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Whole Genome Sequence Analysis of the First Vancomycin-Resistant *Enterococcus faecium* Isolates from a Libyan Hospital in Tripoli

Mohamed O. Ahmed,[1] Asma K. Elramalli,[2] Keith E. Baptiste,[3] Mohamed A. Daw,[4] Abdulaziz Zorgani,[4] Ellen Brouwer,[5] Rob J.L. Willems,[5] and Janetta Top[5]

The purpose of the study was to investigate the molecular characteristics and genetic relatedness of the first reported cases of vancomycin-resistant enterococci (VRE) from the Tripoli Medical Center, Libya. In total, 43 VRE isolates were obtained from various clinical sites throughout the years 2013–2014, including 40 *vanA*-type and 2 *vanB*-type vancomycin-resistant *Enterococcus faecium* isolates and 1 *vanC1*-type *Enterococcus gallinarum*. Of the 42 *E. faecium*, 19 isolates were subjected to whole genome sequencing. Core genome multilocus sequence typing (cgMLST) analysis revealed three sequence clusters (SCs) of clonally related isolates, which were linked to different hospital wards. The first two VRE isolates, isolated early 2013 from patients in the medical intensive care unit, were grouped in SC1 (MLST [ST] 78, *vanB*) and differed in only 3 of 1423 cgMLST alleles. The SC2 ($n = 16$, special care baby unit, neonatal intensive care unit, pediatric surgery ward, and oncology ward) and SC3 ($n = 1$, antenatal ward) were all ST80 *vanA*-VRE, but the single SC3 isolate differed in 233 alleles compared with SC2. Within SC2, isolates differed in 1–23 alleles. Comparison with a larger database of *E. faecium* strains indicated that all isolates clustered within the previously defined hospital clade A1. A combination of Resfinder and mlplasmid analysis identified the presence of resistance genes on different plasmid predicted genetic elements among different SCs. In conclusion, this study documents the first isolates causing outbreaks with VRE in the Libyan health care system. Further surveillance efforts using molecular typing methods to monitor spread of multidrug-resistant bacteria in the Libyan health care system are urgently needed.

Keywords: enterococci, vancomycin-resistant *Enterococcus faecium*, WGS, MLST, Libya

Background

Enterococci are commensals of the gastrointestinal tract of mammals and insects, but have emerged as opportunistic pathogens in hospitals worldwide.[1–4] *Enterococcus faecium* and *Enterococcus faecalis* are an established medical and public health concern associated with persistent infections and microbial resistance, especially *E. faecium*.[2–5] In contrast to *E. faecalis,* population genetics revealed that within *E. faecium* a defined subpopulation (Clade A1) is associated with hospitalized patients, which are often resistant to the most commonly used antibiotics in hospitals, including vancomycin.[3,6] Although vancomycin resistance has been described in *E. faecalis*, *E. faecium* is the main reservoir of gene clusters encoding vancomycin resistance, mainly represented by the *vanA* and *vanB* types.[7,8]

In North Africa, multidrug-resistant (MDR) bacteria are increasingly reported mainly originating from health care settings covering a range of global emerging bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and colistin-resistant carbapenemase-producing Enterobacteriaceae.[5,9,10] Nevertheless, detailed epidemiological studies and/or genomic analyses from this region are limited specifically concerning the occurrence and distribution of MDR enterococci from humans and animals.[5] However, there are some recent reports mainly from health care facilities in North Africa describing vancomycin-resistant enterococci (VRE). The majority of these VRE cases are represented by *vanA*-positive *E. faecium* of multilocus sequence type (MLST) ST18, ST80, and ST78 that belong to the global Clade A1.[11–15]

In this study, we performed whole genome sequencing (WGS) to determine the genetic relatedness of the first 43

[1]Department of Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Tripoli, Tripoli, Libya.
[2]Department of Microbiology, Tripoli Medical Center, Tripoli, Libya.
[3]Department of Veterinary Medicine, Danish Medicines Agency, Copenhagen, Denmark.
[4]Department of Medical Microbiology and Immunology, Faculty of Medicine, University of Tripoli, Tripoli, Libya.
[5]Department of Medical Microbiology, UMCU, Utrecht, The Netherlands.

reported cases of VRE isolated from hospitalized patients throughout 2013–2014 at the Tripoli Medical Center. The Tripoli Medical Center is the largest national Libyan hospital by size and population, providing different medical and health care services to Tripoli as well as the north-west region of Libya.

Materials and Methods

Enterococcal strains

At the Tripoli Medical Center, VRE screening is initiated in response to the identification of multiple infections and the isolation of VRE from clinical samples, such as blood, urine, and wound material. Such screening includes the analysis of rectal swabs obtained from all patients from the ward of the index patient and environmental samples. However, the mobility and screening of health care workers within the hospital are not restricted in response to the identification of a VRE-positive clinical sample.

