 2010). The positive *vanB* amplicons were sequenced for identifying the *vanB* allele type (*vanB1*, *vanB2* or *vanB3*).

2.4. Detection of other resistance genes

Resistance genes for other antimicrobials, including macrolides [*erm*(A), *erm*(B), *erm*(C)], tetracycline [*tet*(M), *tet*(K), *tet*(L)], aminoglycosides [*aph*(3′)-IIIa, *aac*(6′)-*aph*(2″), *ant*(6)-Ia], trimetoprim-sulfamethoxazol [*drfF*, *dfrG* and *dfrK*] and linezolid [*cfr*] were also tested by PCR in all enterococcal isolates which showed resistance or

149 reduced susceptibility for these agents, using primers and conditions as previously
150 described (Cattoir et al., 2009; López et al., 2009).

151 *2.5. Detection of virulence genes*

152 The presence of the virulence genes *gelE*, *cylA*, *asa*, *esp* and *hyl* was studied in the
153 enterococcal isolates containing acquired vancomycin resistance mechanisms. For that,
154 primers and conditions previously described by others were used (López et al., 2012;
155 Vankerckhoven et al., 2004).

156 *2.6. Molecular typing*

157 Vancomycin-resistant *E. faecalis* and *E. faecium* isolates were characterized by the
158 technique of multilocus sequence typing (MLST). For that, internal fragments of seven
159 housekeeping genes were amplified and sequenced (Homan et al., 2002; Ruiz-Garbajosa
160 et al., 2006), and the sequences obtained were analyzed and compared with those
161 included in the databases (http://efaecalis.mlst.net/ and http://efaecium.mlst.net/).
162 Analyzing the combination of the seven obtained alleles, a specific sequence type (ST)
163 was determined. Moreover, pulsed-field gel electrophoresis (PFGE) pattern analysis
164 with *SmaI* restriction enzyme was performed for the two *vanB2*-positive isolates in
165 order to analyze their genetic relatedness, as previously described (López et al., 2012).

166 *2.7. Conjugation experiments*

167 Transference of vancomycin resistant determinants was assayed by conjugation from
168 the *van*A and *van*B2 enterococci as donors to *E. faecalis* strain JH2-2 and *E. faecium*
169 strain GE-1 as recipient strains, using a filter-mating method (López et al., 2010). The
170 antimicrobial resistance phenotype and genotype of transconjugants was analyzed as
171 previously indicated to determine the resistance genes transferred.

172

173 **3. Results**

3.1. Enterococcus *spp. isolates recovered in SB agar plates with vancomycin*

VRE isolates were recovered in 11 of the 155 faecal samples analysed when vancomycin-supplemented SB agar plates (Van-SB) were used. The two colonies isolated in each Van-SB plate studied belonged to the same species and presented identical resistance phenotype. For that, one isolate of each positive sample was maintained and further studied. The characteristics of these 11 isolates are shown in Table 1.

3.2. Detection of VRE with acquired mechanisms of resistance

VRE isolates with acquired vancomycin resistance mechanisms were identified in three samples, two isolates containing the *vanB* gene and one isolate containing the *vanA* gene. The three isolates with acquired vancomycin resistance mechanisms were obtained from faecal samples of *R. rattus.*

The *vanB* and *vanA* positive isolates were obtained from samples collected in the same location (Benalup-Casas Viejas, Cádiz province) in Southern Spain but in two neighbour estates: i) one dedicated to deer farming; and ii) one dedicated to agriculture. The *vanB* gene was found in two *E. faecalis* isolates which were recovered from two rats surveyed in the same day (10th July 2013) in the agricultural estate. After sequencing of the *vanB* gene, it was determined that both isolates harboured the *vanB2* allele. Nevertheless, both sequences showed a nucleotide mutation (G34T) leading the Met11Ile amino acid change. These *vanB2*-positive isolates belonged to sequence type ST6 and showed a multiresistance phenotype that included, in addition to vancomycin (MIC=64-128 μg/mL), also resistance to erythromycin, tetracycline, ciprofloxacin, trimethoprim-sulfamethoxazole and high-level resistance to aminoglycosides (gentamicin, streptomycin, and kanamycin). According to PFGE results, both strains showed closely related patterns since they differed by three bands.

