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Emergence of *vanA Enterococcus faecium* in Denmark, 2005–15

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Objectives: To describe the changing epidemiology of vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis* in clinical samples in Denmark 2005–15 according to species and *van* type, and, furthermore, to investigate the genetic relatedness of the clinical *E. faecium* isolates from 2015.

Methods: During 2005–14, all clinical VRE isolates were tested for the presence of *vanA/B/C* genes by PCR. In 2015, all clinical VRE isolates were whole-genome sequenced. From the WGS data, the presence of *van* genes and MLST STs were extracted *in silico*. Core-genome MLST (cgMLST) analysis was performed for the vancomycin-resistant *E. faecium* isolates.

Results: During 2005–15, 1043 *vanA E. faecium*, 25 *vanB E. faecium*, 4 *vanA E. faecalis* and 28 *vanB E. faecalis* were detected. The number of VRE was <50 isolates/year until 2012 to >200 isolates/year in 2013–15. In 2015, 368 *vanA E. faecium* and 1 *vanB E. faecium* were detected along with 1 *vanA E. faecalis* and 1 *vanB E. faecalis*. cgMLST subdivided the 368 *vanA E. faecium* isolates into 33 cluster types (CTs), whereas the *vanB E. faecium* isolate belonged to a different CT. ST203-CT859 was most prevalent (51%), followed by ST80-CT14 (22%), ST117-CT24 (6%), ST80-CT866 (4%) and ST80-CT860 (2%). Comparison with the cgMLST.org database, previous studies and personal communications with neighbouring countries revealed that the novel cluster ST203-CT859 emerged in December 2014 and spread to the south of Sweden and the Faroe Islands during 2015.

Conclusions: VRE increased in Denmark during 2005–15 due to the emergence of several *vanA E. faecium* clones.

Introduction

Enterococcus faecalis and *Enterococcus faecium* can cause community-acquired and nosocomial infections (e.g. urinary tract infections, intra-abdominal infections, bloodstream infections and endocarditis).

During recent decades, an increase in the occurrence of VRE has been observed in Europe with *E. faecium* being the most dominant species.[1,2] Resistance to vancomycin can be encoded by several gene clusters (*vanA/B/D/E/G/L/M/N*). In Europe, the *vanA* and, to a lesser extent, *vanB* genotypes are most prevalent among clinical VRE isolates.[1,3]

The molecular epidemiology of *E. faecium* isolates has been investigated by several typing methods. For many years, the most commonly used method for the investigation of enterococcal clonality in relation to outbreak situations was PFGE analysis using SmaI as the restriction enzyme.[4] For investigation of population structures and global bacterial epidemiology, MLST has been the gold standard. MLST analysis has previously shown that the

majority of the *E. faecium* isolates causing infections in patients and hospital outbreaks belong to a subpopulation previously called clonal complex 17 (CC17).[5] These are genetically very different from commensal *E. faecium* isolates from healthy humans and *E. faecium* isolates from farm animals.[6] In addition to MLST, WGS can be used for epidemiological studies and to compare population structures of *E. faecium*. Based on WGS data, the analysis of SNPs is now widely used for epidemiological studies.[4,7,8] Inferring the relationship between isolates based on SNPs generally uses variation within the conserved core genome of any given collection of WGS data. Thus, comparisons of isolates analysed at different times require re-analyses. This restricts proper nomenclature and reflects the obtained resolution of the dataset applied. To overcome this limitation, de Been *et al.*[9] developed a core-genome MLST (cgMLST) scheme for *E. faecium* based on 1423 target genes.

The aim of the study was to describe the occurrence of the *vanA* and *vanB* gene clusters among clinical *E. faecium* and *E. faecalis* in Denmark from 2005 to 2015 and to apply MLST and cgMLST to infer the genetic relatedness of *E. faecium* to understand the huge increase in *vanA E. faecium* in Denmark.

Materials and methods

National surveillance of vancomycin-resistant E. faecium and E. faecalis

Since 2005, Danish departments of clinical microbiology (DCMs) located in the five Danish regions (Figure 1a and b) have submitted VRE for surveillance to the Antimicrobial Resistance Reference Laboratory at Statens Serum Institut (SSI). During 2005–15, 1100 VRE isolates were detected at Danish hospitals and submitted to the SSI. The present study only includes vancomycin-resistant *E. faecium* and *E. faecalis* isolates from clinical samples (e.g. blood, tissue and urine) as opposed to screening (faecal) isolates. Only one isolate per patient/per year was included. From 2005 to 2014, all VRE isolates (699 *E. faecium* and 30 *E. faecalis*) received at the Danish reference laboratory were tested for the presence of vancomycin resistance genes *vanA/B/C* by PCR according to Dutka-Malen *et al.*[10]

In 2015, 371 clinical VRE isolates from blood ($n=30$), urine ($n=250$) and other infection sites ($n=91$) originating from 10 of 11 DCMs (representing 95% of the Danish population) were whole-genome sequenced.

