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First report of VanA *Enterococcus gallinarum* dissemination within an intensive care unit in Argentina☆

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Abstract

Enterococcus gallinarum is intrinsically resistant to low levels of vancomycin and has been described as a colonizing microorganism causing bacteraemia and infection among immunosupresed patients. Between August 2000 and February 2001, 15 highly glycopeptide-resistant *E. gallinarum* isolates, one from blood and the remaining from rectal swabs, were recovered in a general hospital of Buenos Aires Province, Argentina. All isolates were characterized by biochemical assays, and displayed MICs of vancomycin in the range 16–128 mg/l and MICs of teicoplanin in the range 16–32 mg/l. In all cases, PCR analysis yield positive results for both *van*C1 and *van*A genes. *E. gallinarum* isolates were classified as two clonal types by *Sma*I-PFGE: clone A (n = 8) and clone B (n = 7) and both harboured a transferable *van*A element.

Keywords: *Enterococcus gallinarum*; vanA; Glycopeptide-resistance; Argentina

1. Introduction

Enterococci have emerged as an increasingly important nosocomial as well as a community-acquired pathogen. Their emergence in the last decades is attributable to the frequent usage of antimicrobial agents [1–4]. Most of human enterococcal infections are caused by *Enterococcus faecalis*, followed by *Enterococcus faecium* [1,4,5]. *Enterococcus gallinarum* and *Enterococcus casseliflavus/flavescens* have been shown to colonize the intestinal tracts of both hospitalized and non-hospitalized individuals [6] and have also been rarely implicated in human infections, such as endocarditis and bacteraemia [6–8], especially in immunocompromised patients.

Vancomycin resistance in enterococci is the result of the expression of two ligases, native and acquired, both able to modify the cell wall precursor and reducing its efficiency [9,10]. The most important genetic determinants involved in glycopeptide resistance mechanisms are the *van*A and *van*B genes, which have been located in mobile elements. The *van*A gene is contained in Tn*1546*, a 10.8-kb transposon that carries the *vanRSHAXYZ* cluster, besides two other genes involved in transposition [10–12]. Diverse insertion sequences (IS*1251*, IS*1542*, IS*1216V*, IS*1476* and IS*3-like*), deletions and point mutations play an important role in the diversity of *van*A elements [10–14].

The *van*C genes, which confer low-level resistance to vancomycin, are native for motile enterococci *E. gallinarum* and *E. casseliflavus/flavescens* [6,9,15] that carry *van*C1 and *van*C2/C3 genes, respectively. Few reports have addressed the clinical and epidemiological significance of VanC enterococci [6,9,16], but some sporadic highly vancomycin-resistant clinical isolates of *E. gallinarum* and *E. casseliflavus*

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carrying *van*A or *van*B genes have been reported worldwide [7,17–19].

In Argentina, the first *E. faecium van*A-positive strain was isolated in 1996 from a blood culture [20]. According to data from the National Surveillance Network (WHONET-Argentina), the prevalence of vancomycin-resistant enterococci (VRE) causing infectious diseases has increased in our country from 0.8% in 1998 to 4.9% in 2002. Although the prevalent VRE in Argentina is *E. faecium* carrying *van*A gene (Corso A, Gagetti P, Rodriguez M, Melano R, Ceriana P, Faccone D, VRE Argentinian Collaborative Group. Abstracts 41st Interscience Conference on Antimicrobial Agents Chemotherapy. Abstr. 509; 2001), a few *E. faecium* VanB isolates have been described [21].

The first *E. gallinarum* with high-level resistance to glycopeptides, named M2686, was isolated from a 64-year-old man hospitalized in the Hospital Interzonal General de Agudos Evita, Buenos Aires Province, Argentina [22]. This isolate was genotypically characterized as VanA and VanC1 by PCR. This was the third clinically significant VRE isolated in this hospital, after two vancomycin-resistant *E. faecium* infections. After this case report, rectal colonization surveillance was immediately performed at the intensive care unit (ICU) of the institution. In this work, we genetically characterized the mechanism conferring high-level resistance to glycopeptides of *E. gallinarum* M2686 and investigated the clonal relationship of the case report and all *E. gallinarum* isolates recovered from the colonization surveillance.