Enterococcal isolates were collected from seven hospital departments and wards throughout the years 2013 ($n=8$) and 2014 ($n=35$) and isolated from various clinical sources, such as blood ($n=6$), urine ($n=10$), umbilical vein catheter ($n=2$), and vagina ($n=1$), as well as from feces ($n=27$) (Table 1).

Samples were initially enriched by Kanamycin esculin azide broth and incubated under aerobic conditions at 37°C for 24 hr. Samples were then plated onto three different media: Kanamycin esculin azide agar, kanamycin esculin azide agar (with 5 mg vancomycin discs) and blood agar and incubated under aerobic conditions at 35°C for 24 hr. Plates were screened for typical white/gray colonies surrounded by black zones, whereby Gram-positive, catalase-negative cocci that hydrolyzed esculin were further selected. The selected colonies were further characterized and tested for antimicrobial susceptibility using VITEK automated identification systems (VITEK-Compact 2; BioMérieux). For the purpose of this study, isolates were additionally confirmed to species level using matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS).

WGS and bioinformatics analysis

In total 19 *E. faecium* isolates were selected for WGS (Table 1). The selection included at least one isolate from each ward. To cover the complete study period, isolates were selected based on isolation date. One representative isolate was randomly chosen when multiple isolates from the same date and ward were present.

Genomic DNA was isolated from overnight cultures in BHI broth using the Wizard Genomic DNA Purification Kit (Promega). Library preparation for sequencing was performed using the Nextera XT Kit and 250 nucleotide paired-end sequencing by Illumina (Miseq). Raw reads were trimmed, assembled into contigs using SPades (vs. 3.6.2) and annotated using PROKKA (vs. 1.11[16]) as previously described.[17] The Illumina MiSeq reads of these 19 *E. faecium* isolates have been deposited in the following European Nucleotide Archive (ENA) public project: PRJEB30640.

To determine the genetic relatedness of isolates, the contigs were analyzed with the *E. faecium* core genome MLST (cgMLST) scheme, which includes 1423 core genes and the 7 genes MLST retrieved based on *in silico* analysis using Ridom SeqSphere+v3.5.0.[18,19] To assign sequence clusters (SCs), we used the threshold criteria from de Been *et al.* that is, strains with ≤20 allele differences that are considered clonally related and most likely belong to a single outbreak, are grouped in SC. Isolates that differ by 21–40 alleles possibly belong to the same outbreak and isolates that differ >40 alleles are most likely unrelated. The cgMLST profiles were clustered with a reference set of 73 *E. faecium* strains as described by Lebreton *et al.*[20] from different sources, isolation time, and countries. A neighbor-joining tree was generated using microreact.*

The identification of resistance genes was performed using ResFinder software[21] and the vancomycin gene clusters were retrieved from the annotated genome. To predict whether the identified resistance genes are plasmid or chromosomally located we used the mlplasmids software.[17] Plasmid containing contigs were aligned using ClustalW (Geneious 8.1.2). The results of the alignment were confirmed by read mapping of all strains against the reference contig (Geneious 8.1.2).

Determination of vancomycin resistance genes

Detection of the *vanA, vanB,* and/or *vanC1* genes was performed by polymerase chain reaction (PCR) for strains that were not subjected to WGS as described previously.[22]

Results

The 43 enterococci isolates were represented by 42 *E. faecium* and 1 *Enterococcus gallinarum*; all were resistant to the majority of tested antibiotics, including vancomycin (Table 1). For the 19 isolates that were subjected to WGS, the presence of the vancomycin resistance gene was determined from the genome sequence using resfinder (see below). For the remaining 24 isolates, PCR revealed that the 23 *E. faecium* isolates contained the *vanA* gene and the single *E. gallinarum* isolate the *vanC1* gene.

Genetic relatedness of E. faecium *isolates*

cgMLST analysis revealed three SCs containing isolates that differ in ≤20 alleles and are therefore clonally related, SC1 (2 isolates), SC2 (16 isolates), and SC3 (1 isolate) (Fig. 1A and Table 1). The two isolates from SC1 (*vanB*-ST78) represented the first VRE isolates (January 2013) from the Tripoli Medical Center and were only found at the medical intensive care unit (Fig. 1B and Table 1). The 16 isolates that grouped in SC2 were all *vanA*-ST80 and recovered from patients from different wards, including the neonatal intensive care unit (NICU), neurosurgery ward (NSW), pediatric oncology (P. ONCO), pediatric surgery ward (PSW), and special care baby unit (SCBU), indicating clonal spread across different wards within a time span of 5 months (Fig. 1B and Table 1).

The first 2 SC2 isolates were recovered from patients in the SCBU and NICU ward, respectively, at the end of November 2013. By the end of April 2014, SC2 isolates were recovered from additional patients in the SCBU ($n=3$) and NICU ($n=6$) ward, respectively. From the beginning of

*https://microreact.org/project/j8OqzluGV.