WGS and assembly

Of the 371 VRE isolates from 2015, 313 isolates were whole-genome sequenced at SSI, whereas 58 isolates from hospitals belonging to the clinical department at Hvidovre Hospital were whole-genome sequenced at their local facility.

Genomic DNA was extracted using a DNeasy Blood & Tissue Kit (QIAGEN, Copenhagen, Denmark) and fragment libraries were constructed using the Nextera Kit (Illumina, Little Chesterford, UK) followed by 251 bp paired-end sequencing at the SSI (MiSeq; Illumina) or 150 bp paired-end sequencing at Hvidovre Hospital (MiSeq; Illumina) according to the manufacturer's instructions. WGS data were assembled using CLCbio's Genomics Workbench v8.0 (QIAGEN, Aarhus, Denmark) using default settings to include only contigs >500 bp.

The ResFinder web server (http://www.genomicepidemiology.org) was used to identify *van* genes in the assembled genome data, using a threshold of 90% minimum sequence identity and 60% minimum length identity cut-off.

MLST was inferred from the WGS using the MLST web server (Version 1.7; http://www.genomicepidemiology.org).

The implementation of the cgMLST scheme by de Been *et al.*[9] in SeqSphere+ v3.4.0 (Ridom GmbH, Münster, Germany; http://www.ridom.de/seqsphere/) was used for further subtyping of the vancomycin-resistant *E. faecium* isolates from 2015. New cluster types (CTs) identified as part of this study were submitted to the cgMLST database through the SeqSphere+ v3.4.0 software suite.

Results and discussion

VRE prevalence over time in the Danish regions

During 2005–15, 1043 *vanA E. faecium*, 25 *vanB E. faecium*, 4 *vanA E. faecalis* and 28 *vanB E. faecalis* were detected at Danish hospitals and submitted to the SSI (Figure 1a). Until 2012, the number of VRE isolated from clinical samples was <50/year, but this number rapidly increased hereafter (Figure 1a and b). From 2009 to 2013, *vanA E. faecium* increased specifically at Aarhus University Hospital located in the Central Denmark Region (Figure 1b). From 2012, an increase in *vanA E. faecium* isolates was observed, primarily at hospitals in the Capital Region, but also from hospitals within the neighbouring Zealand Region and from the Central Denmark Region (Figure 1a and b).

Epidemiology during 2005–14

Subsets of the vancomycin-resistant *E. faecium* isolates from 2005 to 2014 have been characterized in previous studies.[4,8,11,12] During 2005–09, a small outbreak of ST203 *vanB E. faecium* and two ST18 *vanA E. faecium* outbreaks were detected in the Capital Region.[11] From 2009 to 2013, *vanA E. faecium* increased at Aarhus University Hospital; the spread was due to multiple hospital outbreaks, including *E. faecium* isolates belonging to ST18, ST117, ST192, ST203 and ST260 (Table 1).[12]

Pinholt *et al.*[4] investigated *vanA E. faecium* isolates from the Danish hospitals from January 2012 to April 2013. The spread of several *vanA* clones was detected both within and between hospitals. The *vanA E. faecium* clones most often belonged to ST80, ST117 and ST192 (Table 1).[4] The increase in vancomycin-resistant *E. faecium* in the Capital Region during 2012–14 was further studied by Pinholt *et al.*[8] SNP-based analysis of the WGS data divided the *vanA E. faecium* isolates into 13 main clades, 7 small clades and 13 singletons. Most of the isolates belonged to ST80, ST117 and ST192 (Table 1).

