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Original Article

Vancomycin-resistant *Enterococcus faecium* at a university hospital in Taiwan, 2002–2015: Fluctuation of genetic populations and emergence of a new structure type of the Tn*1546*-like element

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Abstract *Background/purposes:* Vancomycin resistance increased significantly to 31.3% among *Enterococcus faecium* in 2006 and remained high thereafter at a university hospital in Taiwan. A longitudinal study was retrospectively conducted to characterize these vancomycin-resistant *E. faecium* (VRE-fm).
Methods: A total of 378 non-repetitive VRE-fm blood isolates collected during 2002–2015 were studied. Multilocus sequence typing, pulsed-field gel electrophoresis, analysis of *van* genes and the Tn*1546* structure, and conjugation experiments were performed.
Results: The majority (78.0%) of the isolates were associated with hospital-acquired infections. Molecular typing revealed nine major pulsotypes and five predominant sequence types (STs): ST17 (33.9%), ST78 (18.3%), ST414 (14.6%), ST18 (10.6%), and ST203 (7.4%). Fluctuation

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resistance

of these prevailing STs among the study years in association with some major pulsotypes was noted. All isolates carried *vanA* genes, except that in four isolates *vanB* genes were found. Among the *vanA*-carrying Tn*1546*-like elements, one predominant structure type (Type I, 55.9%) was noted throughout the study years. Since 2009, another predominant structure type (Type II, 40.1%) has emerged firstly in ST414 and gradually spread to other 11 STs in subsequent years. Isolates carrying these Type II Tn*1546*-like elements have become the most predominant population since 2014, majorly found in ST78 and ST17. Preliminary experiments indicated that plasmids carrying the Type II Tn*1546*-like elements demonstrated ten-fold higher efficiency than those carrying the Type I Tn*1546*-like elements.

Conclusion: Dissemination of some major STs and horizontal transfer of plasmids carrying two major structure types of Tn*1546*-like elements may have together contributed to the increase of VRE-fm infection.

Introduction

Enterococci are one of the commensal flora in the gastrointestinal tract of human and animals.[1] They also frequently cause hospital-acquired infection (HAI), especially those involving blood streams and urinary tracts. *Enterococcus faecalis* (80%) used to be the most predominant *Enterococcus* species, followed by *Enterococcus faecium* (15%—20%) in HAIs in 1990s.[2] During the last decade, however, HAIs caused by *E. faecium* have increased greatly to reach a similar level as those caused by *E. faecalis*.[3]

In 1986, transferable high-level vancomycin resistance was firstly reported in *E. faecium* (VRE-fm) from the United Kingdom and France.[4,5] Up to the present, VRE-fm infection has been reported worldwidely.[6—8] Clonal spread of one or multiple VRE-fm strains may play a major role in the increase of VRE-fm infection. Through multilocus sequence typing (MLST) analysis, HAI-associated strains have been shown to belong to a specific subpopulation, i.e., clonal complex 17 (CC17), which is different from those associated with community and animal populations.[1,9] By using a Bayesian Analysis of Population Structure (BAPS) software, a recent report also indicated that a large proportion (80%) of HAI isolates could be categorized into two subgroups, BAPS 2-1 and BAPS 3-3.[10]

The development of vancomycin resistance is usually through the acquisition of *van* genes. Among the nine *van* genotypes reported so far, *vanA* (80%—90%) and *vanB* (10%—20%) are the most predominant.[11,12] The *vanA* operon usually consists of five genes (*vanHAXYZ*) for glycopeptide resistance, two regulatory genes (*vanRS*), a transposase (*orf1*)/resolvase (*orf2*) region, and is usually carried by a Tn*3*-type transposon, Tn*1546*.[12] Genetic variations, such as deletions and/or addition of some insertion sequences, have been reported in Tn*1546*.[13,14]