TABLE 1. CHARACTERISTICS OF ENTEROCOCCAL SP. STRAIN COLLECTION

Strain	MALDI	Isolation site	Isolation date	Ward	SEX	Antibiogram																			van gene (PCR)	Strain subjected to WGS	MLST	van gene (WGS)	No. of contigs		SC	Type of plasmid-predicted contigs encoding antibiotic resistance genes
						CN	AM	CXM	E	LNZ	DA	MOX	IMP	SXT	TIG	TE	TEC	S	Q	F	LEV	P	VA	AML	CIP					Chromosome predicted	Plasmid predicted		
E9712	*Enterococcus faecium*	Blood	1/20/13	MICU	M	R	R	R	R	S	R	R	R	R	S	S	R	R	S	I	R	R	R	R	R		yes	78	vanB	199	76	SC1	2
E9718	*E. faecium*	Catheter tip (urine)	1/22/13	MICU	M	R	R	R	R	S	R	R	R	R	S	S	R	R	S	R	R	R	R	R	R		yes	78	vanB	204	79	SC1	2
E9719	*E. faecium*	Rectal	11/22/13	SCBU	F	R	R	R	R	S	R	R	R	R	S	S	R	R	S	R	R	R	R	R	R		yes	80	vanA	230	89	SC2	1 and 3
E9715	*E. faecium*	Rectal	11/24/13	NICU	M	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R		yes	80	vanA	152	63	SC2	1 and 3
E9717	*E. faecium*	Rectal	11/24/13	NICU	M	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R		yes	80	vanA	191	82	SC2	1 and 3
E9720	*E. faecium*	Rectal	11/24/13	NICU	F	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R	vanA							
E9721	*E. faecium*	Rectal	11/24/13	NICU	M	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R	vanA							
E9734	*E. faecium*	Rectal	2013	NICU		R	R	R	R	S	R	R	R	R	S	R	R	R	S	R	R	R	R	R	R	vanA							
E9723	*E. faecium*	Rectal	1/26/14	NICU	M	R	R	R	R	S	R	R	R	R	S	R	R	R	S	R	R	R	R	R	R		yes	80	vanA	380	129	SC2	NA
E9728	*E. faecium*	UVC	2/4/14	NICU	M	R	R	R	R	S	R	R	R	R	S	R	R	R	S	R	R	R	R	R	R		yes	80	vanA	199	83	SC2	1 and 3
E9701	*E. faecium*	Rectal	2/9/14	SCBU	M	R	R	R	R	S	R	R	R	R	S	S	R	R	S	I	R	R	R	R	R		yes	80	vanA	140	67	SC2	1 and 3
E9702	*E. faecium*	Rectal	2/9/14	SCBU	M	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R	vanA							
E9703	*E. faecium*	Rectal	2/9/14	NICU	F	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R	vanA							
E9704	*E. faecium*	Rectal	2/9/14	SCBU	F	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R	vanA							
E9705	*E. faecium*	Rectal	2/9/14	NICU	F	R	R	R	R	S	R	R	R	R	S	S	R	R	S	I	R	R	R	R	R		yes	80	vanA	156	69	SC2	1 and 3
E9706	*E. faecium*	Rectal	2/9/14	NICU	M	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R	vanA							
E9708	*E. faecium*	Rectal	2/8/14	SCBU	M	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R		yes	80	vanA	177	79	SC2	1 and 3
E9709	*E. faecium*	Rectal	2/9/14	SCBU	M	R	R	R	R	S	R	R	R	R	S	S	R	R	S	I	R	R	R	R	R	vanA							
E9710	*E. faecium*	Rectal	2/9/14	NICU	F	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R	vanA							
E9711	*E. faecium*	Rectal	2/9/14	NICU	F	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R	vanA							
E9713	*E. faecium*	Rectal	2/9/14	SCBU	M	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R	vanA							
E9716	*E. faecium*	Rectal	2/9/14	NICU	M	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R	vanA							
E9727	*E. faecium*	Rectal	2/9/14	NICU	M	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R	vanA							
E9729	*E. faecium*	Rectal	2/9/14	NICU	F	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R	vanA							
E9730	*E. faecium*	Rectal	2/9/14	NICU	F	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R	vanA							
E9731	*E. faecium*	Rectal	2/9/14	NICU	F	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R	vanA							
E9732	*E. faecium*	HVS (vaginal swap)	3/4/14	ANW	F	R	R	R	R	S	R	R	R	R	S	S	R	R	R	R	R	R	R	R	R		yes	80	vanA	176	60	SC3	2
E9722	*E. faecium*	Blood	3/10/14	NICU	M	R	R	R	R	R	R	R	R	R	S	R	R	R	S	R	R	R	R	R	R	vanA							
E9724	*E. faecium*	Blood	3/10/14	PSW	F	R	R	R	R	S	R	R	R	R	S	R	R	R	S	R	R	R	R	R	R		yes	80	vanA	237	81	SC2	1 and 3
E9737	*E. faecium*	Stool	3/10/14	P. ONCO	F	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R		yes	80	vanA	200	72	SC2	1 and 3
E9743	*E. faecium*	Blood	3/10/14	SBCU	M	R	R	R	R	R	R	R	R	R	S	R	R	R	S	R	R	R	R	R	R	vanA							
E9740	*E. faecium*	Blood	3/12/14	NICU	F	R	R	R	R	S	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R		yes	80	vanA	156	69	SC2	1 and 3
E9742	*E. faecium*	Stool	3/12/14	P. ONCO		R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R	vanA							
E9733	*E. faecium*	Catheter tip (urine)	3/15/14	MICU	M	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R	vanA							
E9744	*E. faecium*	Blood	3/29/14	NICU	M	S	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R		yes	80	vanA	197	75	SC2	1
E9739	*E. faecium*	Stool	4/5/14	P. ONCO	F	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R	vanA							
E9736	*E. faecium*	Urine	4/6/14	NSW	M	R	R	R	R	S	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R		yes	80	vanA	181	69	SC2	1 and 3
E9725	*E. faecium*	Stool	4/13/14	P. ONCO	F	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R		yes	80	vanA	224	93	SC2	1-like and 3
E9741	*E. faecium*	Urine	4/14/14	SCBU	M	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R		yes	80	vanA	168	60	SC2	1 and 3
E9738	*E. faecium*	UVC	4/30/14	NICU	F	S	R	R	R	S	R	R	R	R	S	R	R	R	S	R	R	R	R	R	R	vanA							
E9726	*E. faecium*	Urine	5/8/14	NSW	M	R	R	R	R	R	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R	vanA							
E9735	*E. faecium*	Rectal	12/4/14	NICU	M	R	R	R	R	S	R	R	R	R	S	R	R	R	S	R	R	R	R	R	R		yes	80	vanA	187	66	SC2	1 and 3
	Enterococcus gallinarum	Rectal	2/9/14	SCBU	F	R	R	R	R	S	R	R	R	R	S	R	R	R	S	I	R	R	R	R	R	vanC							

