High diversity of vancomycin-resistant *Enterococcus faecium* isolated in Southern Brazil

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Abstract

Background. Vancomycin-resistant enterococci (VRE) are common in some hospital settings and their clonal spread has been described in different regions of the world. We determined the antimicrobial susceptibility profile and the clonal relationship of VRE isolates recovered from inpatients at three general hospitals of Porto Alegre, Brazil. **Results.** Ninety-four VRE were characterized as *Enterococcus faecium* and exhibited resistance to teicoplanin, ampicillin, ciprofloxacin, and susceptibility to linezolid, quinupristin-dalfopristin and daptomycin. High level resistance to gentamicin was detected in 13.8% of them. All VRE_{fm} harbored *vanA* gene, while 85.1% and 94.7% harbored respectively *esp* and *acm* virulence genes. PFGE profile analysis revealed 23 clonal types including 79 isolates, while 15 isolates exhibited unique pattern type, showing a polyclonal distribution of VRE_{fm} in Southern Brazil. **Conclusion.** These findings contribute to the local understanding regarding the characteristics of the circulating VREs in the region.

Key-words

VRE, *acm* gene, *esp* gene, PFGE, clonal types

Background

The ability of *Enterococcus faecium* to rapidly acquire mobile genetic elements associated to antimicrobial resistance is well-established (Gilmore et al., 2013; Cattoir and Giard, 2014; García-Solache et al., 2016). Vancomycin-resistant *Enterococcus faecium* (VRE_{fm}) has become increasingly common in some hospital settings and their clonal spread has been described worldwide (Freitas et al., 2016; Mahony et al., 2018), including Brazil (Alves et al., 2017; Resende et al., 2014; Sacramento et al., 2017). Most VRE_{fm} isolated from Brazilian hospitals belong to clonal complex 17 (CC-17), i.e. a well-adapted lineage to the hospital environment and responsible for the majority of VRE_{fm} infections worldwide (Top et al., 2008; Palazzo et al., 2011; Alves et al., 2017; Sacramento et al., 2017). VanA-related VRE_{fm} is the most prevalent phenotype around the world and frequently presents virulence factors which facilitates the infection process and multiresistance features that considerably reduce the therapeutic options (Ahmed and Baptiste, 2017).

The aim of this work was to determine genetic relatedness of VRE_{fm}, focusing on virulence and resistance characteristics.

Materials and Methods

Bacterial Strains

Ninety-four vancomycin resistant enterococci from the Gram-Positive Laboratory Microorganism Bank (Federal University of Health Sciences of Porto Alegre, Brazil) were evaluated. The isolates were recovered from clinical samples of patients attended in hospitals of Porto Alegre, Brazil, from September 2012 to April 2017, as part of an epidemiological surveillance study. Only one isolate per patient was considered. The project was approved by the Ethics Committee of Human Research of Federal University of Health Sciences of Porto Alegre, under the number 1.283.544.

Identification of *Enterococcus* species, vancomycin resistance and virulence genes

Primary genus identification was performed through the observation of specific phenotypic characteristics by the respective hospital's microbiology laboratory. The genus confirmation, species identification, detection of the vancomycin resistant determinants *vanA* and *vanB* genes and virulence genes *acm* (adhesin of collagen) and *esp* (enterococcal protein surface) were determined by PCR as previously described (Kariyama et al., 2000; Rathnayake et al., 2012; Kafil and Mobarez, 2015;). Primers used in this study are described in Table 1.

Antimicrobial Susceptibility testing

The antimicrobial susceptibility profile was done using disk diffusion for ampicillin (10 µg), ciprofloxacin (5 µg), gentamicin (120 µg), linezolid (30 µg), quinupristin-dalfopristin (15 µg) and teicoplanin (30 µg) and interpreted according to CLSI 2017 guidelines (Clinical and Laboratory Standards Institute, 2017). Minimum Inhibitory Concentrations (MIC) were determined by Etest® strips (bioMeriéux) for daptomycin. Multidrug resistance (MDR) strains were defined as those presenting resistance to three or more different antimicrobial classes (Magiorakos et al., 2012). *E. faecalis* ATCC 29212 and *E. faecalis* ATCC 51299 were used as quality control.