2. Materials and methods

2.1. Case report and colonization surveillance

In June 2000, a 64-years old man with type II diabetes and general failure was hospitalized at the ICU of the Hospital Interzonal General de Agudos Evita. The patient developed symptoms of aspiration pneumonia and was subsequently treated with ceftriaxone (1 g/12 h) plus clindamycin (600 mg/6 h) for 14 days. After this period, antimicrobial therapy was changed to ceftazidime (1 g/8 h) plus vancomycin (1 g/12 h) for 12 days, because of catheter related bacteraemia due to methicillin-resistant *Staphylococcus aureus*. In August, the patient was transferred to a clinical medical unit (CMU). After 53 days of hospitalization a positive blood culture was obtained during a febrile episode. The isolate, named M2686, was identified as *E. gallinarum* and was characterized as a high-level vancomycin-resistant *Enterococcus* harbouring both *van*C1 and *van*A genes. Because of its susceptibility pattern, antibiotic therapy was changed to ampicillin (2 g/12 h). The patient finally died after 13 days of ampicillin treatment [22].

Because the uncommon genotype of *E. gallinarum* M2686, a rectal swab surveillance was immediately implemented among patients from the ICU. This study was conducted from August 2000 to February 2001. All new patients to the ICU, were also screened for colonization with VRE, and the procedure was repeated weekly. Rectal swabs collected using Stuart media, were enriched in tryptic soy broth for 18 h and subcultured onto bile-aesculin azide agar supplemented with 6 μg/ml of vancomycin. Only black colonies were studied further. During this period, 256 rectal swabs were obtained from 124 patients. Thirty-five VRE were collected, 19 *E. faecium* and 16 *E. gallinarum*. We studied one isolate per patient, a total of 15 VanA-phenotype *E. gallinarum*, 14 from the rectal swab surveillance plus the case report. All the patients, the index case and those that became colonized with vancomycin-resistant *E. gallinarum* were present around the same time. Environmental and health care workers were not screened for VRE.

2.2. Biochemical characterization

Isolates were characterized at species level as *E. gallinarum*, using in-house biochemical methods according to Facklam's scheme [5]. The isolates were tested for bile-aesculin, pyrrolidonyl arylamidase, leucine-aminopeptidase, arginine-dihydrolase, tolerance to 6.5% NaCl, pyruvate, tellurite, mannitol, arabinose, sucrose, sorbitol, sorbose, raffinose and methyl-α-D-glucopyranoside, chain-arrangement in thioglycolate broth, motility and pigment production.

2.3. Susceptibility testing

MICs for ampicillin (Bagó, Argentina), vancomycin (Lilly), teicoplanin (Aventis Pharma), gentamicin (Schering-Plough), streptomycin (Rontag), tetracycline (Phoenix), chloramphenicol (Parke Davis), erythromycin (Lilly) and ciprofloxacin (Roemmers, Argentina) were determined by agar dilution method in Mueller–Hinton agar (Difco Laboratories, Detroit, MI) with a final inoculum of 10^4 CFU/spot. Cultures were incubated in ambient air for 16–20 h (except for vancomycin that were incubated for 24 h) at 35 °C, according to NCCLS recommendations [23]. The following reference strains were included: *E. faecalis* ATCC 29212, *E. faecalis* 51299; *Staphylococcus aureus* 29213.