ANW, antenatal ward; MICU, medical intensive care unit; NICU, neonatal intensive care unit; NSW, neurosurgery ward; P. ONCO, pediatric oncology; PSW, pediatric surgery ward; SCBU, special care baby unit.

CN, gentamicin high-level synergy; AM, ampicillin; CXM, cefuroxime-axetil; E, erythromycin; AMS, ampicillin/sulbactam; LNZ, linezolid; DA, clindamycin; Mox, moxifloxacin; IMP, imipenem; SXT, trimethoprim/sulfa; TIG, tigecycline; TE, tetracycline; TEC, teicoplanin; S, streptomycin high-level synergy; Q, quinupristin/dalfopristin; CXM, cefuroxime; F, nitrofurantoin; LEV, levofloxacin; P, benzylpenicillin; VA, vancomycin; AML, amoxicillin; CIP, ciprofloxacin.

MALDI, matrix-assisted laser desorption ionization; MLST, multilocus sequence typing; PCR, polymerase chain reaction; SC, sequence cluster; UVC, umbilical vein catheter; WGS, whole genome sequencing.

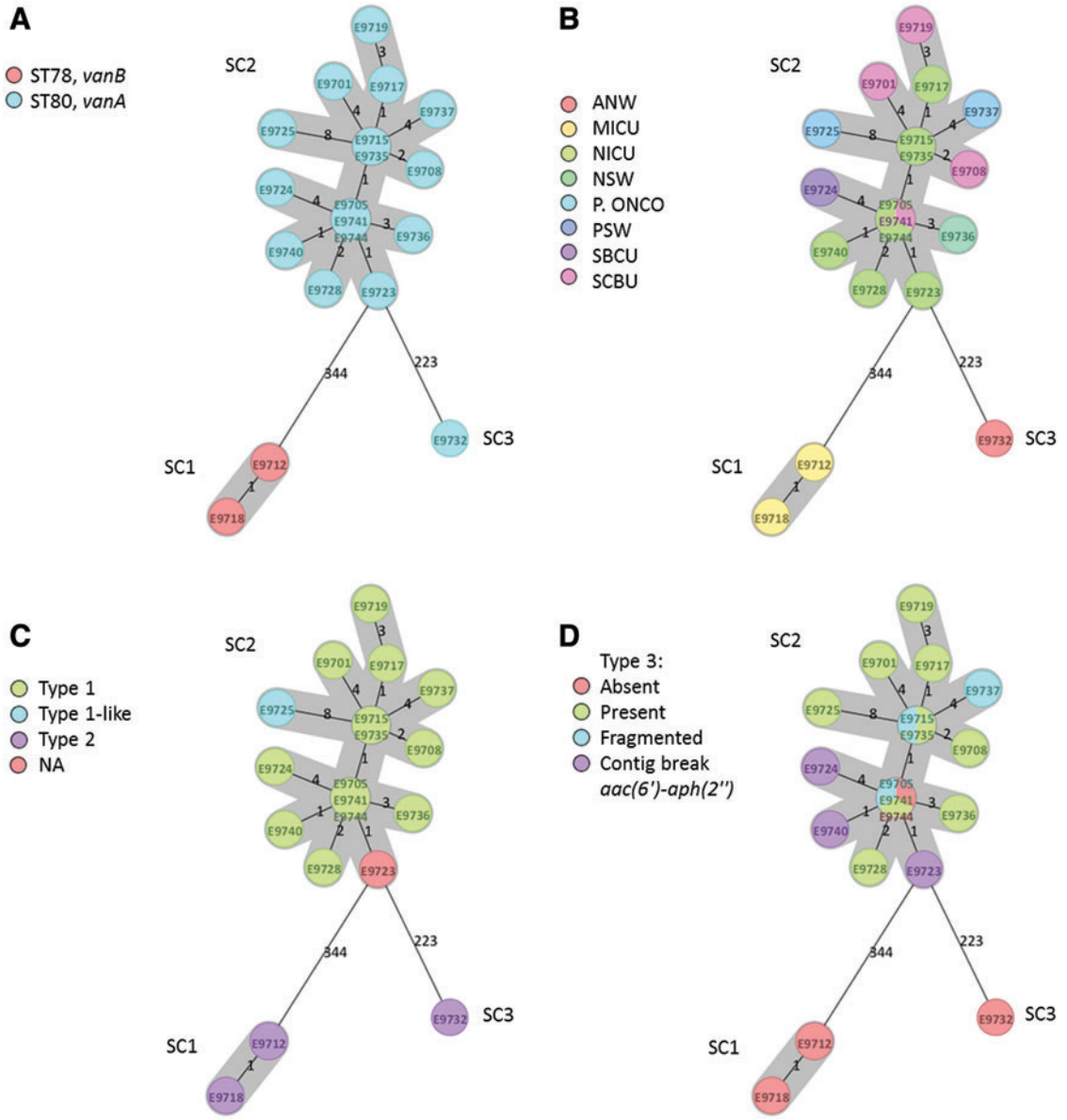

FIG. 1. Minimum spanning tree based on cgMLST profiles, including 1,423 core genes. The distance matrix underlying the network was built from all pairwise allelic profile comparisons, using the pairwise ignoring missing values option in SeqSphere+. Nodes are labeled with the sample ID. The numbers on the connecting lines indicate the number of different alleles between the connected genotypes. **(A–D)** Coloring based on strain characteristic **(A)** MLST and vancomycin resistance gene, **(B)** hospital ward, including ANW, antenatal ward; MICU, medical intensive care unit; NICU, neonatal intensive care unit; NSW, neurosurgery ward; P. ONCO, pediatric oncology; PSW, pediatric surgery ward; SCBU, special care baby unit, **(C)** plasmid-predicted contig 1, **(D)** plasmid-predicted contig 2. cgMLST, core genome multilocus sequence typing. Color images are available online.