In Taiwan, the first VRE was reported in 1996.[15] The prevalence was low initially, but since 2008, several reports have documented the significant increase of VRE-fm infections.[13,16—18] From a national surveillance study performed in 2012, several genetic clusters as well as two major structure types of the Tn*1546*-like element (Type I, 45.5%; Type II, 47.0%) were noted among a collection of 134 VRE-fm isolates identified at 12 hospitals from various regions of Taiwan.[18] The Type II structure type of Tn*1546*-like elements had never been reported elsewhere. Further analysis performed retrospectively at one of the hospitals revealed that the incidence of VRE-fm increased significantly to 31.3% in 2006 and has since remained high or even higher. Isolates carrying the Type II structure type of Tn*1546*-like elements were firstly identified in 2009, and have been increasing since then. To gain a further insight into the molecular epidemiology of VRE-fm in Taiwan, the present study was conducted to characterize a longitudinal collection of VRE-fm blood isolates identified between 2002 and 2015 at this hospital.

Methods

Hospital setting and bacteria

The Chang Gung Memorial Hospital, Linkou (CGMH-LK) is a 3700-bed university-affiliated medical center located in northern Taiwan. All VRE blood isolates identified from the Clinical Microbiology Laboratory have been stored in the Bacteria Bank of this hospital.[19] The first VRE-fm isolate was identified in 2002. Up to 2015, a total of 378 such isolates have been non-repetitively collected and subjected for the subsequent experiments. HAIs were defined according to the public guidelines.[20]

Detection of vancomycin resistance genes

DNA of the isolates was prepared by using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Different vancomycin resistance genes (*vanA*, *vanB*, *vanC*1 and *vanC*2/*C*3) were examined as described previously.[21]

Multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE)

MLST was performed using a published method.[22] Sequence types (STs) were determined through the comparison with a public MLST database (http://pubmlst.org/efaecium/).

The eBURST program provided by the web site was used to perform the clustering analysis for the STs. The BAPS system was also used for analysis.[10]

PFGE was performed as previously described.[23,24] The results were analyzed by BioNumerics (version 6.5, Applied Maths, Austin, Texas). Through the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) analysis, PFGE patterns with more than 80% similarity were categorized into the same pulsotypes.

Molecular analysis of the Tn*1546*-like elements

The Tn*1546*-like elements were detected and analyzed using a published overlapping PCR technique.[25] Nineteen primers (p1 to p19) were used as described previously[25] to amplify 10 overlapping fragments of the Tn*1546*-like elements. P1 is complementary to a portion of the inverted repeats and used with P19 to amplify the tenth fragment of the Tn*1546*. The presence or absence of each of the 10 amplicons was recorded, and the size of each amplicon was compared with that from a Tn*1546* prototype of the reference strain (GenBank accession no. M97297). Amplicons with unexpected sizes were further examined by sequencing analysis.

Conjugation experiments

A filter mating method was performed as previously described.[26] The recipient strain, *E. faecium* BM4105RF, was kindly provided by Dr. van Schaik W. All transconjugants were confirmed by detecting the presence of the *vanA* gene with PCR. The conjugation frequency for each tested isolate was determined by dividing the number of the transconjugant cells (multiplied by the dilution factors) by the number of the recipient cells.

Statistical analysis

Statistical analysis was performed by the χ^2 test or Fisher's exact test when appropriated. A difference was considered statistically significant with a two-tailed *P*-value of less than 0.05.

Results

Epidemiological analysis

A total of 378 non-repetitive VRE-fm blood isolates were studied. The majority ($n = 295$, 78.0%) of the isolates were associated with HAIs, with a significantly increasing trend from 65.2% in 2002–2007 to 87.5% in 2015 ($P < 0.005$). No significant clusters of VRE-fm HAIs were noted throughout the study years.

Blood isolates of VRE-fm were firstly identified in 2002, and the annual numbers were less than three before 2005. Compared to the total blood isolates of *E. faecium*, the annual rate of VRE-fm was low at 4.8%–12.0% during this period (Table 1). The annual number increased suddenly to 15 (31.3%) in 2006 ($P < 0.001$) and has since increased continuously to 52 (46.0%) in 2010. The annual number declined subsequently to 30 (33.7%) in 2013, followed by a reverse trend to reach 64 (56.1%; $P < 0.001$, Table 1 and Fig. 1A) in 2015.

