Genome-based analysis of *Enterococcus faecium* bacteremia associated with recurrent and mixed strain infection

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ABSTRACT

Vancomycin-resistant *Enterococcus faecium* (VREfm) bloodstream infections are associated with high recurrence rates. This study used genome sequencing to accurately distinguish the frequency of relapse and reinfection in patients with recurrent *E. faecium* bacteremia, and to investigate strain relatedness in patients with apparent VREfm and vancomycin-susceptible *E. faecium* (VSEfm) mixed infection. A retrospective study was performed at the Cambridge University Hospitals NHS Foundation Trust (CUH) between November 2006 and December 2012. We analyzed the genomes of 44 *E. faecium* isolated from 21 patients (26 VREfm from 12 patients with recurrent bacteremia, and 18 isolates from 9 patients with putative VREfm/VSEfm mixed infection). Phenotypic antibiotic susceptibility was determined using the Vitek2 instrument. Genomes were compared with those for a further 263 *E. faecium* associated with bacteremia in patients at CUH over the same time period. Pairwise comparison of core genomes indicated that 10 (71%) episodes of recurrent VREfm bacteremia were due to reinfection with a new strain, with reinfection being more likely with increasing time between the two positive cultures. The majority (78%) of patients with a mixed VREfm and VSEfm infection had unrelated strains. More than half (59%) of study isolates were closely related to another isolate associated with bacteremia from CUH. This included 60% of isolates associated with re-infection, indicating acquisition in the hospital. This study provides the first high-resolution insights into recurrence and mixed infection by *E. faecium*, and demonstrates that reinfection with a new strain, often acquired from the hospital, is a driver of recurrence.

INTRODUCTION

Enterococcus faecium is an important cause of bloodstream infections in critically ill and immunocompromised patients (1), and ranks among the ten most common microorganisms associated with healthcare-associated infections in the United States (US) (2). Bacteremia caused by vancomycin-resistant enterococci is associated with increased mortality, costs of care and rates of recurrence compared to vancomycin-susceptible strains (3–5).

Despite the establishment of linezolid and daptomycin as mainstay therapeutic agents for vancomycin-resistant *E. faecium* (VREfm) bacteremia since the early 2000s, recurrence remains a common clinical challenge with rates ranging from 3% to 25% (6–8). Following apparent clearance of infection, recurrent bacteremia can be explained by either relapsing infection with the same strain due to a persistent focus of infection, or reinfection with the same or a different strain. Differentiating between these scenarios is clinically relevant; relapsing infection requires investigation and interventions to deal with a persistent focus of infection, whilst reinfection is more likely to be associated with an underlying susceptibility that increases the risk of invasion due to breakdown of host immune defenses. Surprisingly little is known about the relative frequency of relapse versus reinfection in recurrent *E. faecium* bacteremia, with the literature limited to case reports (9–11) and case series predominantly containing patients with *Enterococcus faecalis* infection (12, 13).

E. faecium bacteremia is polymicrobial in up to 35% of cases due to mixed infection with other bacterial genera or enterococcal species (14). Apparent mixed infection with VREfm and

vancomycin-susceptible *E. faecium* (VSEfm) has been reported in the context of *in-vivo* loss or gain of the *van* transposon by sub-populations of the same strain (15–17). However, whilst carriage of multiple strains of *E. faecium* is presumed to be common (18, 19), the frequency of mixed infection with different *E. faecium* strains is unknown.

Whole-genome sequencing has demonstrated superior discriminatory power compared to traditional bacterial typing techniques such as pulsed-field gel electrophoresis (PFGE) or multilocus sequence typing (MLST) in epidemiological investigations and in studies of the population structure of *E. faecium* causing bacteremia at a local and national level (20–23). Here, we use genome sequencing to gain a better understanding of *E. faecium* bacteremia. Specifically, we investigated strain relatedness in patients with recurrent VREfm bacteremia, and in patients with apparent mixed bloodstream infection with VREfm and VSEfm. In addition, we compared the findings of genome sequencing with antibiotic resistance profile.

