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RESEARCH ARTICLE

VANCOMYCIN-RESISTANT ENTEROCOCCI SEQUENCE TYPES PREVALENT IN HOSPITALS IN TRINIDAD AND TOBAGO

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ABSTRACT

Vancomycin-Resistant Enterococci (VRE) infections are serious healthcare problem worldwide. VRE sequence types prevalent in hospitals in Trinidad & Tobago were delineated after analyzing 45 VRE isolates (*E. faecium* n=38; *E. faecalis* n=7) by multiplex polymerase chain reaction (PCR), pulsed-field gel electrophoresis (PFGE) and multilocus sequence types techniques. All isolates possessed *esp* and *van*A and B genes but no *hyl* genes. PFGE revealed five clones among the *E. faecium* and one clone among the *E. faecalis* suggesting that there has been a horizontal spread or intra/inter hospital spread of the clones at various geographic locations in the country. Sequence types - ST412 (69%), ST736 (23%) and ST203 (8%) were observed. The predominant VRE sequence type in the country is ST412 and belong to clonal complex 17 (CC17) similar to ones observed in the Americas. Since these are associated with nosocomial infections outbreaks, continuous surveillance and infection control measures must remain a priority in hospitals and health care facilities in Trinidad and Tobago in order to prevent any potential of outbreaks by this VRE clonal complex.

INTRODUCTION

Enterococci are established opportunistic pathogens of the normal intestinal microbiota of humans and animals (Top *et al*, 2008). Most Enterococcal infections in hospitals are due to *Enterococcus faecalis* and *Enterococcus faecium* species. Both species are commonly isolated from patients with bacteremia, surgical site infections, urinary tract infections and device related infections (Agudelo *et al*, 2014). Enterococci poses problems to healthcare practitioners worldwide due to their increasing trend of antimicrobial resistance and great adaptability in hospital environments (Top *et al*, 2008).

In recent years, the emergence of multidrug resistant *E. faecium* and *E. faecalis* have increased (Howden *et al*, 2013). The first reported VRE isolates was in Great Britain in 1988 and shortly afterwards they were reported in other European countries and the USA (Uttley *et al*, 1988; Werner *et al*, 2008; Frieden *et al*, 1993). In Latin America, VRE has been reported in Argentina, Brazil, Columbia (Corso *et al* 2007; Panesso *et al*, 2010). Although incidence and prevalence rates of VRE in hospitals in Trinidad and Tobago are not routinely collected, and molecular epidemiology including carriage rates of VRE isolates and infection have been reported (Kissoon *et al*, 2016; Akpaka(a) *et al*, 2016; Akpaka (b) *et al*, 2016) but the sequence types of such isolates have never been described in the country.

Multilocus sequence typing (MLST) has revealed the existence of host-specific geno groups including a specific genetic lineage designated clonal complex 17 (CC17), of *E. faecium* and clonal complex 2 (CC2) of *E. faecalis* associated with hospital-related isolates (Homan *et al*, 2002; Freitas *et al*, 2009). MLST of *E. faecium* and *E. faecalis* is based on identifying alleles from DNA sequences in internal fragments of housekeeping genes. The genes for *E. faecium* (*atpA*, *ddl*, *gdh*, *purK*, *gyd*, *pstS* and *adK*) and *E. faecalis* (*gdh*, *gyd*, *pstS*, *gki*, *arOE*, *xpt*, *yqiL*) are different resulting in a numeric allelic profile with each profile then being assigned a sequence type (ST) (Homan *et al*, 2002; Ruiz-Garbajosa *et al*, 2006)

It has been reported that CC17 is characterized by ampicillin and glycopeptide resistance as well as the presence of a putative pathogenicity island that include *esp* and/or *hyl* genes in the majority of isolates (Willems *et al* 2005; Rice *et al* 2003). These determinants may allow the CC17 to thrive and disseminate in the hospital environment (Rice *et al* 2003).Various STs that belong to clonal complex 17, such as ST412, ST203, ST18 and ST736, have been disseminated worldwide (Wang *et al* , 2014). Likewise, the clonal complex 2 (CC2) and in ST6 described among *E. faecalis* nosocomial isolates have also been disseminated among hospital environments (Freitas *et al*, 2009).

