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Thirty years of VRE in Germany – "expect the unexpected": The view from the National Reference Centre for Staphylococci and Enterococci

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ABSTRACT

Enterococci are commensals of the intestinal tract of many animals and humans. Of the various known and still unnamed new enterococcal species, only isolates of *Enterococcus faecium* and *Enterococcus faecalis* have received increased medical and public health attention. According to textbook knowledge, the majority of infections are caused by *E. faecalis.* In recent decades, the number of enterococcal infections has increased, with the increase being exclusively associated with a rising number of nosocomial *E. faecium* infections. This increase has been accompanied by the dissemination of certain hospital-acquired strain variants and an alarming progress in the development of antibiotic resistance namely vancomycin resistance. With this review we focus on a description of the specific situation of vancomycin resistance among clinical *E. faecium* isolates in Germany over the past 30 years. The present review describes three VRE episodes in Germany, each of which is framed by the beginning and end of the respective decade. The first episode is specified by the first appearance of VRE in 1990 and a country-wide spread of specific *vanA*-type VRE strains (ST117/CT24) until the late 1990s. The second decade was initially marked by regional clusters and VRE outbreaks in hospitals in South-Western Germany in 2004 and 2005, mainly caused by *vanA*-type VRE of ST203. Against the background of a certain "basic level" of VRE prevalence throughout Germany, an early shift from the *vanA* genotype to the *vanB* genotype in clinical isolates already occurred at the end of the 2000s without much notice. With the beginning of the third decade in 2010, VRE rates in Germany have permanently increased, first in some federal states and soon after country-wide. Besides an increase in VRE prevalence, this decade was marked by a sharp increase in *vanB*-type resistance and a dominance of a few, novel strain variants like ST192 and later on ST117 (CT71, CT469) and ST80 (CT1065). The largest VRE outbreak, which involved about 2,900 patients and lasted over three years, was caused by a novel and until that time, unknown strain type of ST80/CT1013 (*vanB*). Across all periods, VRE outbreaks were mainly oligoclonal and strain types varied over space (hospital wards) and time. The spread of VRE strains obviously respects political borders; for instance, both vancomycin-variable enterococci which were highly prevalent in Denmark and ST796 VRE which successfully disseminated in Australia and Switzerland, were still completely absent among German hospital patients, until to date.

1. Introduction

Enterococci are commensals of the intestinal tract of many animals and humans and their dissemination across animal species, genera, orders and classes, is more widespread than expected (Lebreton et al., 2017). At the same time, enterococci are referred to as "modern" nosocomial pathogens that have received wider medical importance due to the broader use of cephalosporins and fluoroquinolones. These latter substances demonstrate moderate or no activity against isolates of the two medically important enterococcal species *E. faecalis* and *E. faecium*.

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Enterococci are capable of frequently exchanging genetic elements, including therapeutically relevant resistance determinants. Vancomycin resistance in clinical *Enterococcus* spp. isolates was first noticed in France in 1986 and shortly thereafter in the UK and the US (Leclercq et al., 1988; Sahm et al., 1989; Uttley et al., 1988). The French isolates were vancomycin-resistant *E. faecium* of the *vanA*-type (Leclercq et al., 1988; Leclercq et al., 1989), whereas the US samples originated from a cluster of related vancomycin-resistant *E. faecalis* isolates of the *vanB*-type (Sahm et al., 1989). Already back then it could be shown, that *vanA*-type vancomycin resistance was plasmid-encoded and *in vitro* transferable (Leclercq et al., 1989). Extensive research of early *vanB* isolates from the US revealed that they were different from the VanB VRE that were predominantly seen in recent years until today. The general composition of the *vanB* element of early US strains represented the *vanB1*-type embedded in transposon Tn*1547*, whereas nowadays the vast majority of worldwide occurring *vanB* isolates harbor *vanB2* (Patel et al., 1998). The latter element is implemented in an Integrative and Conjugative Element (ICE) of the Tn*1549*/Tn*5382*-like type (Dahl et al., 2000; Dahl et al., 1999; Werner et al., 2013). In line with the above findings, the early US VanB isolates generally lacked the ability to transfer the *vanB* element, which is in contrast to most of the newer VanB (*vanB2*) isolates (Dahl et al., 2000; Sivertsen et al., 2014; Woodford, 2001). Last but not least, the worldwide reservoir of *vanB*-type vancomycin resistance is *E. faecium*. Despite these obvious differences, comprehensive studies of these early US *vanB* isolates involving MMH594 and V583 provided a tremendous amount of valuable information about VRE and *E. faecalis* microbiology and pathophysiology (Frankenberg et al., 2002; Hancock and Perego, 2004; Low et al., 2003; Paulsen et al., 2003; Winstedt et al., 2000).