The *vanA* gene was identified in one *E. faecium* isolate. This isolate belonged to a new ST, named as ST915, which presented a new allelic combination. This isolate was recovered from one sample in the deer farm estate on the 9th of July, 2013. This place is 10 km away from the agricultural estate, where the two *vanB2* isolates were obtained. The *vanA* positive isolate was resistant, in addition to vancomycin (MIC=>256 μg/mL) and teicoplanin (MIC=128 μg/mL), to erythromycin and tetracycline. The Tn*1546* structure, carrying the *van*A gene, was characterized in this isolate and was compared with the sequence included in GenBank accession number M97297.1. Our structure presented the IS*1542* structure located on the region between *orf2* and *vanR* (positions 3932-3925) and the IS*1216* sequence was detected inside the *vanXY* intergenic region (positions 8839-8828) (Figure). Moreover, the nucleotide G was found in the position 8234 of gene *vanX*.

None of these three *vanA* or *vanB2* positive isolates showed the virulence genes *esp* and *hyl*. However, both *vanB2* positive isolates presented the virulence genes *gelE* and *asa*, and one of them (C7526) also contained the gene *cylA*. Only transconjugants of the *vanA E. faecium* isolate were obtained using both recipient strains *E. faecium* GE-1 and *E. faecalis* JH2-2 (conjugation frequencies 1.3×10^{-6}/recipient and 5.8×10^{-8}/recipient, respectively), and both transconjugants showed resistance, in addition to vancomycin, to tetracycline and erythromycin.

3.3. Detection of VRE with intrinsic mechanisms of resistance

VRE isolates with intrinsic vancomycin resistance mechanisms were identified in 8 samples analysed (*R. rattus*, 5; *A. sylvaticus, 2; M. arvalis, 1*), and one isolate per sample was selected. Five isolates were identified as *E. casseliflavus* and three isolates as *E. gallinarum*. The range of MICs for vancomycin and teicoplanin in these isolates

223 was 8-16 μg/mL and 1 μg/mL, respectively. Resistance to erythromycin, tetracycline,
224 ciprofloxacin and trimethoprim–sulfamethoxazole was identified in some of them
225 (Table 1).

226 *3.4.* Enterococcus *spp. recovered in SB agar plates without vancomycin*
227 *supplementation*

228 The 155 faecal samples from free-ranging wild small mammals were also analyzed
229 using non antimicrobial-supplemented SB agar plates for enterococci recovery, under
230 non selective conditions. Enterococci isolates were detected in 95 of them. Two
231 colonies with a typical enterococcal morphology were obtained in each positive plate
232 and they were further identified and characterized. A total of 147 enterococci were
233 obtained. Enterococcal species, animal origin, and phenotype and genotype of resistance
234 are shown in Table 2.

235 The most frequent species were *E. faecalis* (38.1%) and *E. faecium* (27.9%) followed by
236 *E. hirae* (14.9%) and *E. mundtii* (13.5%). The *E. durans* and *E. gilvus* species were
237 identified in a low percentage.

238 *E. mundtii* and *E. gilvus* isolates were susceptible to all antimicrobials tested. Among
239 the remaining species, resistance to some antimicrobials was identified. Erythromycin
240 resistance was detected in 12 isolates and in all cases was mediated by the *erm*(B) gene.
241 Trimethoprim-sulfamethoxazole resistance was identified in 11 isolates and one isolate
242 presented the gene *dfrF* and another one the gene *dfrG*. In the remaining trimethoprim-
243 sulfamethoxazole resistant isolates, the responsible gene remained unknown. Regarding
244 tetracycline, 5 isolates showed resistance for this agent and all of them harboured the
245 *tet*(M) gene. One of these isolates contained, in addition to this gene, the *tet*(L) gene.
246 High level of kanamycin resistance was identified in 4 isolates and was encoded by the