March 2014, SC2 isolates were also recovered from the NSW, P. ONCO, and PSW wards, indicating epidemiologically linked spread of the SC2 isolates. Finally, all 23 *vanA* gene containing *E. faecium* strains that were not subject for WGS were recovered on the same wards and in the same time frame, suggesting that they belong to the SC2 group (Table 1).

The single SC3 isolate was also a *vanA*-ST80 but clearly distinct (223 allele difference) from the SC2 isolates. This isolate was recovered from a patient on the antenatal ward.

Finally, we compared the cgMLST profiles obtained from the 19 isolates with a reference set of 73 *E. faecium* isolates from different sources, years, and countries and this revealed that both the ST78 and ST80 isolates from this study clustered among the clade A1 isolates (Supplementary Fig. S1).

Identification of antibiotic resistance genes

Resfinder analysis identified genes encoding for aminoglycoside resistance, including *aac(6′)-aph(2″)* (12/19, 63%), *ant(6)-Ia* (19/19, 100%), *aph(3′)-III* (19/19, 100%), *aph(2″)-If* (1/19, 5%), and *aph(2″)-Ih* (1/19, 5%); glycopeptide resistance, including the *vanA* gene cluster (17/19, 89%) and *vanB* gene cluster (2/19, 11%); macrolide,

lincosamide, and streptogramin B resistance, including *ermB* (18/19, 95%) and *msrC* (19/19, 100%); tetracycline resistance, including *tetL* (16/19, 84%) and *tetM* (2/19, 11%); and trimetroprim resistance, including *dfrG* (17/19, 89%) (Table 2).

According to the mlplasmids prediction only *msrC*, *dfrG*, and the *vanB* gene cluster are chromosomally located and therefore all other resistance genes are plasmid predicted. Analysis of the genomic organization of the chromosomally encoded *vanB* gene cluster in SC1 (ST78) revealed that it was encoded on the integrative conjugative element ICE*Efm1*, encoding for, among other things, the enterococcal surface protein (Esp), which is associated with pathogenicity in *E. faecium*.[23] ICE*Efm1* was not identified among the ST80 isolates that grouped in SC2 and SC3.