Nowadays, 8 types of acquired vancomycin resistance in enterococci have been reported (Werner, 2012a, b), but only *vanA*- and *vanB*-types are widely disseminated and thus received greater public health attention. In contrast, *vanD*- to *vanN*-types occurred only once or a few times worldwide. In Germany, we occasionally encountered *vanD*-type enterococci; a situation which is similar in neighboring countries like the Netherlands (Top et al., 2018). The general reservoir of *vanA*- and *vanB*-type vancomycin resistance in Germany, Europe and worldwide was, and continues to be, *E. faecium* (Guzman Prieto et al., 2016; Rios et al., 2020; van Hal et al., 2017; Werner et al., 2008a).

This review mainly aims at describing the VRE situation in Germany in the past 30 years, from 1990 to 2019. The reader is referred to a tremendous amount of previous papers and review articles for aspects not discussed here in detail such as (i) a detailed description of the genetic composition of the various *van* elements and the functionality of their regulatory and structural components (Arthur and Quintiliani, 2001; Arthur et al., 1996; Courvalin, 2006); (ii) the VRE situation in other parts of the world including other European countries, the Americas, Australia and Asia (Liu et al., 2019; Mahony et al., 2018; Raven et al., 2016; Rios et al., 2020; van Hal et al., 2017; Werner et al., 2008a); (iii) the genetic and mechanistic aspects of mobility and transferability of genetic elements including *vanA* and *vanB* (Arredondo-Alonso et al., 2020; Freitas et al., 2013; Perichon and Courvalin, 2009; Werner, 2012a; Werner et al., 2013; Werner et al., 2011b); (iv) our growing understanding of *E. faecium* and *E. faecalis* population structures (Guzman Prieto et al., 2016; McBride et al., 2007; Neumann et al., 2019; Willems et al., 2005; Zischka et al., 2015) and (v) the pathophysiological aspects of enterococcal and VRE infections (Caballero et al., 2017; Kim et al., 2019; Magruder et al., 2019; McKenney et al., 2019; Stein-Thoeringer et al., 2019).

Although a National Reference Centre (NRC) for Enterococci was first assigned in Germany in 2012, our group served as an enterococcal reference laboratory (human sector) throughout the entire reporting time period covered in this review and for reasons of simplicity we refer to the term NRC throughout the manuscript. The NRC for Enterococci receives isolates on a voluntary basis. Over the years, the network of collaborating laboratories has been growing to more than 150 primary diagnostic laboratories sending strain samples (1000–2000 annually) and serving hundreds of hospitals in all German federal states.