RESULTS

A retrospective review of patients at CUH between November 2006 and December 2012 identified 231 patients with at least one episode of VREfm bacteremia. Of these, 14 patients had at least one episode of recurrence, giving an estimated recurrence rate of 6.1%. We identified twelve patients that had isolates from at least two episodes of VREfm bacteremia available for whole genome sequencing (see Table 1 for patient summary and Dataset S1 for individual isolate information). Ten patients had one recurrence and two patients had two recurrences of bacteremia. To determine the genetic relatedness of isolates causing recurrence we identified

SNPs in the core genome based on mapping to a reference genome. Of the fourteen isolate pairs associated with a recurrent bacteremia, four (from four patients) were closely related (1-7 SNPs, median 1.5 SNPs) to the isolate from the previous episode (Figure 1A, Table 1). This finding is highly indicative of relapse (or reinfection) with the same strain, based on a study that reported a genetic distance between *E. faecium* carried by the same person (within-host diversity) of 6 core SNPs (24). By contrast, 10 isolates (from ten patients) were more genetically distant from the isolate from the previous episode (25-368 SNPs, median 258 SNPs), which is consistent with reinfection by a different strain (Figure 1A, Table 1). The two patients with two recurrences of bacteremia had both an episode of relapse (pairwise SNP difference of 2 or 7 SNPs, respectively), and an episode of reinfection with a new strain (25 or 309 SNPs, respectively) (Table 1). The SNPs acquired between the first and second isolate for the four genetically related isolate pairs were located in different genes in different patients (Table S1). The median time to first recurrence across the study population was 80 days (range 39 to 1578 days), and the second episodes of recurrence occurred 36 and 168 days after the preceding bacteremia. Comparison of the timing of recurrence with the genomic analyses indicated that all isolates from cases of relapse/reinfection with the same strain were isolated within 108 days of each other, whilst recurrences due to reinfection with a different strain were equally likely to occur within 108 days (5/10 episodes) and after 108 days (5/10).

All twelve study patients with recurrent bacteremia had multiple co-morbidities that predispose to VREfm bacteremia (Table 1, Dataset S1). The most probable source for the bacteremia was defined for each case (Table 1). Mucosal translocation (n=7, with possible concurrent

intravascular catheter infection) and intravascular catheters (n=5) were the most common sources of bacteremia for the study patients. There was no clear difference identified between the sources of infection in patients with recurrence due to the same or different strains (Table 1, Table S2). The four episodes of recurrence due to the same strain were associated with presumed persistent intravenous catheter colonization and/or gut carriage (n=3), or failure to eradicate a persistent focus of infection (n=1, chronic pyelonephritis associated with kidney stones) (Table 1, Table S2). A central venous catheter was known to be retained between episodes of bacteremia in 2/10 cases with reinfection with a different strain, meaning that whole genome sequencing was able to refute these as being a persistent focus.

The retrospective review of CUH patients also identified nine patients with putative mixed bloodstream infection with VREfm and VSEfm for whom both isolates were available for whole genome sequencing (Table 1). Pairwise core genome comparison of the 9 VREfm/VSEfm pairs revealed that 7/9 (78%) patients had isolate pairs that were genetically distinct (median 217 SNPs, range 70-381 SNPs) (Figure 1B), which is consistent with true mixed-strain infection. The most common source of infection for patients with true mixed infection was an intravascular catheter (4/7, 57%). The remaining 2 patients had isolates that were identical at the core genome level. Further analysis of the genetic content between these 2 pairs through comparison to the ResFinder database confirmed the variable presence of the *vanRSHAXYZ* genes, which encode vancomycin resistance. There was insufficient sequence adjacent to the *vanA* transposon in the genome assemblies to identify the genetic location of these genes, so differences in gene content between the VREfm and VSEfm in each pair were assessed. In one

isolate pair (Patient 16) the *van* genes had been lost together with 21 genes, including 7 genes best matched to a plasmid (based on BLAST), suggesting they may have been lost/gained together with part of a plasmid (Dataset S2). Two genes labelled as *tetM* and *ermB* were lost alongside the *van* genes in this patient, but both isolates retained a copy of *tet*M and *ermB* and so this may not have affected the wider antibiotic resistance phenotype (Figure 2). In the second isolate pair (Patient 21) an additional 14 genes had been lost with the *van* genes including five genes located adjacent in the genome (Dataset S2) suggesting that *vanA* was not gained/lost as part of a plasmid but may have moved as part of a smaller transposable element.