Analysis of the plasmid-predicted contigs revealed that the resistance genes *ermB*, *ant(6)-Ia*, and *aph(3′)-III* genes were located on a single plasmid-predicted contig in 14 SC2 isolates, designated type-1 (Figs. 1C and 2). Also in the SC2 isolate E9725 *ant(6)-Ia* and *aph(3′)-III* genes were colocated on a single plasmid-predicted contig, but it lacked the *ermB* gene and was therefore designated type 1-like, while in the SC2 isolate E9723, the three resistance genes were identified, but on multiple contigs and, therefore, this strain was excluded from analysis due to a very fragmented genome (Figs. 1C and 2). A multiple alignment generated on the annotated type-1 contigs from the 15 SC2 isolates revealed a conserved gene synteny (Fig. 2 and Supplementary Fig. S2A), which was confirmed by read-mapping of the reads from all strains against the reference contig E9744_17277 (Supplementary Fig. S2A) (data not shown).

In addition to the antibiotic resistance genes, the type-1 contigs encode for plasmid-specific genes like *repB* involved in plasmid replication, a toxin/antitoxin system known for plasmid maintenance and a resolvase and DNA topoisomerase III involved in DNA replication (Fig. 2 and Supplementary Fig. S2A).The annotated type-1 contigs also revealed the presence of an additional antibiotic resistance gene, *sat4*, encoding a streptothricin acetyltransferase, which was not present in the Resfinder database and located between the *ant(6)-Ia* and *aph(3′)-III* genes (Fig. 2). In some strains a contig break was observed downstream the *birA* gene suggesting insertions of insertion sequence (IS) elements at this site (Supplementary Fig. S2A). BLAST analysis revealed that the region encompassing *ant(6)-Ia-sat4-aph(3′)-III* genes and two genes located upstream of *ant(6)-Ia* gene was identical to transposon Tn*5405*, which was first described in *S. aureus* and coagulase-negative staphylococci.[24,25]

The *ermB, ant(6)-Ia*, and *aph(3′)-III* resistance genes were also identified in the two SC1 and single SC3 isolate, but analysis of the multiple alignment revealed that although a large part of these plasmid-predicted contigs, including the Tn*5405* region, were identical to the SC2-type-1 isolates, the region upstream the *ant(6)-Ia* gene was different, including the localization of *ermB* (Supplementary Fig. S2B). Therefore, these plasmid-predicted contigs were designated type-2 (Figs. 1C and 2 and Supplementary Fig. S2B). Read-mapping of the reads from all strains using contig E9721_19929 (Supplementary Fig. S2B) as reference confirmed these findings (data not shown).

In addition to the type-1 and -2 contigs, a type-3 plasmid-predicted contig was identified in 12 SC2 isolates encoding the aminoglycoside resistance gene *aac(6′)-aph(2″),* although in three strains, E9705, E9715, and E9737 this element was very fragmented (Figs. 1D and 2). In three other SC2 isolates (E9723, E9724, and E9740), *aac(6′)-aph(2″)* was at the border of two different contigs, suggesting the insertion of an *IS*-element (Fig. 1D). In one SC2 isolate, E9744, we were unable to identify the type 3 plasmid-predicted contig, whereas this contig was present in two other isolates, E9705 and E9741 belonging to the same SC. This suggests that either E9744 lost this plasmid contig or E9705 and E9741 acquired this plasmid contig.

A multiple alignment of the type-3 contigs revealed a conserved gene synteny encoding for plasmid-specific genes *repE* and *mob* involved in plasmid replication, genes annotated as Type-1 restriction modification DNA specificity domain-containing protein, a toxin/antitoxin system, and several genes annotated as replication protein (Supplementary Fig. S3). The conserved gene synteny was confirmed by read-mapping of all the reads from the strains using contig E9701_25956 (Supplementary Fig. S3) as reference (data not shown). The type-3 contigs were not identified in the SC1 and SC3 isolates (Fig. 1D).

In 17 isolates, the *vanA* gene cluster was located on small plasmid-predicted contigs. Unfortunately, in 15 of the isolates a contig break was observed downstream *vanS*, a well-known insertion site of an *IS*-element,[26] whereas in 2 isolates (E9719 and E9723) the gene cluster was fragmented in 3 contigs (data not shown).

Discussion

In the current study we performed an in-depth WGS analysis of the first isolated VRE strains from in-patients at the Tripoli Medical Center, Libya. WGS identified a cluster of highly related strains, designated the SC2, on six different hospital wards within a time frame of 1 year (the majority within 4 months) as well as the spread of multiple genetic elements encoding for multiple antibiotic resistance genes, which suggests that these SC2 isolates are part of a single outbreak.