Chromosomal Analysis of Genomic DNA by PFGE

Pulsed-field gel electrophoresis (PFGE) was performed as previously described (Saeedi et al., 2002), with the following modifications: agarose plugs were prepared and treated with 1 mg/mL of lysozyme (Sigma Co., 48000U/mg), 5U/mL of mutanolysin (Sigma Co., 3000U/mL). Digestion of chromosomal DNA was achieved with 20 U of Anza™ 22 *SmaI* (Thermo Fisher Scientific®) and restriction fragments were separated using a CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA).

Results were analyzed with Bionumerics software version 7.1 (Applied Maths) using the unweighted-pair group method with arithmetic mean (UPGMA). Dendrogram was constructed using dice coefficients with optimization and tolerance set to 0.5% and 1%, respectively. Clustering above 80% similarity were considered as a clone type (CT) (Alves et al., 2017).

Results

All 94 VRE were identified as *Enterococcus faecium.* Enteroccci were recovered from urine 42.6% (n=40), blood 29.8% (n=28), rectal swab 14.9% (n=14), body fluids 11.7% (n=11) and catheters 1.1% (n=1).

All VRE_{fm} exhibited vancomycin MICs higher than 256 μg/mL and resistance to teicoplanin (all carrying *vanA* gene). They were resistant to ampicillin, ciprofloxacin, and susceptible to linezolid, daptomycin (MIC $\leq$ 4 μg/mL) and quinupristin-dalfopristin. High-level resistance to Gentamicin was detected in 13 (13.8%) isolates. Considering virulence genes, 80 (85.1%) and 89 (94.7%) isolates harbored *esp* and *acm* genes, respectively. Seventy-six isolates carried both genes and one isolate did not possess any of the those.

PFGE defined 23 clone types (CTs) which included 79 of the 94 isolates, and 15 were singletons (Figure 1, Table 2). There was one dominant cluster, CT8, including 17,7% of VRE_{fm}, recovered either from infection (blood, urine) and surveillance cultures.

Discussion

VRE_{fm} has become one of the leading causes of nosocomial infections, especially among severely ill patients (Howden et al., 2013). We described the clonal relationship of 94 VRE_{fm} recovered from inpatients in Porto Alegre, Southern Brazil. Besides vancomycin, all *E. faecium* exhibited resistance to ampicillin and ciprofloxacin, and 13.8% high level resistance to gentamicin.

Around the world, studies have reported the spread of CC-17 (Alves et al., 2017; Brilliantova et al., 2010; López et al., 2012; Palazzo et al., 2011), a lineage that exhibits resistance to most antibiotics clinically used for the treatment of enterococcal infections. It is well adapted to the hospital environment and has been associated with most of the reported hospital outbreaks worldwide (Panesso et al., 2010; Willems et al., 2005). Our isolates showed phenotypic characteristics similar to the CC-17 lineage, such as ampicillin and ciprofloxacin resistance and presence of *esp* gene (Gao et al., 2018). Indeed, most VRE_{fm} harboured *esp* and *acm* genes, both related with biofilm formation and adherence to extracellular matrix, giving *E. faecium* selective advantages in the hospital environment (Hendrickx et al., 2007).

Similar to our findings, Akpaka et al., (2017) performed a study between 2009 to 2014 with twelve hospitals from eight Caribbean countries and they found 31.4% of VRE strains. Among these, 70 were *E. faecium*, harboring *vanA* and *esp* genes, with 100% of resistance to ciprofloxacin, 92.8% resistance to ampicillin and 100% of susceptibility to daptomycin, linezolid and quinupristin/dalfopristin.

In a study performed in 2011 evaluating antimicrobial susceptibility patterns of isolates from 11 countries in Latin America, Brazil presented the highest rate of VRE (27%) (Jones et al., 2013). In 2016, a SENTRY study reported a rate of 71.7% of VRE_{fm} in Brazil (Sader et al., 2016).

