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vanA in *Enterococcus faecium, Enterococcus faecalis*, and *Enterococcus casseliflavus* Detected in French Cattle

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Abstract

The goal of this study was to assess the presence of enterococci species presenting *van*-mediated glycopeptide resistance in French cattle. Fecal samples were collected from healthy and sick animals, and enterococci were screened for vancomycin resistance. Vancomycin resistance was principally encountered in *Enterococcus gallinarum* and *Enterococcus casseliflavus* strains. However, glycopeptide resistance was detected in three different species of enterococci (*E. faecalis, E. faecium*, and *E. casseliflavus*). Molecular characterization of the genetic support proved that they all presented the prototypic VanA element. Interestingly, the *E. casseliflavus* strain displayed a remarkable VanB phenotype/*vanA-vanC* genotype. Transferability, associated resistances, and factors of *vanA* cotransfer were sought. This study proved that acquired *vanA* genes can still be detected in food-producing animals more than a decade after the avoparcin ban. Indeed, calves, which are recurrently exposed to antibiotics in France, may allow the re-emergence of glycopeptide resistance through coselection factors, and this might potentially be concerning for human health.

Introduction

Glycopeptide antibiotics such as vancomycin and teicoplanin are used in the treatment of severe infections caused by Gram-positive bacteria. Enterococci constitute one of the target species, and the emergence of glycopeptide-resistant *Enterococcus faecium* (GRE), in Europe in 1988 (Leclercq *et al.*, 1988; Uttley *et al.*, 1988) and soon afterward in the United States (Sahm *et al.*, 1989), has become of high clinical concern (Bonten *et al.*, 2001). However, the transfer of the *van* genes to multiresistant *Staphylococcus aureus* also represents a major threat, as sporadically reported in the United States since 2002.

Different mechanisms of glycopeptide resistance were described in enterococci (Courvalin, 2006). High-level acquired resistance is principally mediated by the *vanA* gene worldwide and to a lesser extent by the *vanB* gene, which confer a transferable and inducible resistance to both vancomycin and teicoplanin or vancomycin only, respectively. These genes are located on transposons, allowing the intra- and interspecies spread of resistance.

Enterococci that most widely colonize food-producing animals, *Enterococcus gallinarum* and *Enterococcus casseliflavus*, display the intrinsic and chromosomally encoded *vanC* gene that confers a nontransferable low-level glycopeptide resistance. However, enterococci harboring transferable *van* genes were also detected, and even became of high concern when they spread among poultry and pigs (Bonten *et al.*, 2001). Retrospectively, the use of the glycopeptide avoparcin as a growth promoter in Europe was suspected to have selected high-level GRE. With regard to the possible expansion of the GRE reservoir in animals, which might constitute a risk for the human population, avoparcin was banned in all European countries in 1997.

Despite the ban, GRE in pigs and poultry were regularly reported. However, GRE isolated from cattle were scarcely described worldwide (Bonten *et al.*, 2001). In this study, we describe the first *vanA*-presenting *Enterococcus faecalis* and *Enterococcus faecium* isolated from cattle in France. Moreover, we also report an *E. casseliflavus* VanB/*vanA-vanC* isolate, which is to our knowledge the first case of such a complex

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phenotype–genotype association described in food-producing animals.

Materials and Methods

Bacterial isolates

The presence of GRE was assessed by analyzing feces of healthy or sick animals (calves, young beef cattle, and cull cows) in the frame of two studies. First, enterococci were isolated from healthy animals randomly sampled at the time of slaughter during a 2-year (2003–2004) survey, conducted as part of the National Surveillance Program monitored by the French Ministry of Agriculture. Enterococci isolates were sought using the Slanetz and Bartley selective medium (S&B; Oxoid, Dardilly, France). Second, samples from diarrheic bovines collected in separate farms were analyzed in 2006, in partnership with three peripheral veterinary laboratories, as part of a study on the presence of digestive GRE. After enrichment in peptone broth, colonies were selected on S&B containing 6 μg/mL vancomycin.