Antimicrobial resistance is a global challenge and emerging in various North African countries, including Libya with concerning reports of MDR pathogens, including MRSA,[27] extended-spectrum beta-lactamase,[28] and colistin-resistant carbapenem-producing Enterobacteriaceae.[29] Over the last 6 years, the first reports of VRE in North Africa were published, including countries like Algeria, Tunisia, and Morocco.[11–15,30] Except for the study from Morocco, MLST and PCR-based determination of antibiotic resistance genes was performed to determine genetic relatedness of isolates. So far, there are no reports on VRE in Libya, except for one case where VRE was cultured from a war casualty with previous hospitalization history, but this strain was not MLST typed or further characterized.[31]

In the current study, MLST revealed two STs, *vanB*-ST78 and *vanA*-ST80. Both STs belong to the hospital-associated clade A1 that are widely spread throughout Europe and Australia.[32,33] In North Africa, ST78 was first identified in 2010 among vancomycin-susceptible strains isolated from Algeria,[30] while *vanA*-type of vancomycin-resistant ST80 (and other STs) isolates were reported from two studies conducted between 2012 and 2013 in Tunisia.[11,13] This indicates that the *E. faecium* global A1 hospital clade is also present in the North African countries of Libya, Algeria, and Tunisia.

Table 2. Distribution of Antibiotic Resistance Genes Indicated as % of Similarity with Genes from the Resfinder Database

		Aminoglycoside					*Glycopeptide*		*Macrolide, lincosamide, streptogramin B*		*Tetracycline*		*Trimethoprim*
		Gm, Tob, Amk aac(6′)-aph(2″) *(p)*	*Sm* ant(6)-Ia *(p)*	*Km, Neo, Amk, GmB* aph(3′)-III *(p)*	*Gm, Km* aph(2″)-If *(p)*	*Gm* aph(2″)-Ih *(p)*	vanA *(p)*	vanB *(c)*	ermB *(p)*	msrC *(c)*	tetL *(p)*	tetM *(p)*	dfrG *(c)*
	E9701	99.93	100	100			99.9		99.86	98.99	99.85		100
	E9705	99.93	100	100			99.9		99.86	98.99	99.85		100
	E9708	99.93	100	100			99.9		99.86	98.99	99.85		100
	E9715	99.93	100	100			99.9		99.86	98.99	99.85		100
	E9717	99.93	100	100			99.9		99.86	98.99	99.85		100
	E9719	99.93	100	100			99.9		99.86	98.99	99.85	96.54	100
	E9725	99.93	100	100			99.9			98.99	99.85	97.53	100
	E9728	99.93	100	100			99.9		99.86	98.99	99.85		100
SC2	E9735	99.93	100	100			99.9		99.86	98.99	99.85		100
	E9736	99.93	100	100			99.9		99.86	98.99	99.85		100
	E9737	99.93	100	100			99.9		99.86	98.99	99.85		100
	E9741	99.93	100	100			99.9		99.86	98.99	99.85		100
	E9724	Interrupted	100	100	82.57		99.9		99.86	98.99	99.85		100
	E9740	Interrupted	100	100			99.9		99.86	98.99	99.85		100
	E9723	Interrupted	100	100		82.99	99.9		99.86	98.99	99.85		100
	E9744		100	100			99.9		99.86	98.99	99.85		100
SC3	E9732		100	99.87			99.9		100	98.99			100
SC1	E9712		100	100				98.93	100	98.99			
	E9718		100	100				98.93	100	98.99			

Source: Zankari et al.[21]
(p): predicted location plasmid; (c) predicted location chromosome.
Amk, amikacin; Gm, gentamicin; GmB, gentamicin B; Km, kanamycin; Neo, neomycin; Sm, streptomycin; Tob, tobramycin.

