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Long-term carriage and evolution of VREfmLong-term carriage and evolution of vancomycin-resistant *Enterococcus faecium*: a genomic study on consecutive isolates

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Objectives: To determine if vancomycin-resistant *Enterococcus faecium* (VREfm) carriers carry the same VREfm clone after a minimum follow-up of 365 days. For those carrying the same clone, we investigated the genomic evolution per year per genome.

Methods: We used WGS results to assign VREfm clones to each isolate and determine clone shifts. Finally, we calculated distance in core-genome MLST alleles, and the number of SNPs between consecutive VREfm isolates from patients carrying the same VREfm clone.

Results: In total, 44.2% of patients carried the same VREfm clone, and the genomic evolution was 1.8 alleles and 2.6 SNPs per genome per year.

Conclusions: In our population of long-term carriers, we calculated a molecular clock of 2.6 SNPs.

Introduction

Enterococcus faecium is a common nosocomial pathogen, and the cause of both serious invasive infections and colonization.[1] In the Capital Region of Denmark we have seen a rise in vancomycin-resistant *E. faecium* (VREfm) isolates since 2012.[2] In this study, we used WGS to examine consecutive VREfm isolates from patients with positive samples taken a minimum of a year apart. In our setting, they would be considered long-term VREfm carriers. However, we suspect some of these patients to have cleared their initial VREfm colonization and been recolonized with a different clone.

Literature on the natural or spontaneous clearance of VREfm colonization is sparse and results vary greatly. One study showed a natural VREfm clearance of only 33% over a 3 year study period.[3] In a systematic review, 50% of subjects had cleared VREfm colonization at 25 weeks after initial colonization.[4] In a previous clinical trial, investigating the effect of probiotics on eradication of VREfm colonization in an elderly, comorbid and immunocompetent population, the number of participants with spontaneous decolonization was higher than expected: 56% after 4 weeks, and 90% after 24 weeks in the placebo group.[5]

In this study we aimed to determine how many of our patients considered long-term VREfm carriers were true long-term carriers, that is, still carrying the same VREfm clone for at least a 1 year period. Secondly, we investigated how the VREfm isolates evolved in the intestine of the true long-term carriers.

Methods

Selection of patients

The study is a retrospective study with data from 2012 until September 2022 conducted in the Capital Region of Denmark in collaboration between two Departments of Clinical Microbiology. We cover nine hospitals and all GPs in the region. In the Capital Region of Denmark, we perform active surveillance of VREfm, both from clinical samples and screening samples. The first VREfm isolate per patient per year (clinical or screening sample) is routinely sequenced and kept in our database. An exception applies to patients with both a *vanA* and a *vanB* within the same year, in which case, both isolates are sequenced. In hospitals, patients were screened in the case of hospital outbreaks, or upon admission if the patient had had a positive VREfm sample 6 months prior to admission time.[6] We did not perform screening of non-hospitalized VREfm carriers.

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Table 1. Summary statistics of included VREfm carriers with and without shift in clones

	With clone shift		Without clone shift	Total
Patients, *n* (%)	115 (55.8)		91 (44.2)	206
Female sex, *n* (%)	67 (58.3)		49 (53.8)	116 (56.3)
Age (years), median (IQR)	74.0 (64.0–80.0)		72.0 (63.0–77.0)	—
Days between samples, median (IQR)	753.0 (546.5–1083.5)		524.0 (428.5–699.5)	—
MLST	From:	To:	MLST observed:	—
	80	80 (*n* = 15; 13.0%)	17 (*n* = 2; 1.1%)	
	80	1421 (*n* = 10; 8.7%)	80 (*n* = 34; 18.7%)	
	117	80 (*n* = 7; 6.0%)	117 (*n* = 21; 11.5%)	
	203	80 (*n* = 8; 7.0%)	203 (*n* = 60; 33.0%)	
	203	1421 (*n* = 12; 10.4%)	612 (*n* = 2; 1.1%)	
	1421	80 (*n* = 22; 19.1%)	1421 (*n* = 58; 31.9%)	
	Other	(*n* = 41; 35.7%)	1478 (*n* = 1; 0.5%)	
			other (*n* = 4; 2.2%)	
vanA, *n* (%)	165 (71.7)		151 (83.0)	316 (76.7)
vanB, *n* (%)	63 (27.4)		30 (16.5)	93 (22.6)
vanA and *vanB*, *n* (%)	2 (0.9)		1 (0.5)	3 (0.7)