The aim of this study was to perform multilocus sequence type characterization of VRE clinical isolates from tertiary care hospitals in Trinidad and Tobago in order to determine the prevalent sequence types and compare them with others reported elsewhere.

MATERIALS AND METHODS

Bacterial Isolates

Non-duplicate clinical VRE isolates (*E. faecium* n=38; *E. faecalis* n=7) used for this analysis were ones from our previous reports (Kissoon *et al*, 2016; Akpaka (a) *et al*, 2016; Akpaka (b) *et al*, 2016) that were collected during the period March 2010 to February 2012. These were isolates from patients treated in all public hospitals (designated as "H1, H2, H3, H4 and H5") in Trinidad and Tobago. The initial analysis of the bacterial isolates were carried out at the Dept. of Paraclinical Sciences of the University of the West Indies, St. Augustine Campus; Trinidad & Tobago. The study was approved by the Ethics Committee of the University Campus at St. Augustine.

Molecular Analysis

These isolates were subjected to molecular analysis including - multiplex polymerase chain reaction (PCR) and pulsed-field gel electrophoresis (PFGE) to determine the resistant genes, virulent factors and their clonal relatedness to each other as we previously reported (Kissoon *et al*, 2016; Akpaka (a) *et al*, 2016; Akpaka (b) *et al*, 2016).

Multi locus Sequence Typing (MLST)

Based on similar DNA pattern or PFGE profiles, MLST was performed for 16 isolates (*E. faecium* n=13 and *E. faecalis* n=3) to determine their sequence types using methods previously described by Homan *et al*, (2002). Primers of seven housekeeping genes used are shown in Table 1. For the *E. faecalis* isolates, the sequencing analysis was performed according to procedures established by Ruiz-Garbajosa *et al*, (2006).

Table 1 List of *E.faecium* primers used in the study

Housekeeping genes	Primer Sequences (5' - 3')
adk	Forward GAACCTCATTTTAATGGGG
	Reverse TGATGTTGATAGCCAGACG
atpA	Forward CGG TTC ATA CGG AAT GGC ACA
	Reverse AAG TTC ACG ATA AGC CAC GG
ddl	Forward GAG ACA TTG AAT ATG CCT TAT G
	Reverse AAA AAG AAA TCG CAC CG
gyd	Forward CAA ACT GCT TAG CTC CAA GG C
	Reverse CAT TTC GTT GTC ATA CCA AGC
gdh	Forward GGC GCA CTA AAA GAT ATG GT
	Reverse CCA AGA TTG GGC AAC TTC GTC CCA
purK	Forward CAGATTGGCACATTGAAAG
	Reverse TTCATTCACATATAGCCCG
pstS	Forward TTG AGC CAA GTC GAA GCT GGA
	Reverse CGT GAT CAC GTT CTA CTT CC

dk:adenylate kinase; *atpA*: ATP synthase, alpha subunit; *ddl*: D-alanine: D-alanine

PCR amplicons were sequenced using a BigDye Terminator v1.1 cycle sequencing kit (Life Technology, CA, USA) on an ABI 3500xl genetic Analyzer. Sequences were then queried into the MLST databases, that is (http://efaecium.mlst.net/) and (http://efaecalis.mlst.net/) to determine their sequence types (STs). Their genetic relatedness was explored using goeburst (Francisco *et al*, 2009)

RESULTS

Vancomycin-resistant E. faecium isolates

All analyzed vancomycin-resistant *E. faecium* isolates (n=38) possessed the *vanA* genes. Overall the *esp* gene was detected in all isolates while *hyl* genes was not detected in any isolate. The analysis demonstrated five PFGE pattern (Figure 1) among the vancomycin-resistant *E. faecium* isolates. The 38 *E. faecium* isolates from the PFGE analysis showed a heterogeneous pattern associated with a profile of multidrug resistance to different antibiotics and the presence of the *vanA* gene.