MLST genotyping

MLST analysis revealed 24 STs, including five newly identified STs (ST999-ST1003; totally six isolates). Except ST1000 ($n = 1$), all VRE-fm isolates belonged to the CC17 (Fig. 2). Five predominant STs, ST17 ($n = 128$, 33.9%), ST78 ($n = 69$, 18.3%), ST414 ($n = 55$, 14.6%), ST18 ($n = 40$, 10.6%), and ST203 ($n = 28$, 7.4%), were noted, constituting 84.7% of the total VRE-fm isolates studied (Table 1 and Fig. 1A). According to the eBURST analysis (Fig. 2), the two most predominant STs, ST17 and ST78, actually varied by only one single locus. ST203 and ST414 were each differed from ST78 by one and two loci, respectively. ST18 also varied from ST17 by only two loci. Based on the BAPS analysis,[10] these five major STs could be separated into two subgroups, ST17/ST18 in the BAPS 3-3 subgroup and ST78/ST203/ST414 in the BAPS 2-1 subgroup.

Accordingly, some intra-group fluctuations were noted. ST17 was found throughout the study period, with the annual numbers fluctuated between four (in 2009) and 24 isolates (in 2015). ST18, another member of the BAPS 3-3 group, appeared in 2007 ($n = 1$) and reached the peak at 14 isolates in 2009. This ST seemed to replace ST17 during 2009–2010 when ST17 reduced; however, it was replaced by ST17 again after 2011 when its own numbers reduced.

In the BAPS 2-1 group, ST78 appeared suddenly in 2008 ($n = 12$), but soon reduced to 2–6 isolates per year in 2009–2013, and then increased again to 26 isolates in 2015. ST203 was found in 2002–2012 with an annual peak of 7–8 isolates during 2009–2010. The increase of ST203 in 2009–2010, although only for a short period, coincided with the reduction of ST78. ST414 also appeared in 2009 ($n = 6$) and increased rapidly to 23 isolates in 2010. Both ST414 and ST18 reduced substantially after 2011. During the last three years of this study, less than three isolates per year were found in both STs. However, ST78 started to show a rapid increase in 2013 and became the most predominant ST in 2015.

Characterization of Tn*1546*-like elements

All VRE-fm isolates carried a *vanA* gene, except that four isolates were found to carry only a *vanB* gene. Among the *vanA*-carrying Tn*1546*-like elements, two predominant structure types (Type I, $n = 209$, 55.9%; Type II, $n = 150$, 40.1%) were identified (Fig. 1B–C). The remaining isolates ($n = 15$, 4.0%) were found to have various insertions in the Tn*1546*-like element but were not further analyzed.

The most predominant Type I Tn*1546*-like element was present throughout the study period, distributing among 15 STs, including all the prevalent STs, such as ST17, ST18, ST78, and ST203, but not ST414. The annual isolate numbers carrying this structure type increased gradually from 14 (93.3%) in 2006 to 35 (81.4%) in 2009, and then decreased gradually to an average of 15 (34.7%) in 2012–2015 (Fig. 1B).

Table 1 Annual blood isolate numbers of *Enterococcus faecium* and VRE-fm and their distribution among five major sequence types (STs) from 2002 to 2015.