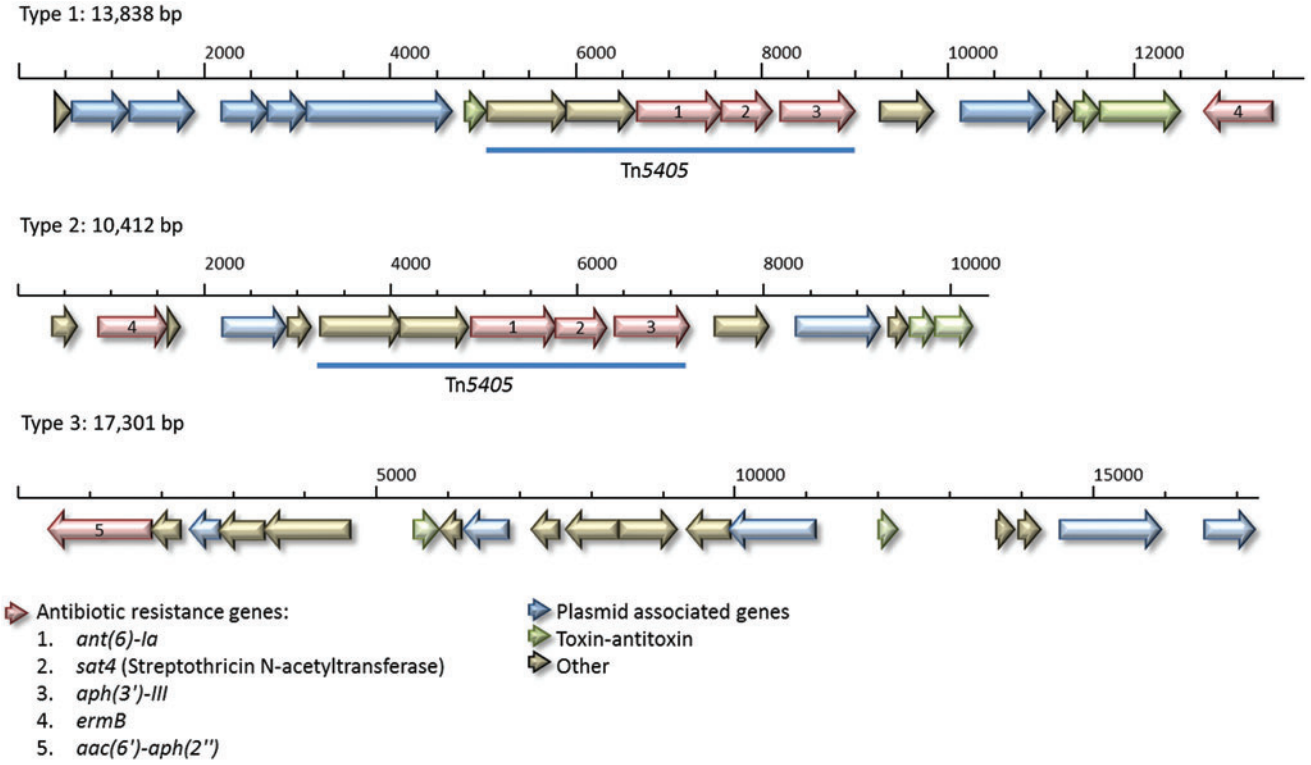

FIG. 2. Genomic organization of three different types of plasmid-predicted contigs encoding antibiotic resistance genes. Color images are available online.

In the present study, cgMLST was performed, which involves the analysis of 1423 genes and revealed that ST80 isolates could be subdivided into two SCs, which differed by 223 alleles. Furthermore, using mlplasmid, we determined colocations of at least four resistance genes, *ermB*, *ant(6)-Ia, sat4,* and *aph(3′)-III*, on a single plasmid-predicted contig. The latter three resistance genes compose, together with the two upstream-located genes, transposon Tn*5405* in *S. aureus*. This transposon is also found on plasmid pJH1 from *E. faecalis*.[24,25] In 2001, Werner *et al.* reported the presence of Tn*5405* in *E. faecium* strains from different sources, including poultry, pig, human, and hospitalized patients.[34] In 2003, the same group identified a genetic element in *E. faecium* encoding Tn*5405* and *ermB,* but observed different genomic organizations of this element, including one configuration with Tn*1546* encoding the *vanA* gene cluster upstream of *ermB*.[35]

The genomic organizations of these elements are similar to the type-1 and -3 plasmid-predicted contigs from the current study. A limitation of short read sequencing is that it cannot be used to reconstruct individual plasmids, while they often contain repetitive elements like IS, which cannot be overspanned by short reads. In this study, we identified the *vanA* gene cluster on different small contigs, which suggests the presence of IS elements. Therefore, using short read sequencing alone, we were not able to link the plasmid-predicted localization of this gene cluster to either type-1 or -3.

The type-2 plasmid-predicted contig was only identified in the *vanB*-ST78 isolates from early 2013. A similar type-2 was found in the single *vanA*-ST80-SC3 isolate that was isolated more than a year later, suggesting transmission of this element and persistence of this type within the hospital.

The limitation of the current study is the lack of systematic collection of strains and epidemiological information on antimicrobial-resistant bacteria as reported in other studies from Libya.[5] Although the enterococci investigated in this study dated back from 2013 to 2014, the results of this study support the ongoing concern about the spread of antimicrobial resistance in Libya and raise further concerns about VREs within the health care system as well as the community. There is an urgent need for strict control measures and in the Libyan health care system through screening both at admission and patients at wards with a high risk of acquiring VRE. Screening can significantly control and minimize the spread of VRE and other nosocomial pathogens within the health care system. National policy guidelines for antimicrobial stewardship and advanced surveillance screening systems involving health care settings for the community and animals are urgently required.

In conclusion, to our knowledge, this study is the first application of WGS to study the epidemiology of VRE in the North African region. VRE from the Libyan health care system are genetically related to the hospital associated clade A1 isolates known from all over Europe. Further WGS-based studies are needed to study interhospital transmission in the north African region. This study was based on isolates from 2013 to 2014 and the results indicate that further surveillance efforts using molecular typing methods to monitor the spread of MDR bacteria in the Libyan health care system are urgently needed.

Disclosure Statement

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3

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Address correspondence to:
Janetta Top, PhD
Department of Medical Microbiology
UMCU
P.O. Box 85500
Utrecht 3508 GA
The Netherlands

E-mail: j.top@umcutrecht.nl