All GRE ($n = 26$) were characterized on the basis of colony morphology, API20 Strep test (Biomérieux, Marcy l'Etoile, France), and species-specific polymerase chain reaction (PCR) (Ke *et al.*, 1999; Depardieu *et al.*, 2004; Jackson *et al.*, 2004).

Antimicrobial susceptibility testing

Antibiotic resistance was determined by disk diffusion following the recommendations of the Antibiogram Committee of the French Society for Microbiology (CA-SFM, 2007a, 2007b). The following antibiotics were tested: ampicillin, streptomycin, gentamicin, kanamycin, vancomycin, teicoplanin, erythromycin, lincomycin, spiramycin, pristinamycin, tetracycline, cotrimoxazole (trimethoprim 25 μg + sulfamethoxazole 23.7 μg), florfenicol, enrofloxacin, and bacitracin. Vancomycin and teicoplanin minimum inhibitory concentrations (MICs) were determined by E-test according to the manufacturer's instructions (AB Biodisk, Solna, Sweden) on Müller-Hinton agar (Biomérieux), following the resistance breakpoints of the CA-SFM (>8 μg/mL for both glycopeptides). *E. faecalis* ATCC 29212 was used as a quality-control strain. The phenotypic copper resistance of selected isolates was tested on plates containing 0, 4, 8, 12, 16, 20, 24, 28, and 32 mM copper sulfate as described by Hasman *et al.* (2006).

Molecular analyses

The *van* genes were detected by PCR using specific primers as described (Depardieu *et al.*, 2004). The VanA elements were analyzed by restriction fragment length polymorphism and restriction analysis as previously described (Palepou *et al.*, 1998), using the CIP 103510 as *vanA*-positive control strain. Point mutations were sought in *vanX* by restriction polymorphism, or in *vanS* by sequencing. The Tn*1546* was analyzed by PCR as described by Oh *et al.* (2007). The presence of the copper-resistance gene *tcrB* (Hasman and Aarestrup, 2002) and the postsegregational killing system (PSK) (Sorum *et al.*, 2006) were investigated by PCR as described by Hasman and Aarestrup and Sorum *et al.*, respectively.

Pulsed-field gel electrophoresis

All *vanC E. gallinarum* and *E. casseliflavus* were characterized by pulsed-field gel electrophoresis (PFGE) after digestion by *Sma*I, according to Garnier *et al.* (2004) with minor modifications. Parameters for electrophoresis for both strains were 6 V/cm at 14°C for 22 h, with pulse time ramps from 0.5 to 15 sec. Differences in PFGE patterns were assessed visually.

Transferability of resistance

Transferability of the *vanA*-mediated resistance was tested by filter and broth mating as described (Lester *et al.*, 2006), using *E. faecalis* JH2-2 as the recipient. For the filter mating, 100 μL of the donor and recipient strains were mixed and placed on a sterile 0.45 μm pore filter on Columbia agar complemented with 5% sheep blood (COS; AES Chemunex, Bruz, France). After 24 h of incubation at 37°C, bacteria were resuspended in NaCl 0.9%, and appropriate dilutions of bacteria were plated on COS agar containing rifampicin, fusidic acid, and vancomycin as selection antibiotics. Transconjugants were observed after 24–48 h of incubation at 37°C. Conjugation was validated by PCR detection of the *vanA* gene on five colonies randomly picked on the selective plate. For broth mating, 0.5 mL of donor and recipient strains were incubated in 4.5 mL of Luria-Bertani broth. Selection was performed as for the filter mating. Transfer frequencies were expressed as the number of transconjugants per donor cell, and the data reported were the average of three different trials.