Year	Total	VRE-fm	VRE-fm among five major STs, No. (%)				
	No.	No.(%)	ST17	ST18	ST78	ST203	ST414
2002	19	1 (5.3)	1 (100)	0	0	0	0
2003	22	2 (9.5)	1 (50.0)	0	0	0	0
2004	28	3 (10.7)	2 (66.7)	0	0	1 (33.3)	0
2005	25	3 (12.0)	3 (100)	0	0	0	0
2006	48	15 (31.3)	7 (46.7)	0	0	3 (20.0)	0
2007	66	22 (33.3)	13 (59.1)	1 (4.5)	0	4 (18.2)	0
2008	66	27 (40.9)	7 (25.9)	5 (18.5)	12 (44.4)	2 (7.4)	0
2009	85	44 (51.8)	4 (9.1)	14 (31.8)	4 (9.1)	7 (15.9)	6 (13.6)
2010	113	52 (46.0)	6 (11.5)	8 (15.4)	2 (3.8)	8 (15.4)	23 (44.2)
2011	83	39 (47.0)	13 (33.3)	6 (15.4)	3 (7.7)	2 (5.1)	14 (35.9)
2012	82	32 (39.0)	10 (31.3)	4 (12.5)	3 (9.4)	1 (3.1)	7 (21.9)
2013	89	30 (33.7)	15 (50.0)	0	6 (20.0)	0	3 (10.0)
2014	90	44 (48.9)	22 (50.0)	2 (4.5)	13 (29.5)	0	2 (4.5)
2015	114	64 (56.1)	24 (37.5)	0	26 (40.6)	0	0
Total	551	378 (40.7)	128 (33.9)	40 (10.6)	69 (18.3)	28 (10.4)	55 (14.6)

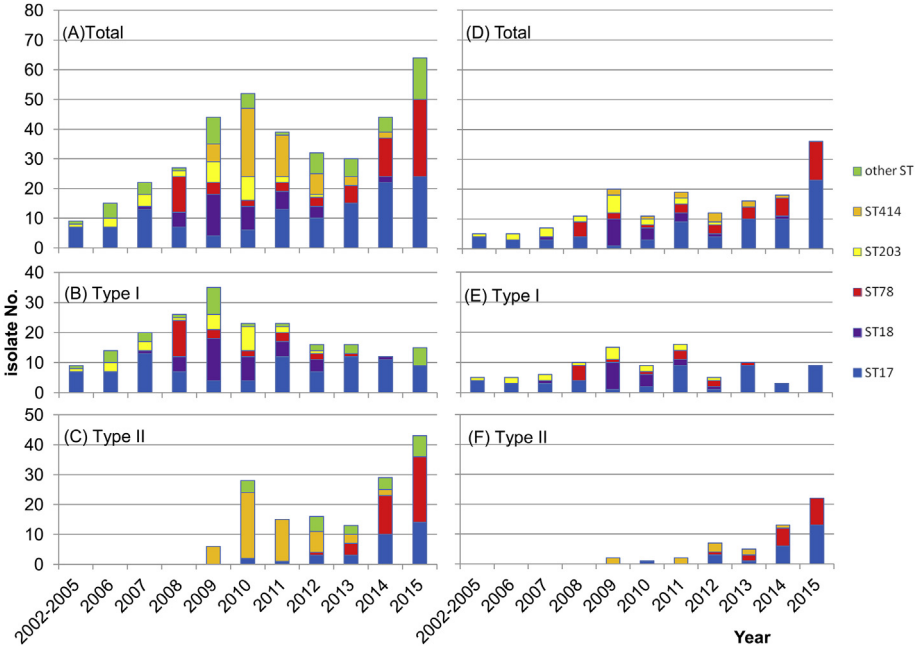

Figure 1. Annual numbers of VRE-fm isolates categorized by sequence types (STs) during 2002—2015. (A) All isolates studied; (B) Isolates carried Type I Tn*1546*-like elements; (C) Isolates carried Type II Tn*1546*-like elements. Panels (D)—(F) are the counterparts of panels (A)—(C), respectively, but for all isolates with more than 80% similarity, only the first isolate was included for analysis. For simplicity, only the five major STs are demonstrated.