Although *E. faecalis* is more prevalent in enterococcal infections, VRE_{fm} has been increasing in Brazilian hospitals. Conceição et al. (2011) observed an increase of 13% in VRE rate in a hospital in Southeastern Brazil between 2006-2009, being 89.5% *vanA-E. faecium*. Another study conducted with 29 isolates from a hospital in Southern Brazil observed that all isolates were VRE_{fm} carrying *vanA* gene and were part of a main clone (Resende et al., 2014).

In our study, VRE_{fm} were classified into 38 types (23 clonal types and 15 singletons), demonstrating a high genetic heterogeneity. A similar polyclonal distribution of VRE_{fm} has also been observed in other studies (Landerslev et al., 2016; Pourshafie et al., 2008; Somily et al., 2016; J. Top et al., 2008).

Finally, our study contributes to the local understanding about the characteristics of the circulating VREs in the region, since there are few publications on this topic in the last 5 years in Brazil.

Acknowledgements

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Table 1. Description of primers used in PCR for the detection of species and vancomycin-resistance genes and virulence factors of *Enterococcus faecium*:

Target gene	Sequence of primer	Amplicon Size (pb)	Reference
Enterococcus faecium	5'-TTGAGGCAGACCAGATTGACG-3' 5'-TATGACAGCGACTCCGATTCC-3'	658	[18]
Enterococcus faecalis	5'-ATCAAGTACAGTTAGTCTTTATTAG-3' 5'-ACGATTCAAAGCTAACTGAATCAGT-3'	941	[18]
vanA	5'-CATGAATAGAATAAAAGTTGCAATA-3' 5'-CCCCTTTAACGCTAATACGATCAA-3'	1030	[18]
vanB	5’-GTGACAAACCGGAGGCGAGGA-3’ 5’-CCGCCATCCTCCTGCAAAAAA-3’	433	[18]
esp	5’-GGAACGCCTTGGTATGCTAAC-3’ 5’-GCCACTTTATCAGCCTGAACC -3’	95	[17]
acm	5’-GGCCAGAAACGTAACCGATA-3’ 5’-AACCAGAAGCTGGCTTTGTC-3’	135	[26]