We evaluated whether the phenotypic antibiotic resistance profile (antibiogram) to 11 drugs (excluding glycopeptides) could be used to distinguish between genetically related and distinct strains from the same patient (Figure 2). Of the 6 isolate pairs that were closely related in the two study collections, four had identical antibiograms, one varied by tetracycline resistance associated with gain/loss of the *tetM* gene, and one varied between susceptible and intermediate resistance to quinupristin-dalfopristin (Figure 2). Of the 17 strain pairs that were genetically different, three had identical antibiograms and the remainder had between 1 and 5 (median 2) differences (Figure 2). The most variable antibiotic was tetracycline (10/17 pairs), followed by high-level resistance to streptomycin (n=7), and nitrofurantoin (n=6). Since 3/7 identical antibiograms (43%) belonged to genetically distinct strains, this suggests that antibiogram does not reliably distinguish between genetically related and distinct strains from the same patient. However, more than one change in the antibiogram was only identified in genetically distinct strains (12/17 genetically distinct pairs).

147

148 The high rate of true mixed VREfm and VSEfm infection and recurrence with a new strain
149 indicates carriage of multiple lineages or the acquisition of new strains over time. Healthcare
150 settings are associated with the acquisition of *E. faecium*, and so we investigated CUH as a
151 putative source by combining the 44 study *E. faecium* genomes with a further 263 *E. faecium*
152 genomes associated with bloodstream infection in 263 patients at the same hospital over the
153 same time period (2006-2012) (Figure S1). Taken together, 26 of the 44 study isolates were
154 closely related to at least one CUH isolate (0-8 SNPs, median 3), including 3 isolates that were
155 closely related to an isolate from another study patient. This included isolates from 6/7 patients
156 infected with different VREfm and VSEfm strains and 6/10 patients with recurrence caused by
157 different strains. The remaining isolates were between 12 and 86 SNPs (median 33 SNPs) from
158 the closest genetic match.

159

160 **DISCUSSION**

161 This study represents the first use of whole genome sequencing in the context of *E. faecium*
162 bacteremia to investigate the relative rates of relapse and reinfection in recurrent infections,
163 and to study mixed infection with VREfm and VSEfm. Although rates of recurrence vary in the
164 literature, the estimated rate of 6.1% identified at CUH is within the range of those reported
165 previously (6–8).

166

167 We found that the majority of patients in our study had a recurrent VREfm bacteremia caused
168 by reinfection with a new strain. This finding supports that of Cheng *et al*. which found

reinfection to be responsible for ~70% of recurrence based on PFGE, although the study focused primarily on *E. faecalis* (12). These reinfections could either be due to persistent carriage of a genetically distinct strain, or reinfection with a newly acquired strain. We found that at least 60% of reinfections were caused by isolates that were genetically closely related to another bacteremia isolate from CUH, suggesting cross-transmission in the hospital. Additionally, the rates of cross-transmission found in this study are likely to be an underestimate, since asymptomatic gut carriage and the environment represent a large reservoir of VREfm and were not sampled in this study. These findings suggest that the emphasis on preventing recurrent VREfm bacteremia should be on infection control and minimizing periods of susceptibility to infection. Further studies will be required to elucidate the role of the environment, staff and patients as sources for these hospital acquisitions to improve infection control.

The results of this study suggest that recurrence with the same strain may be related to time. Episodes of recurrence with the same strain were only identified up to 108 days apart, which concurs with findings by Baran *et al.* based on *E. faecalis* and *E. faecium* from a total of three patients (13). In contrast to our findings for same-strain recurrence, our study showed that bacteremic episodes due to reinfections with a distinct strain were roughly equally likely to occur within and after 108 days of each other, and as early as 57 days apart. Further work will be required using larger sample sizes from multiple centers to determine whether there is a true relationship between the relatedness of *E. faecium* strains causing recurrence and the time between episodes.