2.4. PCR assays and PCR-based restriction fragment length polymorphisms (PCR–RFLP)

Amplifications of *van*A and *van*C1 genes were performed with primer pairs previously defined [15]. DNA templates were prepared by the boiling method and 2 μl of these extracts were used in each PCR assay. Reactions were performed with a Biometra thermal cycler (Whatman Biometra GmbH, Göttingen, Germany) in a final volume of 50 μl containing 20 pmol of each primer, 25 μM of each dNTP, 1.5 mM $MgCl_2$ and 2.5 U of *Taq* polymerase (Promega, Madison, WI, USA). The PCR programme was as follows: 5 min of denaturation at 94 °C; 30 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 42 °C, 30 s of extension at 72 °C; and a final extension step of 5 min at 72 °C. PCRs with specific primers

for 16S ribosomal RNA gene were used as controls of DNA extraction [24]. Amplification of the intergenic *van*S–*van*H region was performed using the specific primers, *van*S-f (forward) and *van*H-r (reverse) described by Brown et al. [25]. The cycling programme was the same as described above, with an annealing temperature of 55 °C and extension time of 2 min. The PCR amplification products were analyzed in 1% agarose gel. *E. faecalis* Tx2403 (*van*A positive), *E. faecium* WHO-3 (*van*A positive), *E. gallinarum* Tx2406 and *E. gallinarum* WHO-11 strains, used as PCR controls, were kindly provided by Barbara Murray (University of Texas at Houston) and Fred Tenover (CDC, Atlanta, GA).

PCR–RFLP was performed on *van*SH amplicons obtained from all 15 isolates, using *Hin*dIII and *Eco*RI enzymes as recommended by the manufacturer (New England Biolabs, Beverly, MA, USA).

2.5. Molecular typing

Enterococcal genomic DNA was prepared as previously described [2] and digested with *Sma*I (New England Biolabs). DNA fragments were analyzed by pulse field gel electrophoresis (PFGE), using 0.8% agarose gels and a CHEF-DRIII apparatus (Bio-Rad Laboratories, CA, USA), under the same conditions described by De Lencastre et al. [2]. Isolates were classified in clonal types according to the Tenover criteria [26]. Briefly, isolates were considered genetically indistinguishable and were assigned to the same clonal type (e.g., type A) if they had identical PFGE profiles. Isolates with differences in one to three bands in their PFGE profiles were considered closely related and were assigned to a subtype (e.g., subtype A1). Isolates whose PFGE profiles differed by more than three bands were considered to be unrelated and were assigned to different clonal types (e.g., A or B).

2.6. Conjugation assays

Biparental conjugations were performed as follows. Cells of both the donor and the recipient strain were mixed on Brain Heart Infusion (BHI) agar in a 1:10 ratio, and the mixture incubated for 18 h at 35 °C. A bloodstream *E. faecium* M95ZAP from an ICU patient, with susceptibility to vancomycin (MIC 0.5 mg/l) and resistance to ampicillin (MIC 256 mg/l), was used as recipient strain. Transconjugant strains were selected on BHI agar supplemented with 32 mg/l of vancomycin plus 16 mg/l ampicillin.

3. Results and discussion

Since the observation of the first case of *E. gallinarum* with high-level resistance to glycopeptides in the Hospital Interzonal General de Agudos Evita [22], a rectal swab surveillance was implemented in the ICU, for 7 months. A total of 14 enterococci showing high-level resistance to glycopeptides were recovered from the surveillance cultures and all were biochemically characterized as *E. gallinarum* and confirmed through the detection of the *van*C1 gene.

Antimicrobial susceptibility profiles of all 15 *E. gallinarum* isolates are summarized in Table 1. All isolates were resistant to vancomycin and teicoplanin having MICs between 16–128 mg/l and 16–32 mg/l, respectively. As expected for *E. gallinarum* isolates, they were susceptible to ampicillin, MIC range 0.5–4 mg/l [6,27]. All strains were susceptibile to tetracycline, chloramphenicol and ciprofloxacin, were resistant to erythromycin and showed high-level resistance to streptomycin. Nine *E. gallinarum* isolates showed high-level resistance to gentamicin with MICs $\geq$1024 mg/l while the remainder had MICs of 4 mg/l. All 15 *E. gallinarum* isolates were positive for the *van*A gene by PCR.