Table 2 Genotypic Characteristics of Vancomycin Resistant *Enterococcus faecium* isolates from Trinidad & Tobago hospitals based on multi-locus sequence typing (MLST).

Area	N	ST	Allelic profiles						
			atpA	ddl	gdh	purK	gyd	pstS	adk
Trinidad	9	412	15	1	1	44	1	20	1
	3	736	1	3	1	44	1	1	1
	1	203	15	1	1	1	1	20	1
Tobago	1	412	15	1	1	44	1	20	1

N = number of strains tested; ST = Sequence types; *Adk* = adenylate kinase; *atpA* = ATP synthase, alpha subunit; *ddl* = D-alanine: D-alanine ligase; *gyd* = glyceraldehyde-3-phosphate dehydrogenase; *gdh* = glucose-6-phosphate dehydrogenase; *pur*K = phosphoribosyl aminoimidazole carboxylase ATPase subunit; *pst*S = phosphate ATP-binding cassette transporter.

Table 3 Genotypic Characteristics of Vancomycin Resistant *Enterococcus faecalis* isolates from Trinidad & Tobago hospitals based on multi-locus sequence typing (MLST)

Area	N	ST	Allelic profiles						
			gdh	gyd	pstS	gki	aroE	xpt	yiqL
Trinidad	3	6	12	7	3	7	6	1	5

N = number tested; ST = Sequence types; *gdh* = glucose-6-phosphate dehydrogenase; *gyd* = glyceraldehyde-3-phosphate dehydrogenase; *pst*S = phosphate ATP-binding cassette transporter, **gki** = putative glucokinase, **aroE** (shikimate 5-dehydrogenase), **xpt** (shikimate 5-dehydrogenase), and **yiqL** = acetyl-coenzyme A acetyltransferase.

Dendogram of Enterococcus faecium isolates from Trinidad and Tobago

The predominant clones were one and three (PFGE-1 and PFGE-3); and these clones occurred in more than half (58%) of the isolates. Clone one was present in two hospitals "H4" and "H3", and these hospitals are located in the southern and northern geographic areas of the country respectively. Clone three was present in four of five hospitals, "H1", "H3", "H4" and "H5" in the country. Clone two and five (PFGE-2 and PFGE-5) was represented by six (16%) and eight (21%) isolates respectively from two hospitals "H3" and "H4". Clone

four (PFGE-4) had two isolates, one from "H3" and the other from "H1" hospitals and both were from the urogenital tract (UGT).

Full MLST of VRE isolates from Trinidad and Tobago

The MLST analysis results are summarized in Tables 2 and 3 and reveals three main sequence types encountered among the *E. faecium* isolates from all hospitals in this twin islands