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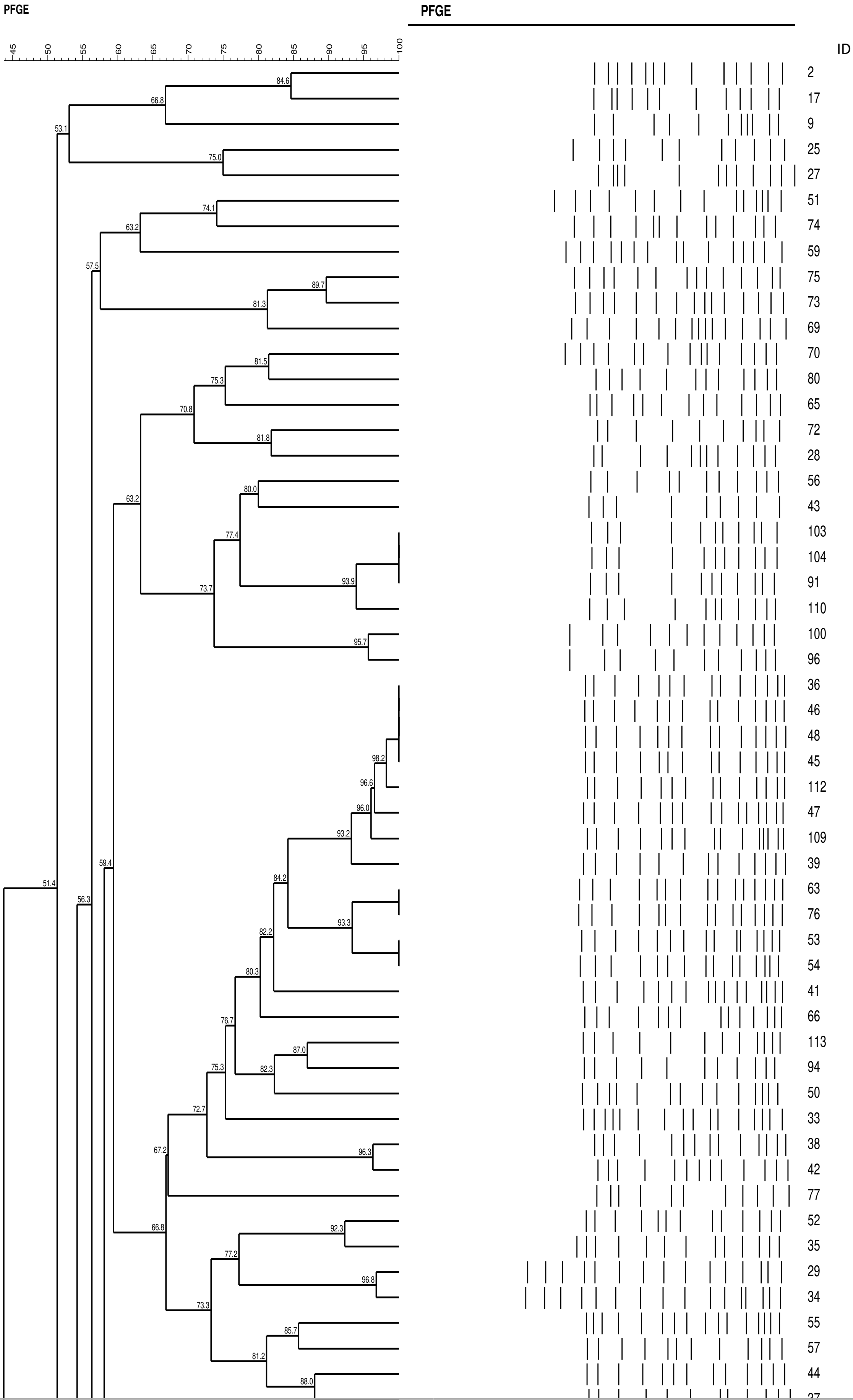
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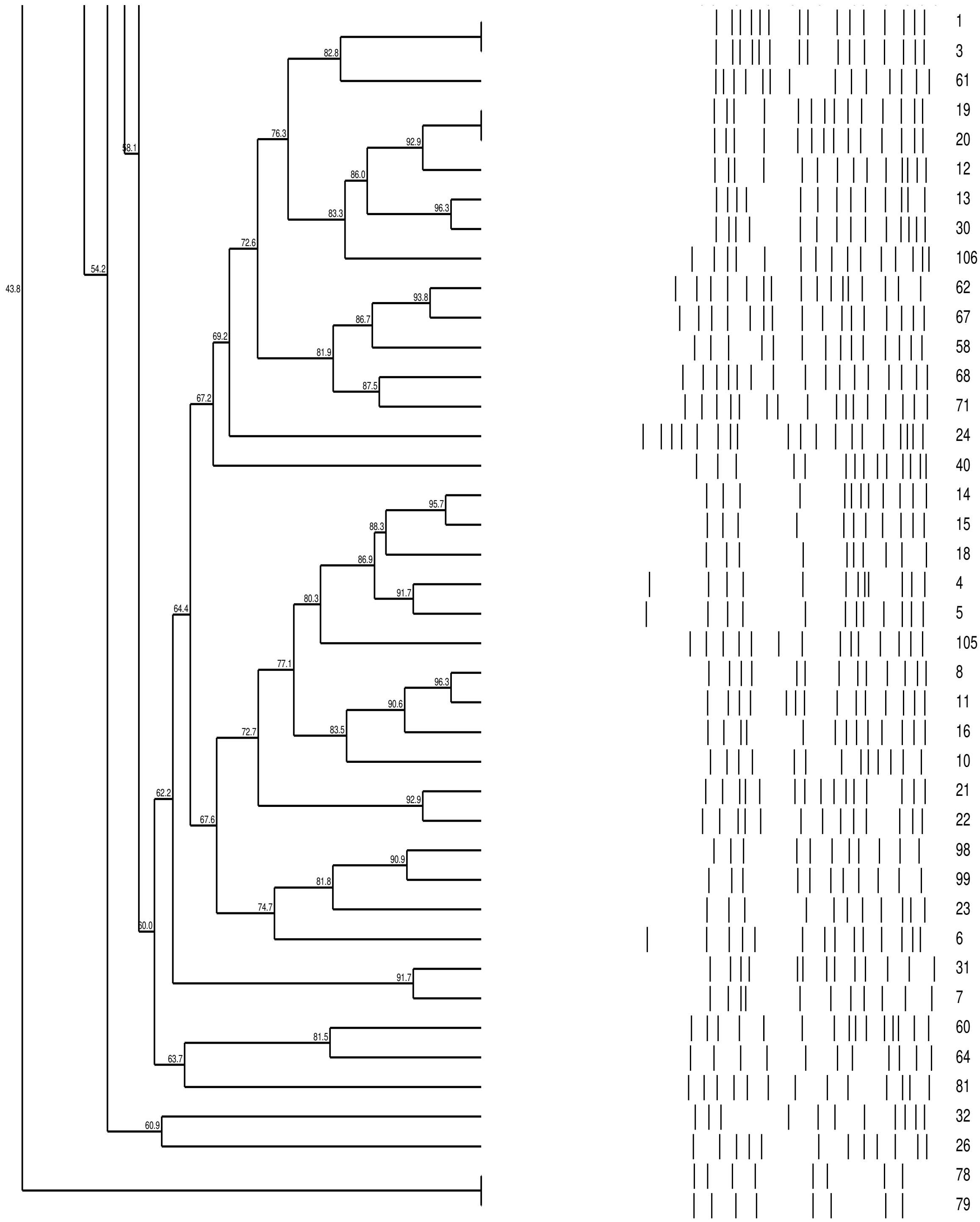