We accessed the database with the objective of identifying patients with repeated VREfm isolates by use of the Danish citizens' personal identification numbers. Patients were included in the study if they had two or more sequenced VREfm isolates a minimum of 365 days apart. We excluded one patient who had both a *vanA* and *vanB* isolate from the same day.

WGS and bioinformatic methods

The VREfm isolates were sequenced on the Illumina MiSeq platform (Illumina Inc., San Diego, USA) as previously described.[7] Raw reads were trimmed using BBDuk (https://sourceforge.net/projects/bbmap/) with the parameters ktrim = r, k = 23, mink = 11, hdist = 1, tbo, qtrim = r and minlength = 30, and assembled with SKESA v.2.2 with default settings except inclusion of the parameter –allow_snps. Only assemblies with a genome size in the range 2.7–3.2 Mb, a minimum depth of coverage of 30, and N50 of minimum 10 000 were included in the study. We used SeqSphere+ v.8.2.0 (Ridom GmbH, Munster, Germany; http://www.ridom.de/seqsphere/) with the previously published scheme for *E. faecium* for the initial bioinformatic analysis.[8] Sequencing reads were aligned, and SNPs were called using the NASP pipeline v.1.1.2.[9] In the pipeline, duplicated regions in the reference were masked using NUCmer v.3.1, reads were subsequently mapped to the *E. faecium* Aus0004 (CP003351.1) reference genome with BWA-mem v.0.7.16 and SNPs were called with GATK v.3.8.0.[10–13] Consensus bases had a minimum coverage of 10 and a minimum proportion of 0.9 for the called base. Pairwise comparisons between isolates of patients were performed using the consensus base matrix. Recombination regions were detected for every clone group and filtered out using Gubbins v.3.2.1.[14]

Analysis of long-term carriage

We included two isolates per patient. Most of the patients had only two sequenced VREfm isolates; however, a subgroup of patients had three or more sequenced isolates. Regarding those patients, the first sequenced isolate was included in the study along with the next isolate after a minimum of 365 days. Thus, all patients contributed with only two isolates each for the primary analysis. The SeqSphere+ software assigned core-genome MLST (cgMLST) to each isolate. Subsequently, the isolates were visualized in a minimum spanning tree (MST), and cgMLST clones were defined with a setting of maximum of 20 alleles between isolates. The MSTs are presented in Figures S1, S2 and S3 (available as Supplementary data at *JAC-AMR* Online). For each patient, we determined if there had been a shift from one clone to another.

Genetic evolution of VREfm isolates over time

For this analysis, patients could contribute with more than two isolates. Concerning the subgroup of patients with three or more sequenced isolates, we included the first isolate as the baseline. The subsequent isolate taken at least 365 days after the baseline isolate was included. Following isolates were included if they were taken a minimum of 365 days after the previous isolate. All included isolates were compared with the baseline isolate in the WGS analysis. An MST was performed as in the primary analysis, and clones were defined for all pairs. We determined whether there had been a shift from one clone to another in each pair of isolates. The pairs without clone shift were identified, and distance matrix was measured to quantify the number of allele differences. Finally, the number of SNPs between pairs were measured, and we calculated the molecular clock for our population.

Statistical analysis

The statistical analysis was performed with R v.4.2.2.[15] We used a mixed-effect linear model to estimate the genetic evolution in the secondary analysis, as the observations were not independent.

Ethics

The study was approved by the Danish Data Protection Agency (P-2022-653), and the Danish Health and Medicines Authority (3-3013-1118/1).