WGS analysis of VRE from 2015

In the present study, all 369 Danish clinical vancomycin-resistant *E. faecium* and the two clinical vancomycin-resistant *E. faecalis* isolates from 2015 were whole-genome sequenced. Both of the *E. faecalis* isolates belonged to ST6, one was *vanA* and the other *vanB* positive, and they were not investigated further. Of the *E. faecium* isolates, 368 were *vanA* positive and 1 carried the *vanB* gene. The majority (89%) of the *vanA E. faecium* isolates were from hospitals located on the Zealand island (the Capital Region and the Zealand Region) (Figure 1b). The vast majority of the *E. faecium* isolates belonged to three STs; ST203 (51%), ST80 (33%, including the *vanB* isolate) and ST117 (10%) (Table 2). The remaining isolates belonged to ST18, ST192 and the novel STs ST1196–ST1201, while one isolate (VRE2011) was non-typeable by MLST, due to a truncated

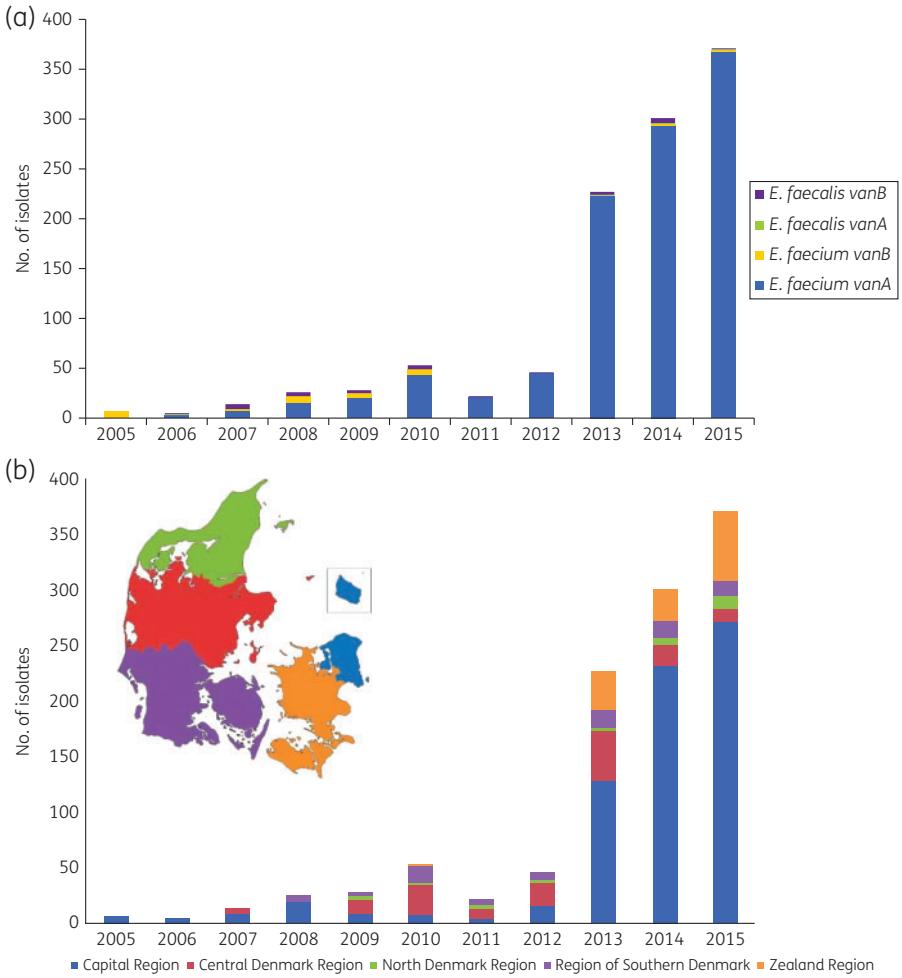

Figure 1. (a) Numbers of vancomycin-resistant *E. faecium* and *E. faecalis* isolates and *vanA* and *vanB* genes from clinical samples, 2005–15, Denmark. (b) Distribution of the clinical VRE isolates according to the five Danish regions, 2005–15. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Table 1. Distribution of STs for Danish vancomycin-resistant *E. faecium* isolates from several studies, 2005–15

Year(s)	ST (number of isolates)	Type of isolates	Region	Reference
2005–08	ST16 (1), ST17 (2), ST18 (24), ST65 (1), ST78 (3), ST80 (1), ST192 (3), ST203 (10), ST275 (2), ST306 (1), ST412 (3)	clinical and screening	all five Danish regions	[11]
2009–13	ST18 (36), ST117 (37), ST192 (26), ST203 (7), ST260 (4), ST665 (1)	clinical	Central Denmark Region	[12]
2012–13	ST18 (7), ST78 (3), ST80 (99), ST117 (94), SLV117 (1), ST192 (34), ST203 (1), ST260 (1), ST323 (1), ST665 (2)	clinical and screening	all five Danish regions	[4]
2012–14	ST18 (5), ST78 (5), ST80 (269), ST117 (125), ST192 (42), ST203 (19)	clinical and screening	Capital Region	[8]
2015	ST18 (5), ST80 (123), ST117 (38), ST192 (6), ST203 (188), ST1196 (1), ST1197 (1), ST1198 (1), ST1199 (1), ST1200 (3), ST1201 (1), non-typeable (1)	clinical	all five Danish regions	this study

purK gene. Recently, Carter *et al.*[13] reported on an outbreak with MLST non-typeable vancomycin-resistant *E. faecium* isolates from Australia; however, these were due to a truncated *pstS* gene.