2. From 1990 to 1999 – The early years or "Much Ado about Nothing?"

Our reference laboratory was made aware of the first VRE cases in Germany in 1990 (Klare et al., 1992). The first VRE isolates originated from colonizations and infections of hospital patients from intensive care units (ICUs) and a liver transplant center in Berlin. Altogether, 4 of the 52 investigated *E. faecium* isolates were vancomycin-resistant and of the *vanA*-type; one isolate originated from a bloodstream infection (Klare et al., 1992). Soon thereafter, additional VRE isolates were submitted from diagnostic laboratories located in Saxony (Leipzig), Brandenburg (Potsdam), Mecklenburg-Western Pomerania (Schwerin) and Saxony-Anhalt (Magdeburg). The increasing prevalence of VRE was quite unexpected and surprising, since up to that point, glycopeptides had not been used or licensed in these regions (former German Democratic Republic) (Witte and Klare, 1995). In order to identify a reservoir of VRE outside hospitals, samples from sewage treatment plants in Berlin, Saxony-Anhalt, Thuringia and Lower Saxony were screened for VRE (Klare et al., 1993). The municipal sewage treatment plants in larger cities revealed samples positive for VRE (*vanA*-type *E. faecium*). In contrast, in smaller and private sewage treatment plants, no VRE could be detected. Further studies confirmed the presence of *vanA*-type *E. faecium* isolates in samples from pig and poultry farms using avoparcin, a glycopeptide antibiotic that was used as growth promoter. In contrast, samples from egg-producing farms, where avoparcin was not licensed, were free of VRE (Klare et al., 1995b). As a consequence of emerging VRE in livestock farms, food samples were tested positive for VRE as well, including pork and chicken and poultry carcasses and thighs (Klare et al., 1995a). Similar findings were published in neighboring European countries (Aarestrup, 1995; Aarestrup et al., 1996; Bager et al., 1997; Schouten et al., 1997; van den Bogaard et al., 1997; van den Braak et al., 1998). During the 1990s, a certain amount of healthy people in the general community in Germany were colonized with VRE (6-12%) (Klare et al., 1995a; Zimmermann et al., 1998), which was in accordance with observations from other European countries (Endtz et al., 1997). A hypothesis of a common gene pool between animal, food and human colonization and/or infectious isolates derived from several findings and facts. First, several studies showed that across all sectors, VRE identified were *E. faecium* and of the *vanA*-type. Second, VRE contained plasmid-located resistance determinants (Werner et al., 2011b; Werner et al., 1999). Third, VRE isolates from different sources revealed similar *vanA* elements resembling Tn*1546*-like structures (Jensen et al., 1998; Werner et al., 1997; Willems et al., 1999). Danish researchers identified both a point mutation in Tn*1546* specific for *vanA*-positive *E. faecium* isolates from pig or pork and a point mutation associated with isolates from chicken and poultry; most importantly, both variants were identified in *vanA*-type VRE from humans (Jensen, 1998). The overwhelming amount of evidence indicating a link between avoparcin use and VRE prevalence led to a ban of avoparcin, first in single European countries like Denmark (1995) and Germany (1996) and finally in the entire European Union (1998). As a further consequence, the use of all antimicrobial growth promoters was finally stopped in the EU (Witte, 1998, 2000). Soon after the discontinued use of avoparcin in commercial animal husbandry, colonization rates of VRE among the general German community significantly dropped (Klare et al., 1999). Similar decreasing trends were described for food animals in Denmark (Bager et al., 1999) and outpatients in the Netherlands (van den Bogaard et al., 2000). Further evidence for the success of the discontinued use of antimicrobial growth promoters was provided by a prevalence study carried out between 1999 and 2000 among the healthy community in Southern Germany, demonstrating that rectal VRE colonization dropped below the limit of detection (Lietzau et al., 2006).

All these studies documented a large reservoir of VRE strains in

livestock, food and as colonizers in the general community. Additionally, mobile *vanA* resistance elements of similar structure were detected in isolates from all sectors including human VRE patients. Contrary to this situation, infections and outbreaks with VRE during the early and mid 1990s remained rare in Germany (hospital outbreaks became officially noticeable with the amendment of the Infection Protection Act in 2000). As the situation was not properly surveyed at that time, information is scarce and only available from individual publications and from aggregated data of antibiotic resistance surveillance studies performed by the Paul Ehrlich Society. The Paul Ehrlich Society has run multicenter resistance surveillance studies since 1975, but with continued, reliable and comparable sampling only from 1995 onwards[2]. Data and isolates from a total of 33 medical microbiological laboratories from Germany (n = 21), Switzerland (n = 7) and Austria (n = 5) were included for the study performed in 1995. Altogether it could be shown that 3 of 78 clinical *E. faecium* isolates (from suspected or proven infections) and none of 760 *E. faecalis* isolates were VRE, revealing a positivity rate of 3.8 % and 0%, respectively. All three VRE were resistant to teicoplanin indicating the VanA phenotype. A further resistance surveillance study performed in 1998 by the Paul Ehrlich Society comprising 29 laboratories (20 centers from Germany, 6 from Switzerland and 3 from Austria) found 4 VRE among 78 *E. faecium* isolates (5.1%). Again, all showed the VanA phenotype. In this study, there was a single VRE isolate (VanA phenotype) among 757 *E. faecalis* isolates (resistance rate, 0.1%).

During the early 1990s, about 300 enterococcal isolates were sent annually to the NRC for strain typing and characterization. The vast majority was VRE and almost all VRE isolates were *vanA*-type *E. faecium* (*vanB*-type *E. faecium* per year: n<5) (Witte and Klare, 1995). In most isolates *vanA*-type resistance was mediated by large conjugative plasmids demonstrating a narrow host range within *E. faecium* (Werner et al., 2011b; Werner et al., 1999). In terms of clonal association and genomic content, little is known about these early VRE isolates from 1990 to 1995. However, information derived from subsequent Multilocus Sequence Type (MLST) analysis suggested that clinical *E. faecium* isolates of that time originated from animal or commensal human sources (unpublished data; see also chapter 4) (Lebreton et al., 2013).