The Type II Tn*1546*-like element appeared in 2009, all carried by the ST414 isolates ($n = 6$, 14.0%). The annual number of isolates carrying this structure type increased significantly to 28 (53.8%; $P < 0.00005$) in 2010. Other than the ST414 ($n = 22$), this structure type was also found in several other STs: ST17 and ST233 ($n = 2$ each), ST341 and ST479 ($n = 1$ each). During 2012—2013, the proportions of isolates carrying the Type II Tn*1546*-like element ($n = 15$, 46.8%) were about the same as those carrying the Type I Tn*1546*-like element ($n = 16$, 51.6%). However, in 2014—2015, isolates carrying the Type II Tn*1546*-like element ($n = 36$, 66.7%) increased rapidly and replaced those carrying the Type I Tn*1546*-like element ($n = 14$, 25.0%; $P < 0.00005$) to become the major structure type among the VRE-fm studied (Fig. 1B—C). By 2015, the Type II Tn*1546*-like element has been found in 12 STs. ST414

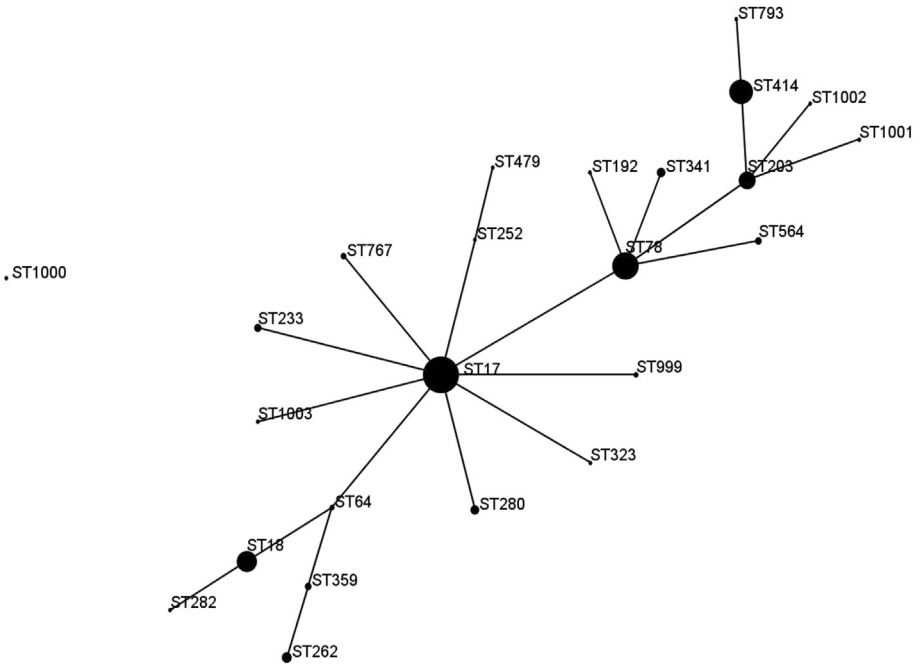

Figure 2. eBURST analysis of the 24 sequence types (STs) identified among the 378 isolates studied. Each dot presents a ST, and the size of each dot corresponds to the number of isolates in the respective ST.

(n = 36, 83.7%) were the most predominant, but mainly found in 2010–2011. Later in 2014–2015, the major ST has become ST78 (n = 35, 48.6%) (Fig. 1C).

PFGE analysis of VRE-fm isolates

PFGE analysis revealed 129 pulsotypes among the five major STs (320 isolates), with nine major pulsotypes A-I identified in 112 (35.0%) isolates (Fig. S1–5). For ST17, four major pulsotypes A-D were identified in 34 (26.6%) isolates, and another 56 pulsotypes were found in the remaining 94 isolates (Fig. S1). For ST18, two major pulsotypes E and F were found among the total 17 pulsotypes (Fig. S2). ST78 (24 pulsotypes), ST203 (17 pulsotypes) and ST414 (11 pulsotypes) isolates each consisted of only one major pulsotypes G, H and I, respectively (Fig. S3–5).