Figure 1. PFGE dendrogram and PFGE profile images of 94 *vanA E. faecium* from Porto Alegre, Brazil.

Table 2. Description of 94 VRE_{fm} clinical isolates from Porto Alegre, Brazil, recovered from Sept-2012 to Apr-2017.

Strain ID	**Source**	**Date**	**PFGE**	**Resistance Profile**	**Virulence Profile**
2	Urine	Oct-12	CT1	AMP, CIP, TEI, VAN	*esp+, acm+*
17	Urine	May-13	CT1	AMP, CIP, TEI, VAN	*esp+, acm+*
69	Blood	Dec-16	CT2	AMP, CIP, TEI, VAN	*esp+, acm+*
73	Urine	Jan-17	CT2	AMP, CIP, TEI, VAN	*esp+, acm+*
75	Urine	Jan-17	CT2	AMP, CIP, TEI, VAN	*esp+, acm+*
70	Body Fluids	Dec-16	CT3	AMP, CIP, TEI, VAN	*esp+*
80	Urine	Apr-17	CT3	AMP, CIP, TEI, VAN	*acm+*
28	Urine	May-15	CT4	AMP, CIP, TEI, VAN	*esp+, acm+*
72	Urine	Jan-17	CT4	AMP, CIP, TEI, VAN	*esp+, acm+*
43	Body Fluids	Sep-15	CT5	AMP, CIP, TEI, VAN	*esp+, acm+*
56	Body Fluids	Mar-16	CT5	AMP, CIP, TEI, VAN	*esp+, acm+*
91	Rectal Swab	Sep-14	CT6	AMP, CIP, TEI, VAN	*esp+, acm+*
103	Rectal Swab	Dec-14	CT6	AMP, CIP, TEI, VAN	*esp+, acm+*
104	Rectal Swab	Jan-15	CT6	AMP, CIP, TEI, VAN	*esp+, acm+*
110	Rectal Swab	Jun-15	CT6	AMP, CIP, TEI, VAN	*esp+, acm+*
96	Urine	Nov-14	CT7	AMP, CIP, TEI, VAN	*esp+, acm+*
100	Rectal Swab	Dec-14	CT7	AMP, CIP, TEI, VAN	*esp+, acm+*
36	Blood	Aug-15	CT8	AMP, CIP, TEI, VAN	*esp+, acm+*
39	Blood	Aug-15	CT8	AMP, CIP, TEI, VAN	*esp+, acm+*
41	Blood	Sep-15	CT8	AMP, CIP, TEI, VAN	*esp+*
45	Blood	Oct-15	CT8	AMP, CIP, TEI, VAN	*esp+, acm+*
46	Urine	Nov-15	CT8	AMP, CIP, TEI, VAN	*esp+, acm+*
47	Blood	Oct-15	CT8	AMP, CIP, TEI, VAN	*esp+, acm+*
48	Blood	Oct-15	CT8	AMP, CIP, TEI, VAN	*esp+, acm+*
53	Urine	Jan-16	CT8	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*
54	Blood	Jan-16	CT8	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*
63	Blood	Oct-16	CT8	AMP, CIP, TEI, VAN	-
66	Blood	Nov-16	CT8	AMP, CIP, TEI, VAN	*esp+*
76	Urine	Jan-17	CT8	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*
109	Rectal Swab	May-15	CT8	AMP, CIP, TEI, VAN	*esp+, acm+*
112	Rectal Swab	Jul-15	CT8	AMP, CIP, TEI, VAN	*esp+, acm+*
50	Body Fluids	Dec-15	CT9	AMP, CIP, TEI, VAN	*acm+*