cgMLST analysis

Using the cgMLST scheme with the suggested threshold of ≤20 alleles difference published by de Been *et al.*,[9] the 368 *vanA E. faecium* isolates subdivided into 33 CTs (Table 2 and Figure 2).

Table 2. Description of the 368 *vanA E. faecium* and 1 *vanB E. faecium* according to MLST and cgMLST, 2015, Denmark

MLST (number of isolates)	CT (number of isolates)
ST18 (5)	CT863 (1), CT864 (1), CT870 (1), CT876 (1), CT884 (1)
ST80 (123)[a]	CT14 (82), CT16 (2), CT860 (7), CT866 (13), CT867 (1), CT868 (1), CT869 (1), CT871 (12), CT878 (1), CT880 (1)[a], CT881 (1), CT992 (1)
ST117 (38)	CT24 (23), CT861 (1), CT862 (1), CT872 (2), CT873 (5), CT874 (2), CT875 (1), CT877 (1), CT882 (1), CT883 (1)
ST192 (6)	CT15 (3), CT46 (1), CT865 (2)
ST203 (188)	CT20 (1), CT859 (187)
ST1196 (1)	CT866 (1)
ST1197 (1)	CT871 (1)
ST1198 (1)	CT14 (1)
ST1199 (1)	CT885 (1)
ST1200 (3)	CT877 (2), CT879 (1)
ST1201 (1)	CT24 (1)
Non-typeable (1)	CT860 (1)

[a]The single *vanB E. faecium* isolate belonged to ST80-CT880.

In total, 187 (99%) of the 188 ST203 *E. faecium* isolates belonged to a new CT, which was designated CT859 upon submission to the cgMLST server through the SeqSphere+ software, whereas a single ST203 isolate belonged to CT20. ST203-CT20 *vanA E. faecium* isolates have previously been detected in the Capital Region in Denmark in 2013 and 2014, corresponding to Group 8b in the publication by Pinholt *et al.*[8] Clinical *E. faecium* isolates belonging to ST203-CT20 have also been reported from Germany from 2013 to 2016 (http://www.cgMLST.org). In 2015, *vanA E. faecium* isolates belonging to ST203-CT859 could be detected in all five Danish regions, but the majority were detected in the Capital Region ($n=137$; 73%) and the Zealand Region ($n=45$; 24%). During 2015, ST203-CT859 *vanA E. faecium* were also detected from the Faroe Islands and from the southern part of Sweden. The presence was most likely due to patient transfer, as patients with ST203-CT859 *vanA E. faecium* had been transferred from hospitals in the Capital Region to the Faroe Islands and to Sweden on multiple occasions (Shahin Gaini, National Hospital Faroe Islands, personal communication; Petra Edquist, Public Health Agency of Sweden, personal communication). Four *vanA E. faecium* isolates constituting Group 8c in the paper by Pinholt *et al.*[8] all belonged to ST203-CT859 and were first detected during December 2014, but subsequently detected in clinical samples at three different hospitals within 12 days (Mette Pinholt, Hvidovre University Hospital, Denmark, personal communication). In total, 51% (187 of 368) of the Danish *vanA E. faecium* from 2015 belonged to the new CT859 variant, which has not been reported in the cgMLST database outside Denmark (accessed 25 January 2017). Arguably, the lack of non-Danish ST203-CT859 isolates in the cgMLST database could partly be explained by the fact that this database is relatively new and relies on availability of WGS data, which may currently be obstructive for some laboratories. Therefore, it may not reflect the true global diversity of *E. faecium* variants, which could mean that this variant may be present outside Denmark, Sweden and the Faroe Islands, but until more data are made available, we have no evidence to support this hypothesis. In conclusion, the apparent rapid emergence in Denmark remains an enigma and studies are currently ongoing to investigate the national epidemiology of this specific CT.