We also identified that the majority of patients with mixed VREfm and VSEfm bacteremia were infected with two genetically distinct strains. This differs from the finding by Cardenas et al. that four patients had closely related VREfm and VSEfm strains associated with bacteremia based on MLST (16). The true mixed infections in our study frequently varied in antibiotic resistance profiles. Whilst most cases of mixed VREfm and VSEfm would be detected during routine disc susceptibility testing, this variation in antibiotic resistance profiles could complicate treatment in cases that go undetected. Although the numbers are low in our study, it was interesting to note that true mixed VREfm and VSEfm infections were commonly suspected to originate from an intravascular source, suggesting that central venous catheters may become colonized with multiple strains of *E. faecium*.

The results of our study suggest that antibiogram lacks accuracy in predicting the genetic relatedness of strains. The utility of antibiograms for determining the relatedness of *E. faecium* has not previously been evaluated, but our finding that a pair of identical strains could vary in their resistance to antibiotics is consistent with the fact that *E. faecium* has a highly mobile genome, with many resistance genes carried on mobile genetic elements.

Our study may have implications on future evaluation of VREfm treatment efficacy. There are currently no randomized controlled trials to define the optimal antibiotic for the treatment of VREfm bacteremia. Current knowledge is based on retrospective observational studies comparing linezolid to daptomycin, where recurrent infection is often defined as one of the outcome measures in the absence of bacterial typing results (6, 8, 25). These studies imply that

213 early recurrence (often assessed at 30 or 60 days after treatment completion) is caused by true
214 relapse (6, 8), or rely on phenotype such as identical antibiograms to infer relapse (25). Our
215 results show that in the absence of prospective randomized studies or bacterial genotyping, one
216 needs to question whether a recurrent infection is indeed due to ineffective therapy as opposed
217 to underlying confounding from patient related factors conferring increased susceptibility to
218 reinfection. Future studies should address this issue.
219
220 This study has several limitations. We did not sequence multiple colonies from the same sample
221 to assess diversity, meaning that apparent reinfections could have been mixed infections at the
222 outset. The study samples were retrieved from frozen stock and it is not possible to know
223 whether these were originally created from a single or multiple colonies. It is not possible to
224 differentiate between relapse and reinfection by the same strain, introducing an element of
225 uncertainty into our classification. The true level of mixed infection will be higher than we
226 report here, since we only assessed patients with a VREfm and VSEfm mixed infection and
227 analysed one colony of each from every bacteremic episode. Finally, the rate of recurrence
228 identified here may be an underestimate since repeat cultures were not taken systematically.
229
230 In conclusion, the findings of this study show that the majority of VREfm recurrences and mixed
231 VREfm and VSEfm infections are due to different strains, and that antibiogram lacks accuracy in
232 determining genetic relatedness. This has important implications for infection control as it
233 highlights the importance of reducing cross-transmission in vulnerable patient groups.
234

MATERIALS AND METHODS

A retrospective study was conducted at the Cambridge University Hospitals NHS Foundation Trust (CUH), a tertiary referral center in the United Kingdom with 1,170 beds and 350,000 occupied-bed-days per year. The rate of vancomycin resistance in *E. faecium* bacteremia isolates at CUH is high (>60%), approaching rates reported in the US (2) and historical data from 2001 suggests that 32.6% of patients at CUH in high-risk wards carry VRE (either *E. faecium* or *E. faecalis*) (26).

All patients with VREfm bloodstream infection between November 2006 and December 2012 were identified using the diagnostic microbiology laboratory database. These cases were evaluated to identify all patients with (i) recurrence of VREfm bloodstream infection, and/or (ii) putative mixed VREfm and VSEfm bloodstream infection. Recurrence was defined as a blood culture that was positive for VREfm taken >30 days after the index culture from a patient with intervening negative blood cultures and/or resolution of clinical signs of infection. Putative mixed VREfm and VSEfm infection was defined as the isolation of VREfm and VSEfm from the same blood culture or different cultures taken within 48 h of the index sample.