247 *aph*(3´)-IIIa gene. Ciprofloxacin resistance was only identified in two isolates and
248 linezolid resistance was found in one isolate but the *cfr* gene was not detected.
249
250
251 **4. Discussion**
252 VRE isolates were detected in 11 wild small mammal faecal samples when Van-SB
253 plates were used. Different percentages of VRE with acquired and intrinsic mechanisms
254 have been previously found in wild animals, from no detection of VRE isolates in
255 buzzards (Radhouani et al., 2012), and low VRE rates (2%) in Iberian wolves and
256 lynxes (Gonçalves et al., 2011) up to percentages similar to the ones found in our study
257 (about 6%) in crows and other wild birds (Oravcova et al., 2013; Silva et al., 2011).
258 These variations may be due in part to differences in the methodologies applied and
259 especially to difficulties in survey design when wild animals are studied. Moreover, our
260 study was focused on samples taken in a long period of time and this could be affecting
261 detected prevalences.
262 Three VRE isolates with acquired mechanisms of resistance from three different
263 samples were identified. Two *E. faecalis* isolates harboured the *vanB2* gene and one *E.*
264 *faecium* presented the *vanA* gene. The detection of the *vanB2* gene is highly relevant
265 since it is the second time that this gene is found in enterococcal isolates from animals
266 (Torrres et al., 2003) and the first time in wild animals. There have been several
267 outbreaks and clinical cases related to *vanB2* positive isolates, both *E. faecalis* and *E.*
268 *faecium*, in the last years in Spain (López et al., 2012; Nebreda et al., 2007). Our two
269 isolates belonged to the lineage ST6, which is included in the hospital adapted clonal
270 complex CC2 (López et al., 2012). A recent study about the population structure of
271 human *E. faecalis* in Europe showed as *E. faecalis* belonging to CC2 seems to be very

prevalent acting as agent causing human infections in Spain and in The Netherlands (Kuch et al., 2012). Moreover, it seems that ST6 (CC2), may be particular adept at acquiring exogenous genes through recombination, being able to contain *vanA* or *vanB* genes (McBride et al., 2007).

Interestingly, in Spain, one *E. faecalis vanB2* ST6-CC2 clone was identified in clinical samples of patients of three geographically related hospitals (López et al., 2012). The three hospitals were located in the same city (Zaragoza) and this city and the region in which our *vanB2* positive isolates were detected are separated by more than 700 km in a straight line. All isolates detected in that study presented a multiresistance phenotype, being a serious problem for public health. Our *vanB2* isolates showed a similar multiresistance phenotype (resistance to macrolides, tetracycline, quinolones, and high level of aminoglycosides) and also presented resistance to trimethoprim-sulfamethoxazole encoded by the gene *dfrF*. Moreover, our two *vanB* positive isolates presented the virulence genes *gelE* and *asa*. In the study of López et al., the gene *gelE* was also found in all their clinical isolates (López et al., 2012). Interestingly our two isolates presented closely related patterns with three different bands and only one of them contained the virulence gene *cylA*. None of the two *vanB2* positive isolates showed the virulence genes *esp* and *hyl*. The genes *gelE*, *asa* and *cylA* seems to be most commonly found in clinical *E. faecalis* isolates than in *E. faecium* isolates (López et al., 2012; Vankerckhoven et al., 2004).

Our two *vanB2*-isolates were identified in two samples from *R. rattus*. *Ratus rattus* and *R. norvegicus* are widely distributed in wild small mammals in Spain (Palomo et al., 2007). Both species may widely interact with other wildlife, livestock and humans since they display an extremely flexible feeding behavior and are well-adapted to humanized environments. Additionally, *R. rattus* is spread all over the world and it is included in