The 122 *vanA E. faecium* isolates belonging to ST80 were subdivided into two already known CTs (CT14 and CT16), as well as nine new CTs (860, 866–869, 871, 878, 881 and 992). The *vanB* isolate belonged to a separate CT (CT880). Eighty-two of the 122 ST80 *vanA* isolates belonged to CT14, which was detected in all five Danish regions. *vanA E. faecium* isolates belonging to ST80-CT14 were also detected in the Capital Region in 2013 (http://www.cgMLST.org), but the cgMLST database did not contain any non-Danish ST80-CT14 isolates.[9] Two *vanA E. faecium* isolates from the Capital Region belonged to ST80-CT16; this type has previously been detected in Denmark in 2012, as well as in Germany between 2013 and 2015 (http://www.cgMLST.org).

The 38 *E. faecium* isolates belonging to ST117 were subdivided into CT24 ($n=23$; 61%) and nine new CTs (861, 862, 872–875, 877, 882 and 883) by cgMLST. CT24 were detected from hospitals in the Zealand Region, the Capital Region and the Central Denmark Region (Figure 1b). Isolates belonging to CT24 were also detected in 2010 in Danish hospitals as was observed when comparing WGS data from the ST117 *E. faecium* isolates from Aarhus University Hospital in the Central Denmark Region.[12] Furthermore, *E. faecium* isolates belonging to ST117-CT24 have been reported from clinical samples from Sweden in 2014 and Germany in 2014 and 2015 (http://www.cgMLST.org).

Six *vanA E. faecium* belonged to ST192. Three of these belonged to CT15, which previously has been detected in the Capital Region in June 2013 (http://www.cgMLST.org).[9] One of the ST192 *vanA E. faecium* isolates belonged to CT46, which has also been reported from Germany in 2014 and 2015; http://www.cgMLST.org). The remaining ST192 *vanA E. faecium* isolates belonged to CT865, a new CT. The five ST18 *vanA E. faecium* isolates belonged to five different cgMLST CTs (CT863, CT864, CT870, CT876 and CT884) and were therefore not considered clonally related. Eight of the *vanA E. faecium* isolates belonged to new STs (ST1196–ST1201). ST1196–ST1198 were single-locus variants of ST80; the three isolates belonging to these new STs sub-clustered to already known