Fourteen patients fulfilled the criterion for recurrence and 10 patients fulfilled the criterion for putative mixed VREfm and VSEfm infection, with no overlap of cases between the two. Seven of the patients had mixed infection with other bacterial species, as shown in Dataset S1. Cross-referencing these 24 patients with the bacterial freezer archive identified 44 isolates from 21 patients (12 patients with recurrence and 9 patients with mixed VREfm and VSEfm infection),

who were the basis for this study. Clinical data for the 21 cases were collected from paper and computerized medical records using a standardized proforma, including the suspected focus of infection, underlying comorbidities, and dates of positive and negative blood cultures. Neutropenia was defined as a polymorphonuclear leukocyte count of less than 500/μl within 24 hours of the onset of bacteremia. The focus of infection was defined based on clinical, radiological and microbiological features. Bacteremia was determined to be secondary to an intravascular device if i) a positive intravascular catheter tip semi-quantitative culture yielded more than 15 cfu of *E. faecium* with identical antibiogram as the blood culture isolate (definite), or ii) if no other focus of infection was identified in the presence of an intravascular catheter, and/or clinical signs of sepsis improved after line removal (probable). For neutropenic patients with no definite clinical focus, mucosal translocation was presumed to be the origin of the bacteremia based on the Centers for Disease Control and Prevention definition (http://www.cdc.gov/nhsn/pdfs/pscmanual/4psc_clabscurrent.pdf). In cases of recurrent infection, a focus was considered persistent if there was an unresolved deep source of infection or if a potentially infected intravascular catheter was not removed between episodes of bacteremia. Ethical approval for the study was obtained from the local Research Ethics Committee (reference no. 13/EE/0044) and the need for informed consent was waived.

Twenty-one of the 44 isolates had been sequenced previously (27). For the 23 new *E. faecium* isolates sequenced here, bacteria were cultured on Columbia Blood Agar (CBA, Oxoid) for 48 hours at 37°C in air. Phenotypic antimicrobial susceptibility testing for all 44 isolates was performed using the Vitek2 instrument (bioMérieux, Marcy l'Etoile, France) with the AST-P607

card. DNA was extracted using the QIAxtractor (QIAgen) and sequencing performed on an Illumina HiSeq2000. Sequence reads were assembled using Velvet and annotated using Prokka. The pangenome was estimated using Roary (28) with a 98% ID cut-off. The *van* gene in the two patients with genetically related VREfm and VSEfm was extracted from the Roary pan genome and compared to the *vanA* gene extracted from a *vanA* transposon (accession number M97297) and the *vanB* gene extracted from Aus0004 (accession number CP003351) using BLAST. The presence of antibiotic resistance genes was determined using an in-house curated version of the ResFinder database (genes listed in Dataset S3) (29) and ARIBA (https://github.com/sanger-pathogens/ariba/wiki).

Sequence data for an additional 263 *E. faecium* associated with bloodstream infection in 263 patients at CUH between November 2006 and December 2012 and belonging to the hospital-adapted clone of Clade A based on whole genome sequence analysis were taken from Raven et al. (27). These 263 genomes together with the 44 study genomes were mapped to *E. faecium* Aus0004 (ENA accession number CP003351) using SMALT (http://www.sanger.ac.uk/science/tools/smalt-0). Mobile genetic elements (identified based on annotation and PHAST (30)) and recombination events (identified using Gubbins (31)) were removed to identify the core genome. A maximum likelihood tree was created using RAxML based on single nucleotide polymorphisms (SNPs) in the core genome. Pairwise SNP differences were calculated based on SNPs in the core genome.

Accession numbers are listed in Dataset S1.

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FIGURE LEGENDS

Figure 1. Genetic relatedness of isolates from the same patient with recurrent bacteremia (A), or mixed infection with VREfm and VSEfm (B). A) Right hand side: Graph showing the pairwise core genome SNP difference between *E. faecium* isolates cultured from the same patient more than 30 days apart, and timing of episodes. Red indicates isolate pairs that were closely related (1-7 SNPs) based on genome sequence data. Left hand side: expansion of the area of the graph under 100 SNPs. B) Graph showing the pairwise core genome SNP difference between VREfm and VSEfm isolates cultured from the same patient within 48 hours.