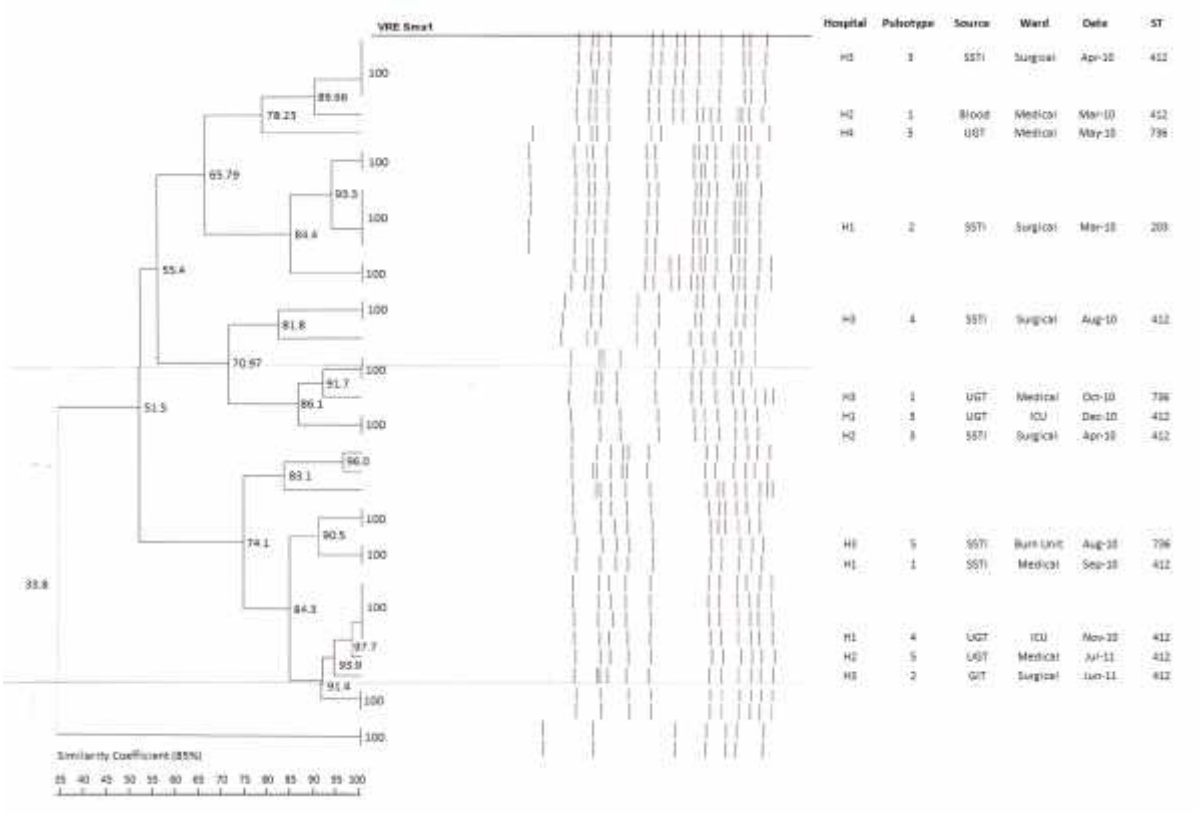

Figure 1 Dendogram of *Enterococcus faecium* isolates from Trinidad and Tobagog predominant PFGE 1 and 3

Vancomycin-resistant E. faecalis isolates

The vancomycin-resistant *E. faecalis* isolates (n=7) possessed the *vanB* and *esp* genes, no *hyl* gene and had an identical PFGE pattern indicating they belong to the same clone (Figure 2). The cluster analysis was also achieved by the Bionumerics software (Applied Maths, Austin TX, USA). Percentages of similarity determined using the DICE correlation coefficient and dendograms (Figures 1 and 2) were produced via the unweighted pair group method with arithmetic mean clustering (UPGMA). All these *E. faecalis* isolates belong to the ST6 clone

Dendogram of Enterococcus faecalis isolates from Trinidad and Tobago

These sequence types are ST412 69% (9/13); ST736, 23% (3/13) and ST203, 8% (1/13). The ST412 was observed in isolates from hospitals H1, H2, H3 and H4; ST736 was seen in isolates from hospital H3 and H4; while ST203 was only seen in hospital H1. The ST203 is a single-locus variant of ST412 and ST736, a double variant of ST412. Thus the MLST data suggest that all *E. faecium* isolates in this study belong to one clonal complex, CC17 (Figure 3).

DISCUSSION

This study represent to the best of our knowledge the first time sequence typing of VRE isolates from Trinidad and Tobago