At the time of the first VRE outbreaks in Berlin hospitals, a VRE screening within a University clinic revealed colonization rates among various populations between 2% (no risk factors) and 16% (ICU patients with risk factors) (Wendt et al., 1999). The evidence and knowledge available at that time led to the hypothesis that the VRE clusters among Berlin hospital patients were fed by a community reservoir of VRE and the subsequent selection of these clones in the stationary setting by various factors.

A surveillance study performed in 1997 at 22 diagnostic laboratories in North-Rhine Westphalia revealed a prevalence of 1.5% vancomycin resistance among clinical enterococci. However, the majority of the 730 isolates tested were *E. faecalis* (n = 648; 89%) followed by *E. faecium* (n = 72; 10%). As the prevalence of vancomycin resistance differed significantly between the two species with 0.5% for *E. faecalis* (n = 3) and 11% for *E. faecium* (n = 8) (Reinert et al., 1999), the overall prevalence reported back then should be interpreted with caution. Interestingly, by using macrorestriction pattern analysis, it could be shown that isolates from local outbreaks in the federal states Hesse, Lower Saxony and North-Rhine Westphalia exhibited an indistinguishable pattern suggesting a supra-regional spread of an epidemic VRE strain (Reinert et al., 1999).

In the following two years, the dissemination of an epidemic VRE strain was confirmed on the basis of macrorestriction pattern analyses (Klare et al., 2003). Corresponding VRE isolates originated from hospital patients from Bavaria, Baden-Wuerttemberg, Hesse, North-Rhine Westphalia, Lower Saxony, Hamburg, Schleswig-Holstein, Mecklenburg Western Pommerania, Saxony, Brandenburg and Berlin. Based on the available information about patient transfers, the spread of this specific clonal type across various Berlin clinics could be reconstructed; however, there was no data available to prove a country-wide spread of a single VRE clone from Southern to Northern Germany within a few years. Plasmid analyses identified a similar but not identical plasmid pattern (Klare et al., 2003). With the introduction of MLST typing soon thereafter (Homan et al., 2002), this strain type was assigned as ST117. The latter represents a classical hospital-associated MLST type that nowadays is widely prevalent among hospital patients in Germany, Europe and worldwide (Arslan et al., 2013; Eisenberger et al., 2020; Falgenhauer et al., 2019a; Hammerum et al., 2017; Zhu et al., 2009). Preliminary and unpublished whole genome-based analyses showed, that the circulating ST117 epidemic VRE strain belonged to the cgMLST type CT24 and had a chromosomally-located *vanA* gene cluster (Bender et al., unpublished). Already back in the 1990s we discovered the ST117/CT24 strain type to be a strong producer of bacteriocin, hence inhibiting the growth of commensal *E. faecium* and *E. faecalis* isolates. We suggested that bacteriocin production might suppress the competing commensal enterococcal flora, thus allowing preferred intestinal settlement and subsequent overgrowth of this hospital clone (unpublished data). In the beginning of the 2000 years, this epidemic strain type suddenly disappeared and other strain variants emerged, first in the Southwestern part of Germany (see next chapter).

3. From 2000 to 2009 – The second decade or "The calm before the storm?"

In the early 2000s, modern approaches for strain typing like AFLP (Amplified-fragment length polymorphism), MLST and MLVA (Multiple Locus Variable number of tandem repeat Analysis) substituted fragment-based strain typing by comparing genomic macrorestriction patterns (Homan et al., 2002; Top et al., 2004; Top et al., 2008; Willems et al., 2000). Software tools like eBURST (https://www.mlst.net/eburst/) or goeBURST (http://www.phyloviz.net/goeburst/) were introduced for both analyzing these novel datasets and reconstructing the *E. faecium* population structure (Francisco et al., 2009; Turner et al., 2007). These technological advances completely changed the understanding of the population structure of *E. faecium* and the role of multidrug resistant *E. faecium* strains affecting human populations at risk in hospitals worldwide. It became evident that *E. faecium* isolates from colonizations in animals and humans were microbiologically different from those observed in hospital infections and causing VRE outbreaks. The newly introduced term of a clonal complex CC1 (or CC17) designated a group of hospital-associated *E. faecium* strain types that could be distinguished from colonizing *E. faecium* variants of animals and humans. Although this term is already outdated today, it has been used for at least 15 years (see also chapter 4).