The association between the prevalence of STs and pulsotypes was further analyzed. For ST17, pulsotypes C (n = 10) and D (n = 8) were found in 2006–2007, and may contribute to the prevalence of ST17 in the initial stage of this study. Pulsotypes A (n = 9) and B (n = 7) were found in 2013–2014 and 2012–2013, respectively, and may contribute to the prevalence of ST17 in the later stage of this study. The major pulsotypes E (n = 7, in 2009–2012) and F (n = 9, in 2008–2011) of ST18 and the pulsotype H (n = 9, in 2009–2010) of ST203 all coincided with the prevalence of the individual ST in the respective period. The presence of pulsotype I (n = 26, in 2009–2012) may be associated with the high proportion of ST414 in 2009–2012. Similarly, the emergence of pulsotype G (n = 27) appeared to have contributed to the significant increase of ST78 during 2013–2015.

To exclude the effect associated with clonal spread, for all isolates with more than 80% similarity, only the first isolate was included for a further analysis. As shown in Fig. 1D–F, isolates carrying the Type I plasmids have been fluctuated between 3 and 16 isolates among the study period. However, isolates carrying the Type II plasmids appeared to have increased significantly since 2014, mainly found in ST17 and ST78.

Conjugation results

To examine whether the plasmids carrying either Type I or Type II Tn*1546*-like elements were self-transferrable, three each of representative isolates were selected from major STs, ST17, ST78 and ST414, for the conjugation assay. All the *vanA*-carrying plasmids were found conjugative. The conjugation frequency from VRE-fm isolates carrying Type I Tn*1546*-like elements were within the range of $0.9–4.7 \times 10^{-7}$. Those carrying Type II Tn*1546*-like elements demonstrated a ten-fold higher conjugation frequency at $1–1.2 \times 10^{-6}$, except those from the ST414 isolates. All ST414 isolates studied carried only Type II Tn*1546*-like elements, and their conjugation frequency ($0.8–6.1 \times 10^{-7}$) was similar to those found in isolates carrying Type I Tn*1546*-like elements.

Discussion

A significant increase of VRE-fm from 2002 to 2010 was demonstrated herein at one single university hospital in Taiwan, as also noted globally.[27,28] The observed high prevalence (78.0%) of hospital-acquired VRE-fm infection was also similar to those reported in other countries.[28,29] The finding suggests that implementation or reinforcement of some infection control measures is required to control the rapid increase of the VRE-fm infection. Starting

from late 2010, a hand hygiene campaign was launched at this hospital. Later, in early 2011, using chlorhexidine for skin preparation was incorporated into the disinfection procedure for central-line care.[30] Coincidently, the increasing trend of VRE-fm isolates reversed rapidly. Previous reports have demonstrated that improvement in hand hygiene could greatly reduce the VRE acquisition by up to 47%.[27,31] Similarly, using chlorhexidine skin preparation could significantly decrease the risk for infection by endemic methicillin-resistant *Staphylococcus aureus* or VRE and reduce the incidence rate of catheter-related bloodstream infection.[32,33] The reduction of VRE-fm at this hospital may serve as another successful example for the importance of hand hygiene in controlling VRE infections. However, a reverse increasing trend of VRE-fm isolates with distinct clones was noted in 2014—2015. A previous report using a large-scale comparative genomics strategy demonstrated that the increase of VRE-fm may involve both *de novo* generation from individual patient's bowl and nosocomial transmission.[34] More studies are needed to elucidate the problem underlying the increase of VRE-fm at this hospital.