Results

A total of 1503 fecal samples were analyzed, 917 coming from the survey on healthy cattle and 536 from diarrheic bovines. In the survey, 218 enterococcal isolates were detected (recovery rate, 22.5%), among which 9 were GRE (9/967, 1%). In parallel, 17 GRE (17/536, 3.2%) were directly recovered from the sick animals.

Eight *E. casseliflavus* harboring the *vanC2* gene and 15 *E. gallinarum* with the *vanC1* gene were isolated (Table 1). Seven out of these 8 *E. casseliflavus* were recovered from healthy animals, while 14 out of the 15 *E. gallinarum* came from sick cattle. PFGE profiles showed no genetic relatedness (data not shown).

In parallel, three strains presented the GRE phenotype (Table 1).

E. faecalis H356_2 was isolated from a healthy calf in 2003 at the Chapin slaughterhouse, Ille-et-Vilaine. The strain presented a high-level resistance to vancomycin and teicoplanin (MICs >256 mg/L), and the *vanA* gene was detected. Additional resistances to kanamycin, erythromycin, lincomycin, spectinomycin, pristinamycin, tetracycline, and cotrimoxazole were identified as well.

E. faecium S8346 was isolated from a diarrheic calf on farm in Moselle, in 2006. High-level resistance to vancomycin and teicoplanin (MIC >256 mg/L) coincided with the presence of the *vanA* gene. S8346 strain also displayed resistances to ampicillin, streptomycin, kanamycin, erythromycin, lincomycin, spectinomycin, tetracycline, and enrofloxacin.

E. casseliflavus S8702 was isolated from a diarrheic calf in Seine-Maritime, in 2006. This strain presented a VanB phenotype (high-level vancomycin resistance [MIC > 256 mg/L] and susceptibility to teicoplanin [MIC = 3 mg/L]), associated with a *vanA*/*vanC2* genotype. No additional antibiotic resistance was associated, but the *tcrB* gene was detected. The presence of this gene conferred a slightly reduced susceptibility to copper, since bacterial growth was observed on plates containing 11 mM of copper, instead of 6 mM for the *E. faecalis*

Table 1. Species, Genotypes, and Antimicrobial Resistance of Glycopeptide-Resistant *Enterococcus faecium* Strains Isolated From Cattle

Strains	Species	Genotype[a]	MICs[b] (mg/L)		Additional resistances
			Vancomycin	Teicoplanin	
Healthy animals					
H95	*Enterococcus casseliflavus*	*vanC2*	8	1.5	L
H236	*E. casseliflavus*	*vanC2*	8	1.5	L
H344	*E. casseliflavus*	*vanC2*	6	2	L, E
H402	*E. casseliflavus*	*vanC2*	8	2	L, Tc
H430	*E. casseliflavus*	*vanC2*	6	2	L
H447	*E. casseliflavus*	*vanC2*	6	1.5	L
H537	*E. casseliflavus*	*vanC2*	8	1.5	L, E, Sp, Tc
H143	*Enterococcus gallinarum*	*vanC2*	16	1.5	L
H356_2	*Enterococcus faecalis*	*vanA*	>256	>256	L, E, Sp, K, Pt, SXT, Tc
Sick animals					
S8552	*E. gallinarum*	*vanC1*	12	1.5	L, B
S8555	*E. gallinarum*	*vanC1*	12	1.5	L, B, Sp
S8556	*E. gallinarum*	*vanC1*	8	1.5	L
S8557	*E. gallinarum*	*vanC1*	12	1.5	L
S8561	*E. gallinarum*	*vanC1*	12	1.5	L, Rif
S8565	*E. gallinarum*	*vanC1*	12	1.5	L
S8568	*E. gallinarum*	*vanC1*	12	1.5	L
S8569	*E. gallinarum*	*vanC1*	12	1.5	L
S8570	*E. gallinarum*	*vanC1*	8	1	L, B
S8572	*E. gallinarum*	*vanC1*	12	1.5	L, B
S8573	*E. gallinarum*	*vanC1*	12	1.5	L
S8698	*E. gallinarum*	*vanC1*	12	1.5	L, B
S8700	*E. casseliflavus*	*vanC1*	8	1.5	L
S8703	*E. gallinarum*	*vanC1*	12	1.5	L, B
S9011	*E. gallinarum*	*vanC1*	16	1.5	L, B
S8346	*E. faecium*	*vanA*	>256	>256	Am, Sm, L, E, Sp, Tc, K, Enr
S8702	*E. casseliflavus*	*vanC2, vanA*	>256	3	–