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94	Urine	Nov-14	CT9	AMP, CIP, TEI, VAN	*esp+, acm+*
113	Rectal Swab	Aug-15	CT9	AMP, CIP, TEI, VAN	*esp+, acm+*
38	Urine	Aug-15	CT10	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*
42	Urine	Sep-15	CT10	AMP, CIP, TEI, VAN	*esp+, acm+*
35	Urine	Jul-15	CT11	AMP, CIP, TEI, VAN	*esp+, acm+*
52	Urine	Dec-15	CT11	AMP, CIP, TEI, VAN	*esp+, acm+*
29	Urine	May-15	CT12	AMP, CIP, TEI, VAN	*esp+, acm+*
34	Urine	Jul-15	CT12	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*
37	Urine	Aug-15	CT13	AMP, CIP, TEI, VAN	*esp+, acm+*
44	Urine	Sep-15	CT13	AMP, CIP, TEI, VAN	*esp+, acm+*
55	Urine	Jan-16	CT13	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*
57	Urine	Apr-16	CT13	AMP, CIP, TEI, VAN	*esp+, acm+*
1	Blood	Sep-12	CT14	AMP, CIP, TEI, VAN	*acm+*
3	Blood	Oct-12	CT14	AMP, CIP, TEI, VAN	*esp+, acm+*
61	Body Fluids	Nov-16	CT14	AMP, CIP, TEI, VAN	*esp+, acm+*
12	Catheter	Apr-13	CT15	AMP, CIP, TEI, VAN	*esp+, acm+*
13	Blood	May-13	CT15	AMP, CIP, TEI, VAN	*acm+*
19	Urine	Jun-13	CT15	AMP, CIP, TEI, VAN	*esp+, acm+*
20	Blood	Jul-13	CT15	AMP, CIP, TEI, VAN	*esp+, acm+*
30	Blood	May-15	CT15	AMP, CIP, TEI, VAN	*esp+, acm+*
106	Rectal Swab	Mar-15	CT15	AMP, CIP, TEI, VAN	*esp+, acm+*
58	Urine	Mar-16	CT16	AMP, CIP, TEI, VAN	*esp+, acm+*
62	Urine	Oct-16	CT16	AMP, CIP, TEI, VAN	*acm+*
67	Urine	Nov-16	CT16	AMP, CIP, TEI, VAN	*esp+, acm+*
68	Urine	Dec-16	CT16	AMP, CIP, TEI, VAN	*acm+*
71	Urine	Dec-16	CT16	AMP, CIP, TEI, VAN	*acm+*
4	Urine	Oct-12	CT17	AMP, CIP, TEI, VAN	*esp+, acm+*
5	Blood	Oct-12	CT17	AMP, CIP, TEI, VAN	*esp+, acm+*
14	Blood	May-13	CT17	AMP, CIP, TEI, VAN	*esp+, acm+*
15	Body Fluids	May-13	CT17	AMP, CIP, TEI, VAN	*esp+, acm+*
18	Blood	May-13	CT17	AMP, CIP, TEI, VAN	*esp+, acm+*
105	Rectal Swab	Jan-15	CT17	AMP, CIP, TEI, VAN	*esp+, acm+*
8	Urine	Feb-13	CT18	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*
10	Urine	Mar-13	CT18	AMP, CIP, TEI, VAN	*esp+, acm+*
11	Urine	Apr-13	CT18	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*
16	Blood	May-13	CT18	AMP, CIP, TEI, VAN	*acm+*