Table 1
Susceptibility to antimicrobial agents and molecular typing of 15 *E. galtinarum* isolated in a general hospital from Argentina

Isolate[a]	Date of isolation	MIC (mg/l)									*Sma*I-PFGE types
		VAN	TEC	AMP	TET	CHL	GEN	STR	CIP	ERY	
M2686	02-Aug-00	64	32	4	0.5	8	1024	>2048	0.5	>1024	A
M2685	12-Sep-00	64	32	4	1	8	1024	2048	0.5	>1024	A
M2695	03-Oct-00	64	32	4	0.5	4	1024	>2048	0.5	>1024	A
M2696	03-Oct-00	64	32	4	1	8	1024	>2048	0.5	>1024	A
M2715	17-Oct-00	64	32	4	1	8	1024	>2048	1	>1024	A
M2716	17-Oct-00	64	32	4	1	8	1024	>2048	1	>1024	A
M2725	07-Nov-00	64	16	4	0.5	4	1024	>2048	0.5	>1024	A
M2749	19-Dec-00	128	16	4	0.5	4	2048	>2048	1	>1024	A
M2723	05-Sep-00	32	16	0.5	0.5	8	>2048	>2048	0.5	>1024	B
M2724	24-Oct-00	32	16	1	1	8	4	>2048	1	>1024	B
M2747	29-Nov-00	16	16	0.5	0.5	8	4	>2048	1	>1024	B
M2746	02-Jan-01	32	16	0.5	1	4	4	>2048	1	>1024	B
M2765	02-Jan-01	32	16	0.5	1	8	4	>2048	1	>1024	B
M2753	09-Jan-01	32	16	0.5	0.5	4	4	>2048	1	>1024	B
M2764	I6-Jan-O1	64	16	1	1	8	4	>2048	1	>1024	B

[a] M2686 was isolated from a blood culture in CMU. The remaining isolates were collected from rectal swab in ICU.

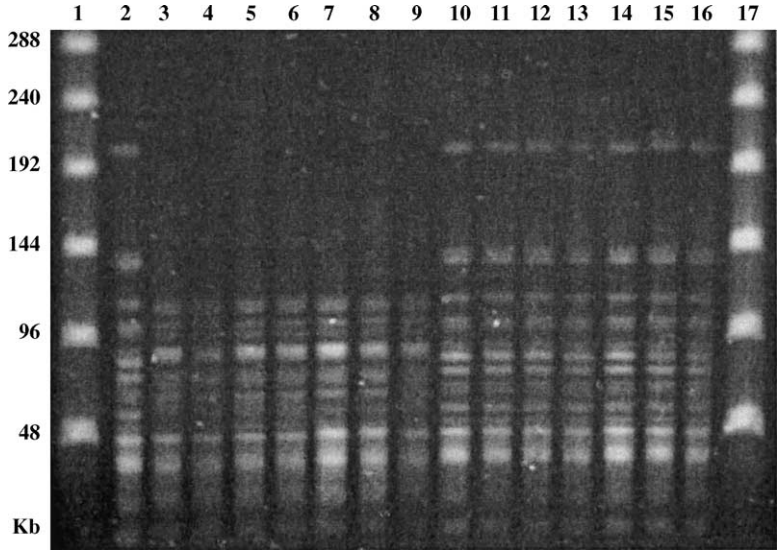

Fig. 1. *Sma*I restriction endonuclease patterns obtained by PFGE for *E. gallinarum* isolates. Lanes 1 and 17, lambda ladder (numbers indicate size in kb); lane 2, M2686; lane 3, M2723; lane 4, M2624; lane 5, M2747; lane 6, M2746; lane 7, M2765; lane 8, M2753; lane 9, M2764; lane 10, M2685; lane 11, M2695; lane 12, M2696; lane 13, M2715; lane 14, M2716; lane 15, M2725 and lane 16, M2749.

Isolates were analyzed by PFGE, after restriction of genomic DNA with *Sma*I enzyme (Fig. 1). Two clonal types were distinguished, named A and B, representing eight and seven isolates, respectively (Table 1). Clone A was present from August to December 2000, but four of eight isolates were detected in October (Table 1). Clone B was detected during September 2000 to January 2001, most isolates being found during January (Table 1). In conclusion, clones A and B were disseminated simultaneously in the ICU. VanA and VanB *E. gallinarum* strains have been previously documented in other countries, but to our knowledge they have not been recorded as being disseminated in a ward [4,7,17,19]. Raffinose fermentation in *E. gallinarum* is usually positive [16], but in the seven isolates belonging to clone B the fermentation was negative after 7 days incubation.