Figure 2. Comparison between strain similarity by genome sequence data and antibiogram. Left hand side indicates the strain relatedness based on genome sequence data, and the patient. Two patients with both relapse and reinfection occur twice in the list. Middle block indicates changes in antibiotic resistance (red, defined as a change from susceptible to resistant; green, defined as a change from resistant to susceptible; white, no change) between isolates from the same patient.

Table 1. Clinical and isolate details for the patient cohort.

	Pt	Age **	Gender	Co-morbidities ***	Number of isolates	Presumptive source of infection			Year of first isolate	Number of days		Number of SNPs#			Interpretation
						1st	2nd	3rd		1st to 2nd	2nd to 3rd	1st to 2nd	2nd to 3rd	1st to 3rd	
Recurrence	1	67	Male	SOM	3	Urinary	Urinary	Urinary	2010	54	36	25	2	25	Reinfection/Relapse
	2	50	Male	HM, SCT	2	MT/IV	MT/IV	-	2007	1578	-	234	-	-	Reinfection
	3*	56	Male	HM	2	MT/IV	MT/IV	-	2009	61	-	268	-	-	Reinfection
	4*	24	Female	Congenital neutropenia	3	IV	MT/IV	MT/IV	2012	108	168	7	311	309	Relapse/Reinfection
	5	44	Female	SOT, ESRD, DM	2	IV	Unknown	-	2009	777	-	299	-	-	Reinfection
	6	42	Female	Alcoholic liver disease	2	IV	IV	-	2009	215	-	282	-	-	Reinfection
	7	13	Male	HM, SCT, ESRD	2	MT/IV	IV	-	2007	1484	-	232	-	-	Reinfection
	8	41	Female	HM, SCT, ESRD	2	MT/IV	MT/IV	-	2011	57	-	64	-	-	Reinfection
	9	10	Female	HM	2	IV	MT/IV	-	2010	80	-	1	-	-	Relapse
	10	0	Male	HM	2	IV	MT/IV	-	2010	59	-	348	-	-	Reinfection
	11	15	Female	HM	2	MT/IV	MT/IV	-	2010	39	-	1	-	-	Relapse
	12	39	Male	HM	2	MT/IV	MT/IV	-	2012	104	-	282	-	-	Reinfection
Mixed VREfm & VSEfm	13	59	Female	SOM, HM	2	IV	-	-	2012	2	-	329	-	-	Genetically distinct
	14	72	Male	SOM, HM	2	MT	-	-	2012	0	-	119	-	-	Genetically distinct
	15	62	Male	HM, SCT	2	IV	-	-	2011	0	-	217	-	-	Genetically distinct
	16	50	Male	HM, SCT	2	MT/IV	-	-	2009	0	-	0	-	-	Genetically related
	17	56	Male	HM	2	IV	-	-	2008	0	-	18	-	-	Genetically distinct
	18	59	Male	SOM	2	IA - biliary	-	-	2008	0	-	381	-	-	Genetically distinct
	19	63	Male	HM, SOT	2	IV	-	-	2009	1	-	203	-	-	Genetically distinct
	20	19	Male	HM, SCT	2	MT/IV	-	-	2010	0	-	154	-	-	Genetically distinct
	21	48	Female	ESRD, LC	2	Lung and IA	-	-	2010	0	-	0	-	-	Genetically related

* Patients with recurrence with a different strain for whom a central venous catheter was retained between episodes of bacteremia.

** Age at time of first bacteremia

*** Co-morbidities identified across all episodes of bacteremia in the study (breakdown by bacteremic episode in Dataset S1)

Number of SNPs based on mapping to a reference genome (*E. faecium* Aus0004)

Abbreviations: Pt = patient, SOM = solid organ malignancy, HM = hematological malignancy, SCT = stem cell transplant, SOT = solid organ transplant, ESRD = end stage renal disease, DM = diabetes mellitus, LC = liver cirrhosis, MT = mucosal translocation, IV = intravascular, IA = intra-abdominal