[a]*van*-specific PCRs were validated using reference strains (two).
[b]MICs were systematically performed on the required quality-control strains.
MICs, minimum inhibitory concentrations; Am, ampicillin; B, bacitracin; Enr, enrofloxacin; E, erythromycin; K, kanamycin; L, lincomycin; Pt, pristinamycin; Rif, rifampicin; Sp, spiramycin; Sm, streptomycin; SXT, cotrimoxazole; Tc, tetracycline; PCR, polymerase chain reaction.

AU2 ▶ ATCC 29212 reference strain. Moreover, the PSK system was also identified by PCR.

PCR amplification followed by enzymatic digestion indicated that all three strains presented an element similar to the prototypic Tn*1546* described by Palepou *et al.* (1998). The sequential PCR on the Tn*1546* performed according to Oh *et al.* (2007) led to the same amplification for the three vanA-
AU2 ▶ containing strains as well as for the CIP 103510 control strain. The *vanRSHAX* genes were further detailed by sequencing of the *vanS* gene, and by restriction analysis of *vanX* with the *Dde*I enzyme. No mutations were detected in the *vanS* and *vanX* genes, and the length of the PCR fragments showed no major genetic rearrangements, demonstrating the conservation of the *vanRSHAX* organization.

Transferability of the *vanA*-mediated resistance was tested, but could not be detected from S8346 and S8702 strains (frequency $<10^{-8}$ per donor cell). Conversely, H356_2 transferred the *vanA* determinant at a frequency of 4×10^{-4} transconjugants per donor. Only cotrimoxazole cotransferred with the *vanA* gene.

Discussion

In this study, both intrinsic *vanC*-mediated and acquired *vanA*-mediated resistances were detected in enterococci isolated from French cattle.

The presence of *E. gallinarum* and *E. casseliflavus* harboring the *vanC* gene was expected in cattle. However, surprisingly, *E. gallinarum* were nearly all isolated from sick cattle, whereas *E. casseliflavus* mainly came from healthy carriers. Yet, all isolates came from different farms and, according to their nonidentical PFGE patterns, should not have spread clonally. This discrepancy has never been reported before and thus remains unexplained. Therefore, further investigations on sick and healthy cattle would be needed, and particularly on samples collected in a same period of time. On the other hand, the nontransferability of the *vanC* elements, and the low-level resistance conferred, usually leads to a lack of interest for these species. Yet, this might change with the emergence of *vanA-vanC* genotypes as described in human (Camargo *et al.*, 2004) and now in cattle (*E. casseliflavus* S8702), as well as with the recent report of *vanA-vanC1 E. gallinarum* in human (Merquior *et al.*, 2008).

Three GRE were also described here. They were recovered from different species (*E. faecalis*, *E. faecium*, and *E. casseliflavus*), but all presented the prototypic Tn*1546* and conserved *vanRSHAX* genes.

The *E. faecalis* H356_2 showed a high transfer rate—proving the dissemination capacities of the VanA element, but no cotransfer of erythromycin and/or tetracycline resistance was observed. This stands in partial contradiction with the

hypothesis that persistence of vancomycin resistance is due to coselection through the use of these antibiotic classes (Aarestrup, 2000). Yet, coselection might occur through the use of other antimicrobials such as cotrimoxazole, which is the unique antibiotic that cotransferred with *vanA* in our experimental setting. It is also to note that cotrimoxazole is the second most widely used drug after tetracyclines in veterinary medicine in France. However, whether the cotransfer of the VanA-element might occur through another mechanism than antibiotic coselection cannot be excluded. Further, horizontal transfer of the *van* genes between poultry and cattle should not be underestimated when different animal species are reared within the same farm.