To see if the high-level resistance to glycopeptides was transmissible, three independent conjugation assays were performed. *E. gallinarum* M2715, representative of clonal type A, and *E. gallinarum* M2753 and M2723, representatives of clonal type B, were conjugated with *E. faecium* M95ZAP. Transconjugants obtained after mating were confirmed to be *E. faecium* and harboured the *van*A gene. Therefore, the *van*A element present in each clonal type was successfully transferred from *E. gallinarum* clinical isolates to *E. faecium* M95ZAP strain. *E. faecium* transconjugant strains obtained from mating assays with *E. galliuarum* M2723 and M2715 did not show high-level resistance to gentamicin. Therefore, the gentamicin resistance determinant could be on a separate genetic element from the vanA element. This fact could explain, in part, why most of the clonal type B isolates did not display high-level resistance to gentamicin (Table 1).

Transposon Tn*1546* was the first element described carrying the *van*A cluster [11]. Tn*1546* is highly heterogeneous, because of the occurrence of deletions, insertions and point mutations [2,10,14]. Although these events resulted in different *van*A elements, they could be derived from a unique ancestral Tn*1546* [14]. The insertion sequence IS*1251* has been found in the intergenic *van*S–*van*H region, mainly in isolates from the United States [2,13,14]. A few transposons harbouring this sequence have also been found in European isolates [10]. Therefore, IS*1251* may be a useful epidemiological marker from United States isolates [10]. De Lencastre et al. [2] reported that the occurrence of IS*1251* is indicative of the presence of a larger transposon (∼26 kb), named Tn*5482* [2,13]. Here, we observed that all 15 *E. gallinarum* isolates yielded an 1871-bp amplimer when *van*S-f and *van*H-r primers were used, suggesting the presence of an IS*1251*-like element (data not shown). The presence of IS*1251* in American countries other than United States has not been reported to date. The analysis of the 1871-bp amplimer by PCR–RFLP resulted in fragments with the same sizes as those expected from Tn*1546*::IS*1251* (Accession numbers: Tn*1546*, M97297 and IS*1251*, L34675) (Fig. 2). Recently, the first molecular characterization of *van*A elements on *E. gallinarum* isolates from Italy was reported [28], but none of them carried the IS*1251*. Our results suggest the presence of Tn*5482*-like elements in *E. gallinarum* isolates from Buenos Aires, and suggest the possibility of using the IS*1251* insertion in the intergenic *van*S–*van*H region as a potential molecular marker for an 'American type Tn*1546*'. Further studies will be conducted in order to characterize the *van*A elements in these *E. gallinarum* isolates.

Enterococcus vanC species are rarely documented in clinical infection or secondary transmission. Therefore, few reports have addressed the clinical and epidemiological significance of enterococci vanC species [9,16]. VanC enterococci can be particularly troublesome, since in vitro tests may indicate vancomycin susceptibility even when treatment failure