Results

Analysis of long-term carriage

Of 6689 patients with at least one positive VREfm sample in the database, 206 patients had at least two sequenced VREfm isolates a minimum of 365 days apart and were included in the analysis. Age and sex for the subgroups are listed in Table 1. For 115

(55.8%) patients the second VREfm isolate belonged to a different clone than the first VREfm isolate, and for 91 (44.2%) both isolates belonged to the same clone. The median number of days between samples was 753.0 (IQR 546.5–1083.5) in the subgroup with clone shift, and 524.0 (IQR 428.5–699.5) in the subgroup without clone shift (Table 1). The distribution of the major STs did not differ between the subgroups, but in the subgroup with clone shift most of the shifts were to an ST80 clone (Table 1).

Genetic evolution of VREfm isolates over time

A total of 223 pairs of isolates were included in the analysis of the genetic evolution of VREfm isolates over time. Of these, 123 pairs had shifted from one clone to another, and 100 pairs belonged to the same clone. The further analysis of the 100 pairs without clone shift revealed a significant estimate of 1.8 alleles per year per genome (95% CI 1.3–2.3, $P<0.001$). The SNP analysis resulted in a significant estimate of 2.6 SNPs per year per genome (95% CI 1.8–3.4, $P<0.001$), constituting the molecular clock for the included isolates (Figure S4). The average percentage of compared genome between all isolate pairs was 80.5% (SD = 0.02).

Discussion

In our cohort, 55.8% of the patients had acquired a different VREfm clone when compared with the baseline sample, and 44.2% still carried the same clone at the time of the second sample. The subgroup without clone shift could be long-term carriers of VREfm. Regarding the subgroup with different VREfm clones, recolonization most likely explains long-term carriage of VREfm; however, *in vivo* horizontal gene transfer from other gut commensals is also possible. Not surprisingly, the median number of days between samples was higher in the subgroup of patients with a clone shift. There was no difference in age between the two subgroups. Rohde and colleagues[16] investigated the risk factors of VREfm colonization and found them to be age, recent stay in a long-term care facility, recent hospital admission, recent proton pump inhibitor/antiacid treatment, previous colonization with MDR organisms and antibiotic treatment at the time of VREfm colonization or in the 6 months prior to detection. In the analysis of genetic evolution, we found the genetic evolution to be 1.8 alleles per year, and 2.6 SNPs per year, constituting the molecular clock. In previous studies of multiple individuals, the molecular clock of VREfm has been estimated as between 3.41 and 5 SNPs per year.[17,18] In our study we report the molecular clock from a collection of genomes from the same individuals, and we believe this could be the explanation for a lower SNP accumulation.

The study is limited by the retrospective observational study design. We do not have data on duration of hospital stay and comorbidities. In a previous randomized clinical trial we found the VREfm carriers to be a homogeneous group with a high Charlson comorbidity index of 4.[5] The patients included in the study have only been retested on hospital readmission. Therefore, we cannot determine the time of decolonization based on these data. Since we observe clonal spread of VREfm, we cannot rule out recolonization with the same clone for the group of patients without clonal shift within the 524 day observation period.[19,20] Another limitation is that only one isolate, recovered by picking one random colony, per sample is sequenced. A recent study on immunosuppressed patients suggests they can be carriers of multiple clones at the same time. The authors of that study sequenced 10 colonies per sample and showed that 33.3% of patients carried two VREfm clones.[21]

The study contributes to the existing knowledge of long-term VREfm carriage and the genetic evolution of the bacteria in carriers. The molecular clock of VREfm should be further investigated in future WGS studies to fully understand the genetic evolution of VREfm.

Conclusions

In conclusion, just over half of the patients with a positive VREfm sample more than a year after their initial sample had acquired a different VREfm suggesting recolonization. We estimated the molecular clock of VREfm in long-term carriers with the same VREfm clone, to be 1.8 alleles per year per genome, and 2.6 SNPs per year per genome.

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Transparency declarations

None to declare.

Data availability

All isolates are uploaded in a BioProject. Please refer to Table S1 for BioProject number and metadata for the isolates.

Supplementary data

Figures S1 to S4 and Table S1 are available as Supplementary data at *JAC-AMR* Online.

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