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21	Blood	Jul-13	CT19	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*
22	Urine	Jul-13	CT19	AMP, CIP, TEI, VAN	*esp+, acm+*
23	Rectal Swab	Jul-13	CT20	AMP, CIP, TEI, VAN	*esp+, acm+*
98	Rectal Swab	Nov-14	CT20	AMP, CIP, TEI, VAN	*esp+, acm+*
99	Rectal Swab	Dec-14	CT20	AMP, CIP, TEI, VAN	*esp+, acm+*
7	Blood	Dec-12	CT21	AMP, CIP, TEI, VAN	*esp+, acm+*
31	Blood	May-15	CT21	AMP, CIP, TEI, VAN	*esp+, acm+*
60	Body Fluids	May-16	CT22	AMP, CIP, TEI, VAN	*acm+*
64	Urine	Oct-16	CT22	AMP, CIP, TEI, VAN	*esp+, acm+*
78	Blood	Feb-17	CT23	AMP, CIP, TEI, VAN	*esp+, acm+*
79	Blood	Apr-17	CT23	AMP, CIP, TEI, VAN	*esp+, acm+*
6	Blood	Dec-12	*Singleton*	AMP, CIP, TEI, VAN	*esp+, acm+*
9	Body Fluids	Feb-13	*Singleton*	AMP, CIP, TEI, VAN	*esp+, acm+*
24	Body Fluids	Aug-13	*Singleton*	AMP, CIP, TEI, VAN	*esp+, acm+*
25	Blood	Aug-13	*Singleton*	AMP, CIP, TEI, VAN	*esp+, acm+*
26	Urine	Aug-13	*Singleton*	HLG, AMP, CIP, TEI, VAN	*acm+*
27	Rectal Swab	Aug-13	*Singleton*	AMP, CIP, TEI, VAN	*acm+*
32	Urine	May-15	*Singleton*	AMP, CIP, TEI, VAN	*esp+, acm+*
33	Urine	May-15	*Singleton*	AMP, CIP, TEI, VAN	*esp+, acm+*
40	Blood	Sep-15	*Singleton*	AMP, CIP, TEI, VAN	*esp+, acm+*
51	Blood	Dec-15	*Singleton*	AMP, CIP, TEI, VAN	*esp+, acm+*
59	Urine	Apr-16	*Singleton*	AMP, CIP, TEI, VAN	*acm+*
65	Urine	Oct-16	*Singleton*	AMP, CIP, TEI, VAN	*acm+*
74	Body Fluids	Jan-17	*Singleton*	HLG, AMP, CIP, TEI, VAN	*esp+*

77	Urine	Feb-17	*Singleton*	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*
81	Body Fluids	Apr-17	*Singleton*	HLG, AMP, CIP, TEI, VAN	*esp+, acm+*

PFGE, pulsed-field gel electrophoresis; CT, clonal type; HLG, high level of gentamicin; AMP, ampicillin; CIP, ciprofloxacin; TEI, teicoplanin; VAN, vancomycin; *esp*, enterococcal protein surface gene; *acm*, collagen adhesin gene.

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