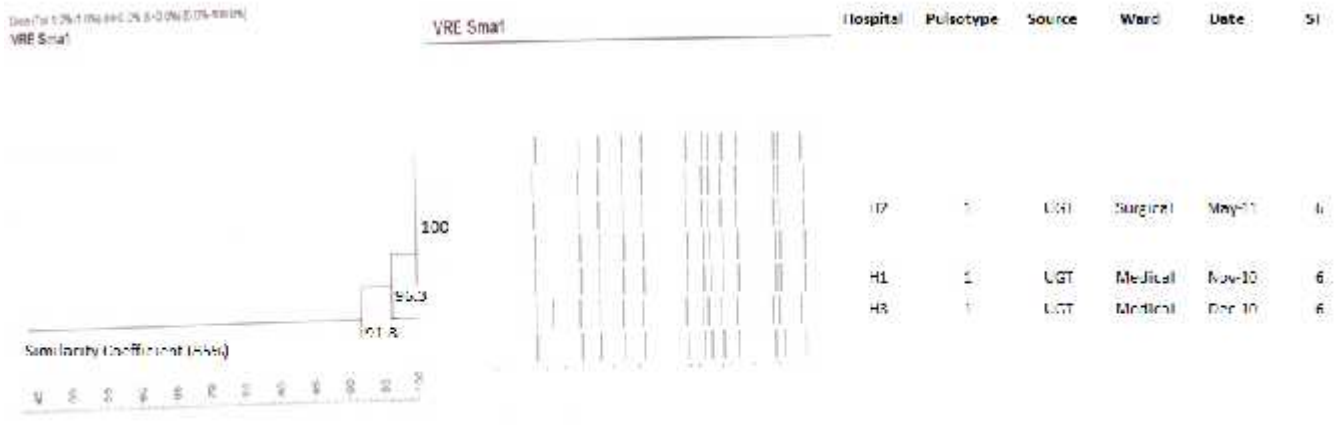

Figure 2 Dendogram of *Enterococcus faecalis* isolates from Trinidad and Tobago showing a single PFGE type

This analysis provides a baseline picture of the prevailing enterococci sequence types which will lay the foundation for future studies in this field in the country.

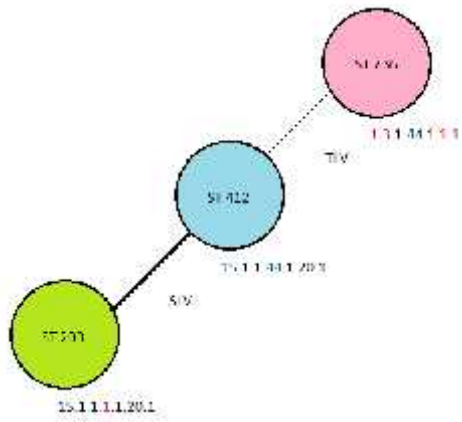

Figure 3 Multilocus sequence tpes results of VRE isolates from Trinidad and Tobago where darker links represent less allelic differences than lighter links. ST203 is a single-locus variant (SLV) of ST412 and ST736, a double variant of ST412 as indicated by the single and double thick lines.

Published reports have shown that PFGE and MLST analyses are effective methods for the molecular characterization of VRE clinical isolates (Kuriyama T *et al*, 2003 and Weng PL *et al*, 2013).

The presence of a dominant vancomycin resistant *E. faecium* clone (Clone 1 and 3) in major public hospitals in Trinidad & Tobago shows that their spread has occurred not only within individual hospitals but also between hospitals at various geographic locations in the country. This is in agreement with other studies that have documented the spread of vancomycin resistant *E. faecium* and *E. faecalis* among hospitals (Del Campo *et al*, 2001). The spread of same clones in different institutions in the country may suggest that some strains possesses bacterial factors that enhance their spread within hospitals. Similar to our results that detected esp gene in all VRE isolates encountered in our analysis, other researchers have identified the *esp* gene encoding a surface protein associated with virulence for *E. faecium* and *E. faecalis* residing on a pathogenicity island (Agudelo *et al* 2014; Homan *et al* 2002; Ruiz-Garbajosa *et al*, 2006).

The most prevalent sequence types of VRE in hospitals in Trinidad and Tobago is ST412. This was identified in various wards of all major hospitals, while ST203 was found in only hospital "H1". This finding or result is similar to those by Panesso *et al*; which showed that ST412 was the most frequent ST among VREs isolated in 4 countries in South America, indicating the presence of the strain associated with the hospital environment (Panesso D *et al*, 2010). Also our results were similar to those from Mexico, Canada, Greece and France (Ochoa *et al*, 2013; McCracken *et al*, 2013; Damani *et al*, 2010; Bourdon *et al*, 2011).