VRE is known to spread through two major pathways, clonal dissemination of the VRE isolates themselves and/or lateral transfer through plasmids or transposons of resistant elements among enterococcal isolates.[12,35] In the present study, all but one isolate studied belonged to CC17, a globally distributed, nosocomial-related lineage.[36,37] Similar information was also demonstrated through the BAPS analysis. The five major STs in the present study belonged to two subgroups, BAPS 2-1 and BAPS 3-3, which consisted of the majority of HAI isolates.[10] However, our results also demonstrated that the most prevalent ST has been changing over time and were usually associated with the presence of some particular major pulsotypes. Clonal spread appears to play a major role for the observed increase of overall VRE infection. On the other hand, we also found the predominance (98.9%) of the *van*A gene among the isolates studied. Therefore, lateral transfer of resistance genes may also contribute greatly to the increase of VRE. This is especially clear when the clonal effect was excluded for the epidemiological analysis. In addition, as mentioned above, despite that some infection control measures during 2010—2011 had stopped or even reversed the increasing trend of VRE infection in this hospital, isolates carrying the newly emerged Type II Tn*1546*-like element could still be found in almost a half of the VRE isolates in the following years. Even the isolates carrying the original Type I Tn*1546*-like element, although reduced substantially after 2010, remained at a level much higher than those observed before 2005. According to a recent national surveillance study conducted in 2012, ST17, ST414, ST78, and ST18 were among the top five predominant STs in Taiwan.[18] The prevalence of ST78 and ST414 were also documented in other reports from Taiwan.[38] According to the PFGE-based dendrograms, non-HAI isolates appeared rather similar to HAI isolates, suggesting that some particular strains have been spreading across this island country. The effort of infection control measures should be reinforced in all acute care or long-term care facilities to control the further spread of such multidrug-resistant pathogens.

Through the BAPS analysis, it is interesting to note the existence of some intra-group fluctuation of the five major STs. Even excluding the clonal effect, the trend of intra-group fluctuation was still obvious. In the BAPS 2-1 group, ST78 appeared suddenly in 2008 for unknown reasons, as also observed in other countries.[7,39] However, as this ST reduced, ST203 that carried the Type I Tn*1546*-like element started to increase. Another member of the BAPS 2-1 group, ST414 that carried the Type II Tn*1546*-like element, also appeared parallel but increased much significantly. Both ST203 and ST414 reduced subsequently and again replaced by ST78. However, unlike the ST78 firstly found in prior years, the newly appeared ST78 carried the Type II Tn*1546*-like element and demonstrated very different pulsotypes with less than 60% similarity, compared to the early isolates of ST78. To our knowledge, this phenomenon of intra-group fluctuation has never been reported. As indicated above, clonal spread may explain the increase of the major STs among some specific periods of years. However, the reason for the once prevalent clones to reduce and be replaced by another closely related clone(s) remains unclear. The pan-genome of *E. faecium* has been indicated to be essentially unlimited in size, suggesting that the organism can efficiently obtain and integrate exogenous DNA in its own gene pool, thereby adding to its own competency over the changing environment.[40] Such a natural adaptability may therefore facilitate some efficient clones to dominate when appropriate. Still, the actual mechanism underlying this phenomenon of fluctuation waits for further studies.

The emergence of the Type II Tn*1546*-like element is a warning sign that warrants for close monitoring. Since being introduced together with the clonal spread of ST414, plasmids carrying this structure type of Tn*1546*-like elements have been gradually spread to isolates of other STs, especially the most predominant ST17 and ST78. If excluding the clonal effect, infections associated with VRE-fm carrying the Type I Tn*1546*-like element were generally kept at a background level of less than 15 isolates per year in this hospital. However, the increase of isolates carrying this Type II Tn*1546*-like element seemed to beyond the efficacy of the current infection control measures. At present, we only know such plasmids showed much higher conjugation efficiency than those carried the Type I Tn*1546*-like element. More studies are required to reveal the associated features of plasmids carrying this special structure type of Tn*1546*-like element.

In summary, the 378 VRE-fm blood isolates from hospitalized patients over the 14 years demonstrated the fluctuation of some predominant STs. The increase of STs in some particular periods of study years was generally associated with the presence of various pulsotype clusters. Plasmids carrying the Type II Tn*1546*-like element further facilitated the spread of the most predominant ST17 and ST78. Molecular epidemiology regarding the genotypes of glycopeptides resistance determinants as well as the genetic relatedness of VRE-fm isolates is of great importance to act as the reference for developing control strategies. Future studies on the monitoring of the prevailing resistant clones and the investigation of mechanisms underlying the expansion of some particular clones and the spread of plasmids carrying the Type II Tn*1546*-like element are also essential in controlling this problematic pathogen.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jmii.2018.08.008.