The *E. faecium* S8346 displayed multiple antibiotic resistances associated with the prototypic VanA element. Globally, the presence of multiresistant strains should be monitored, irrespective of the presence of van genes, since they might narrow the already small therapeutic arsenal against *Enterococcus* spp. Yet, resistance to tetracycline and macrolides—which was also detected in the *E. faecalis* H356_2 strain as well as in three *E. casseliflavus* and one *E. gallinarum*—was not unexpected. Indeed, these antibiotic families are still used for the treatment and prevention of disease, and such enterococcal resistances have already been reported in cattle or food products (meat and milk) (MARAN, 2005; Jung *et al.*, 2007; Kaszanyitzky *et al.*, 2007). On the contrary, the detection of beta-lactam resistance was of higher concern because ampicillin remains the first-line treatment in human.

Finally, the *E. casseliflavus* S8702 presented the complex VanB/*vanA-vanC* pattern. Phenotype–genotype discrepancies have already been reported in human enterococci (Song *et al.*, 2006). Likewise, the *vanA-vanC* genotype has been described in human *E. gallinarum* and *E. casseliflavus* (Dutka-Malen *et al.*, 1994; Camargo *et al.*, 2004), but, to our knowledge, such a complex association had never been described in an *E. casseliflavus* isolated from animals. Moreover, no VanS mutations or major genetic rearrangements of the Tn*1546* can explain the mechanism of the VanB phenotype in the *vanA* genetic background (Song *et al.*, 2006). Similarly, no detectable insertion or deletion in the Tn*1546* was detected, thus leaving this specific phenotype–genotype association unexplained, as already described in *E. faecium* (Gu *et al.*, 2009). As mentioned above, S8702 also harbors the *tcrB* and PSK system genes. On the one hand, copper sulfate, which is used as an additive, was shown to act as a selective factor of conjugation and transfer of the resistance to glycopeptide and macrolides (Hasman and Aarestrup, 2002), and *tcr* genes were already detected in *E. casseliflavus* isolated from pigs (Hasman *et al.*, 2006). On the other hand, it has been suggested that PSK systems might be involved in the long-term persistence of *vanA*-containing plasmids in *E. faecium* isolated from poultry. However, the true relevance of these mechanisms is still debated (Hasman and Aarestrup, 2005; Sorum *et al.*, 2006).

In conclusion, we isolated three different species of enterococci presenting a *vanA*-mediated glycopeptide resistance in cattle. Even if both studies were not conducted over the same period of time, the global prevalence of GRE (3/1503; 0.2%) is obviously not significant. However, one should note that all three strains were isolated from calves, both in healthy and sick contexts. In France, calves are recurrently exposed to antibiotics and, by far, harbor the most elevated resistance among cattle, including to extended-spectrum beta-lactamases (Madec *et al.*, 2008). Therefore, despite the ban of avoparcin usage since 1997, it remains crucial to prevent any GRE re-emergence in food-producing animals, that is, in calves, but also in broilers and pigs (Kempf *et al.*, 2008), that would now result from the use of other antibiotics than glycopeptides.

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Disclosure Statement

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No competing financial interests exist.

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AU1: "GRE" has been expanded as "glycopeptide-resistant *Enterococcus faecium*." Please check.
AU2: Please expand ATCC and CIP.
AU3: Disclosure Statement accurate? If not, please amend as needed.
AU4: In Ref. "CA-SFM, 2007a," please mention the language.
AU5: In Ref. "CA-SFM, 2007b," please mention the language.
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