297 the list of 100 most invasive species of the world (Lowe et al., 2000). The presence of
298 antimicrobial resistance in wild rodents has been previously demonstrated (Gilliver et
299 al., 1999). Different antimicrobial resistance determinants have been found in
300 Enterobacterias in these animals (Gilliver et al., 1999; Guenther et al., 2010, 2013).
301 Interestingly, in two recent studies, extended-spectrum-betalactamase producing *E. coli*
302 strains has been detected in urban rats (*R. norvegicus*) (Guenther et al., 2010; Guenther
303 et al., 2013). According to our results, it would be very interesting to study if *R. rattus*
304 and other rodents can be also acting as reservoirs of vancomycin resistance mechanisms
305 and their possible role in the emergence of infections caused by *vanB2*-positive isolates
306 in hospital environments.
307 Regarding the *vanA* gene, several studies have identified *vanA*-positive *Enterococcus*
308 spp in wild animals in percentages very variable (0%-13.5%) (Goncalves et al., 2011;
309 Mallon et al., 2002; Oravcova et al., 2013, 2014; Poeta et al., 2007; Radhouani et al.,
310 2010, 2011, 2012; Santos et al., 2013; Silva et al., 2011; 2012). The *vanA* gene has been
311 detected in these studies in *E. faecalis*, *E. faecium*, *E. hirae*, and *E. durans* (Oravcova et
312 al., 2014; Silva et al., 2011, 2012), being *vanA*-containing *E. faecium* the most frequent
313 (Oravcova et al., 2013; Radhouani et al., 2010; Silva et al., 2011, 2012). The reservoir
314 for *vanA* gene in humans seems to be also *E. faecium*. Interestingly, in hospital
315 environments in Europe *vanA*-positive *E. faecium* is the most prevalent one (Werner et
316 al. 2008). Our *vanA* positive *E. faecium* isolate belonged to a new ST (ST915).
317 According to its allelic combination (5-2-13-6-1-1-1), this ST would be a double-locus
318 variant of ST26 (CC26). CC26 *E. faecium* isolates have been previously detected in
319 animals, mainly in poultry samples (Cha et al., 2012), but not in humans. However, the
320 Tn*1546* structure identified in our *vanA* isolate was previously described in one *E.*
321 *faecium* clinical isolate in Spain (López et al., 2010). In the referred study, this structure

was called type VI. This transposon is usually located in conjugative plasmids which contain other antimicrobial resistance genes, specially *erm*(B) and *tet*(M) genes, associated with co-selection processes (López et al., 2010). Both genes were identified in our *vanA E. faecium* isolate and the transconjugants of this *vanA* isolate showed resistance in addition to vancomycin to these antimicrobials (tetracycline and erythromycin).

Among the 147 vancomycin susceptible enterococci isolates detected in SB plates (without vancomycin supplementation), the most frequent species were *E. faecalis* and *E. faecium*, being those species the most commonly found among free-living animals by others (Radhouani et al., 2011, 2012; Santos et al., 2013). Most of our *Enterococcus* spp. isolates, recovered in non-antimicrobial supplemented plates, were susceptible to all tested antimicrobial agents. However, resistance to some antimicrobials, such as erythromycin, trimethoprim-sulfamethoxazole, tetracycline, ciprofloxacin, linezolid, and high level resistance to kanamycin, was identified in some of them. In our study, *E. faecium* and *E. hirae* were the species which showed higher percentages of resistance. Similar results were found in one study carried out in faecal enterococci from wild boar, being *E. faecium* followed by *E. hirae* isolates the most resistant ones (Poeta et al., 2007).

In conclusion, the identification of VRE with acquired resistance mechanism (*vanA* and *vanB2*) in *R. rattus* is highly remarkable. To the best of our knowledge, there is no previous report on the *vanB2* gene in enterococci isolates from wild animals and it would be the second time in enterococci from animals. Rats could be acting as carriers and donors of these clinically important resistance mechanisms. More studies are necessary to know the possible role of these animals in the emergence of infections in

hospital settings and to detect the actual spread of these vancomycin resistance mechanisms in different ecosystems.

Conflicts of interest

There are no conflicts of interests to be declared.

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Figure caption. Structure of Tn*1546* found in our *vanA* positive *E. faecium* isolate.