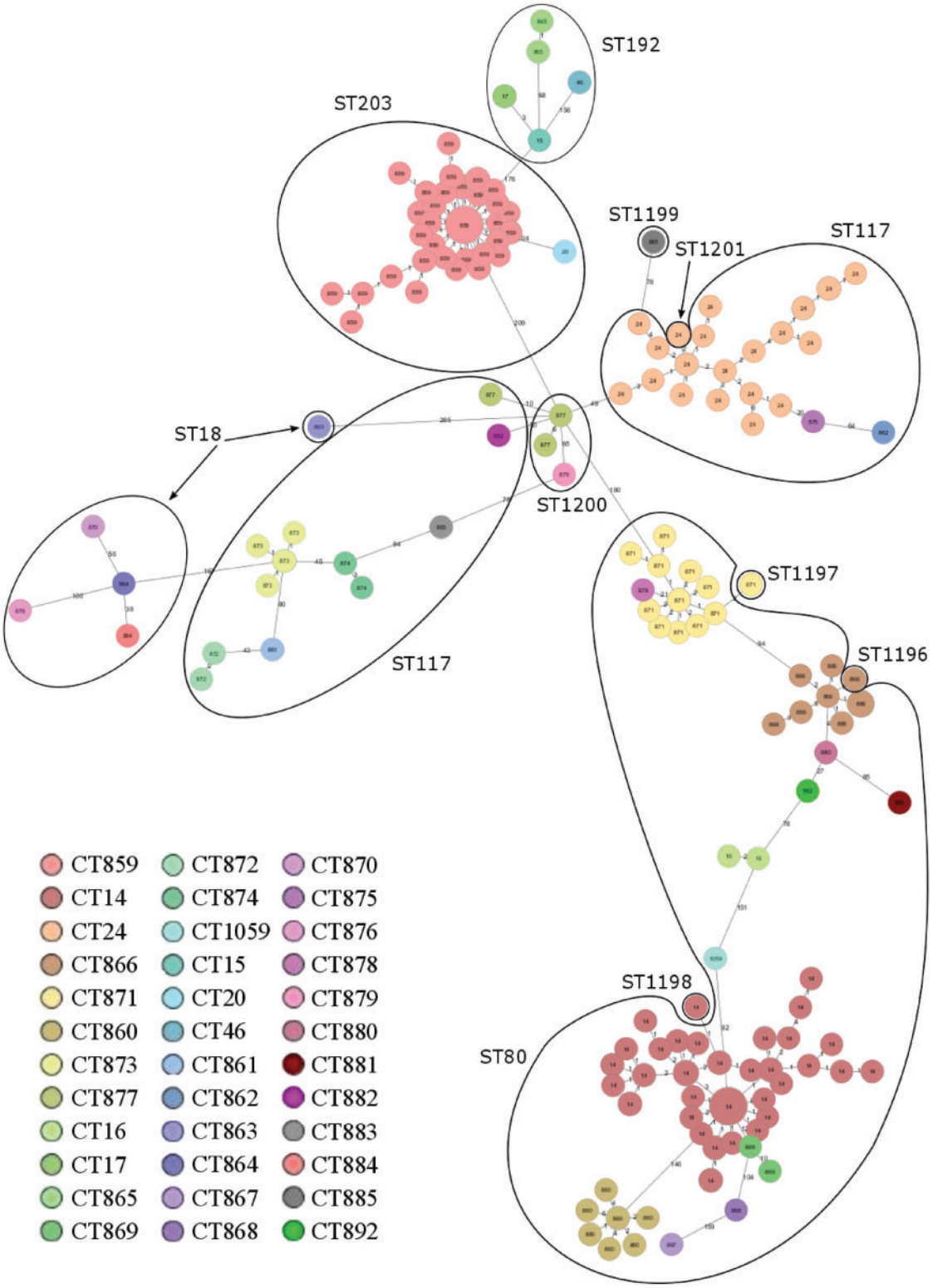

Figure 2. cgMLST (based on 1423 alleles) and MLST (based on 7 alleles) for 369 *E. faecium* isolates from 2015. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

CTs (CT14, CT866 and CT871) together with other isolates belonging to ST80. The isolate belonging to ST1199, a single-locus variant of ST117, belonged to a new CT, CT885. The three isolates belonging to ST1200, another single-locus variant of ST117, were subdivided into CT877 ($n=2$) and a new CT, CT879 ($n=1$). The isolate belonging to ST1201, a third single-locus variant of ST117, belonged to CT24 similar to other ST117 isolates.

Our data highlight the usefulness of cgMLST for sub-clustering of isolates beyond conventional MLST and, in combination with future records in the cgMLST scheme, will produce a powerful tool for the detection of emerging clones and outbreaks across regional and national borders.

Increased use of antimicrobial agents

In the last decade, *vanA E. faecium* isolates submitted from Danish DCMs have increased rapidly. The significant increase in *vanA E. faecium* between 2012 and 2015 is highly regional and linked to the Capital Region and the Zealand Region and follows a similar regional increase in nosocomial *Clostridium difficile* CD027 infections.[14] As *C. difficile* infections are often treated with vancomycin, a result of this could be a stronger selection for VRE at the hospitals in these regions.[15] Isaac *et al.*[16] recently showed that the human intestinal microbiota can change drastically during oral vancomycin treatment. The clinical relevance of the observed microbiota changes were further demonstrated in mice, which developed analogous microbiota alterations. During vancomycin treatment, mice were highly susceptible to intestinal colonization by a vancomycin-resistant *E. faecium* strain.[16] Furthermore, in the last 20 years the use of cephalosporins has increased in Danish hospitals.[15,17] Similar to the mice study with vancomycin, mice treated with cefuroxime were also colonized with a CC17 ampicillin-resistant *E. faecium* isolate.[18] So the increased use of cephalosporins might have contributed to an increase in the number of infections caused by *E. faecium* and stresses the fact that a rational antibiotic prescription pattern is an important prevention measure.

VRE can be carried in the intestine for a long period without any symptoms of infection and likewise persist in the hospital environment. This challenges infection control measures consisting of cleaning and hand hygiene, as well as screening for VRE and isolation of patients.

Conclusions

During 2005–15 *vanA E. faecium* increased dramatically in Denmark. In the period 2009–13, several *vanA E. faecium* outbreaks were detected in the Central Denmark Region. Since 2013 *vanA E. faecium* has become endemic in the Capital Region and the Zealand Region. In 2015, a new type, ST203-CT859, emerged and spread across Danish borders. Further studies are ongoing to investigate the cause of the spreading capacity of ST203-CT859 *E. faecium*. Besides ST203-CT859 *E. faecium, E. faecium* belonging to several other CTs were detected; many of these have been detected before, both inside and outside of Denmark.

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Transparency declarations

None to declare.

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