The ST412 clone carries the type 44 allele of the *purK* gene, which has been reported to be related to the epidemic vancomycin resistant *E. faecium* strains (Willems *et al*, 2005). Significantly, there has never been any history or report of VRE outbreaks in hospitals in Trinidad & Tobago before. This ST412 including other types (ST203 and ST736) seen in VRE isolates from hospitals in the country have been associated or linked with nosocomial outbreaks in other places (Ruiz-Garbajosa *et al*, 2006), but this has not been the case in Trinidad and Tobago. Delineation of ST412 clone among the hospitals located in different areas of the country indicates a horizontal transmission. This emphasizes the need for the application of strict infection control measures such as the isolation of infected patients, increased environmental cleaning, improved hand hygiene and better antibiotic policy.

One hospital located in the northern area of Trinidad Island and designated as "HI", had a unique clone ST203 which was not found in other hospitals. This ST203 has been described in other studies in USA, Canada, and South America (Galloway-Peña *et al*, 2009; McCracken *et al* 2013; Panesso D *et al*, 2010). The ST412 and ST203 belong to the clonal complex 117 (CC17) with the purK1 allele. The clonal complex 17 has been resolved into two different subgroups, one of which harbors ST17 and ST18, while the second harbors ST78. ST17, ST18 and ST203 are the major groups in the genetic linkage of *E. faecium*; they are dispersed worldwide and have been associated with outbreaks (Leavis *et al*, 2006: Ruiz-Garbajosa *et al*, 2006).

On the other hand, the MLST analysis of three selected vancomycin-resistant *E. faecalis* isolates revealed one sequence type, ST6. The presence of ST6 is associated with clonal complex 2 (CC2). CC2 is commonly reported among nosocomial isolates and represents hospital adapted complexes [16]. Our result was similar to what was found or reported in Canada (McCracken *et al*, 2013) and Portugal (Freitas *et al*, 2009).

In our study ST6 was resistant to multiply antibiotics such as ciprofloxacin, gentamycin and erythromycin and possessed the putative virulence *esp* gene. This was similar to other results by Freitas *et al* (2009). Studies elsewhere have confirmed that the VRE isolates like the ones we encountered in our analysis (ST6, 203, 412) belong to CC17 and CC2, clonal groups associated with worldwide nosocomial infections. Despite the fact that these clonal complexes are associated with worldwide nosocomial infections, there has never been any report of VRE outbreaks in Trinidad and Tobago.

The PFGE has been previously considered as the "gold standard" for the study of hospital outbreaks because of its high degree of isolate differentiation (Kuhn *et al*, 1995), however, MLST has emerged as an important tool to study the long term epidemiology and the population structure and patterns of evolutionary descent (Feil *et al*, 2004). From the PFGE typing investigation, we found that VRE isolates with PFGE patterns designated as "A1" and "A2" were clonally related (100%) with similar DNA banding patterns, but were of different ST types. Similar results have been reported by Weng *et al* in which a difference was noted in ST but PFGE patterns were similar (Weng *et al*, 2013). These differences have been attributed to changes in the nucleotides of the house keeping genes analyzed. Also the possibility of having the *esp* gene may contribute to the increased conjugation frequency which could lead to changes in nucleotides of the MLST genes and that can give rise to a new ST, which could not be detected by PFGE.

CONCLUSION

The small number of VRE isolates analyzed in this study is a major limitation and may be insufficient to generalize conclusions about the several sequence types in Trinidad &Tobago. Nevertheless, these data constitute an important contribution to the genetic knowledge and characteristics of VRE isolates from Trinidad & Tobago. Our study still delineated VRE sequence types from the country to mainly belong to ST412, and to a lesser extent ST736 and ST203. These sequence types characterized as CC17 and CC2, associated with nosocomial outbreaks are similar to ones also observed in the region. Therefore, continuous surveillance and infection control measures must remain an important practice in hospitals and health facilities in Trinidad & Tobago in order not trigger the outbreaks of these complexes.

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