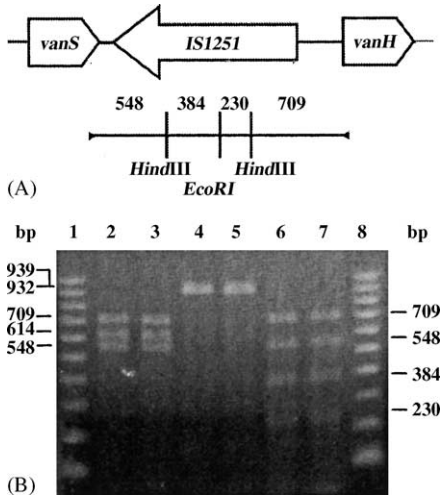

Fig. 2. PCR–RFLP of 1871-bp *van*SH amplicon. (A) Location of the 1871-bp *van*SH amplicon into Tn5482. *Hin*dIII and *Eco*RI sites are shown and the numbers indicate sizes (in bp) of restriction fragments as deduced from the reported sequence (12). (B) Separation of restriction fragments in 2% agarose gel. Lanes 1 and 8, 100-bp ladder; lanes 2, 4 and 6, *van*SH amplicon from *E. gallinarum* M2753 restricted with *Hin*dIII, *Eco*RI or both, respectively; lanes 3, 5, and 7, *van*SH amplicon from *E. gallinarum* M2715 restricted with *Hin*dIII, *Eco*RI or both, respectively. Sizes of restriction fragments (indicated by arrows) are shown on the left and right edges.

with vancomycin was reported in vivo [6]. The low prevalence reported for these species may be due, to its real low frequency as pathogen as well as to the inability of automated systems to identify these species. Moreover, they would be underestimated in colonization studies because not all *van*C enterococci isolates can grow in the bile-aesculin screening with 6 μg/ml vancomycin [3,5,23,27]. The motility test is a simple assay that can help to detect these species, but it may take up to 3 days and its performance varies with the composition of the media [5,9]. In addition, non-motile strains of *E. gallinarum* and *E. casseliflavus* [5,9] have also been reported. The use of methyl-α-D-glucopyranoside reagent has shown promising results [5]. Therefore, the use of a combination of antibiotype, or MICs, with some biochemical methods, such as motility and methyl-α-D-glucopyranoside, may be a reasonable choice for routine procedure to identify these VanC *Enterococcus* species [9]. *E. gallinarum* isolates have been obtained from different sources such as blood culture, digestive tract, urogenital tract or perianal swabs and stool [1,7,9,16], but only in a few cases has this species been associated with infectious diseases, like endocarditis [6,8] or bacteraemia [7,27]. At present, there is only one report dealing with clonal dissemination of *E. gallinarum* in a long-term care facility (Kapala M, Armstrong-Evans M, Willey BM, Berntson A, Nusinowitz S, Low DE, McGeer A. Abstracts 38th Interscience Conference on Antimicrobial Agents Chemotherapy; 1998. Abstr. 34), but this phenomenon may be more frequent than described. In the present report, the high level resistance to glycopeptides displayed made the detection of *E. gallinarum* clinical isolates easy.

The increasing prevalence of highly glycopeptide-resistant enterococci in Argentina was due to the emergence of *E. faecium* carrying *van*A gene (Corso A, et al. Abstracts 41st ICAAC. Abstr. 509; 2001). The Hospital Interzonal General de Agudos Evita was one of the centrees involved in such emergence. After vancomycin-resistant *E. gallinarum* isolates were detected in the hospital, the infection control measures were reinforced: notification and education to hospital staff personal, isolation of patients colonized with VRE in private rooms and enhancing hand washing between patients with iodopovidone. In addition to these measures, equipment and surfaces were cleaned with 5.25% sodium hypocholorite, medical devices with 70% ethanol and clinicians were asked to make a voluntary reduction in the use of vancomycin. All new patients admitted to ICU were screened for VRE colonization but in all cases they were free of VRE. These results suggest a low probability of community acquisition of VRE, through animal colonization or food contamination. The index case and all the patients those that became colonized with vancomycin-resistant *E. gallinarum* were linked in time, suggesting person-to-person spread. However, we cannot discard the VRE cross transmission through surfaces or equipment, as they were not screened. Unfortunately, the efficacy of the infection control measures could not be evaluated because the surveillance cultures of VRE were interrupted due to economical restrictions.

The CDC does not recommend infection control initiatives for patients infected or colonized with ‘motile’ enterococci. However, our findings suggest that *E. gallinarum* is capable of capturing the genetic elements responsible of high-level resistance to glycopeptides and to transfer them to *E. faecium*. This is the first report of VanA *E. gallinarum* dissemination in an Argentinean hospital. Therefore, strict control measures should be taken in hospitals where bacteria act as reservoir of unusual genotypes of resistance.

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