Resistance studies of the Paul Ehrlich Society performed in 2001, 2004, and 2007 demonstrated an increase in vancomycin resistance rates and frequencies among clinical *E. faecium* isolates from German, Austrian and Swiss hospitals (https://www.p-e-g.org/resistenz/database/). Vancomycin resistance rates among *E. faecium* isolates were 2.7% in 2001, 13.5% in 2004 and 11.2% in 2007. According to the study design, a predefined number of enterococcal isolates (*E. faecalis* and *E. faecium*) from clinical infections was collected, among others. While the number of *E. faecium* isolates steadily increased from n = 110 in 2001 to n = 250 in 2007, the number of *E. faecalis* isolates dropped, indicating that not only the rates but also the overall number of *E. faecium*/VRE infections increased. These findings were in line with reports from other European countries at that time demonstrating an increasing amount of *E. faecium* infections, which were associated with common hospital-associated strain types of ST17, ST18 and ST203 (Lester et al., 2008; Top et al., 2007; Top et al., 2008). Additional data supporting these early findings were provided from studies like KISS (https://www.nrz-hygiene.de/surveillance/kiss/) and SARI (http://sar

[2] https://www.p-e-g.org/berichte-der-studien.html

i.eu-burden.info/) that were coordinated at the National Reference Centre for Surveillance of Nosocomial Infections in Germany (see also next chapter) (Kohlenberg et al., 2009).

The early years of the 2000s were characterized by a generally low VRE prevalence in Germany; nevertheless, several VRE hotspots were described by local and temporary outbreaks (Klare et al., 2012b). Unfortunately, only a limited number of these outbreaks were published, leaving little data available to a broader audience. Analysis of certain VRE outbreaks back in these days suggested screening and isolation as well as intensified hand hygiene and environmental cleaning as effective infection control measures for high risk populations and wards (Knoll et al., 2005; Schmidt-Hieber et al., 2007). In contrast, other studies described self-limiting VRE outbreaks with decreasing VRE trends, even without the implementation of stringent measures (Borgmann et al., 2004). The NRC was involved in analyzing some of these VRE outbreaks by performing strain typing and characterization. Several isolates that were involved in smaller and larger outbreaks in the early 2000s were characterized in a descriptive study published in 2005 (Klare et al., 2005). By using MLST, ST203 (*vanA*) could be assigned to most of the VRE outbreak clones (Klare et al., 2005). Almost all of these ST203 (*vanA*) isolates were associated with patients in various hospitals in the federal state of Baden-Wuerttemberg in 2003 and 2004.

Experiences with larger VRE outbreaks, first starting in the South-Western parts of Germany, resulted in the publication of two infection prevention and control consensus papers in 2006 and 2007 (Von Baum et al., 2006; Vonberg et al., 2007). In general, the suggested bundle centered on hygiene measures that were reported to be effective against MRSA. The first guideline paper was based on experiences with the early VRE outbreaks in Baden-Wuerttemberg and focused mainly on VRE outbreak management (Von Baum et al., 2006), while the second recommendation suggested measures for routine surveillance situations and was elaborated by a working party of the German Society for Hygiene and Microbiology (DGHM) (Vonberg et al., 2007). For many years, these two papers proposed strict measures to guide VRE outbreak management and infection prevention in Germany (Mutters et al., 2013a; Mutters et al., 2013b).

During the second half of this decade, the NRC noticed a shift in the prevalence of distinct strain types and a wider distribution of VRE across Germany (Klare et al., 2012b; Werner et al., 2008c). These observations were in line with results of the resistance surveillance studies of the Paul Ehrlich Society (see above). The *vanA* type remained the most common *van* genotype and novel clonal variants such as ST17, ST18 and ST78 became prevalent; in contrast, Berlin hospitals were dominated by *vanA*-type VRE isolates of ST202. More detailed molecular analyses of certain hospital outbreaks revealed that VRE outbreaks were typically oligoclonal (Borgmann et al., 2004). Thus, the identification of type strains that were responsible and representative for such outbreaks and for defining the general public health burden was quite ambiguous (Abele-Horn et al., 2006; Borgmann et al., 2007). Molecular investigations revealed that *vanA*-type gene clusters were mostly located on self-transmissible, conjugative plasmids and thus could potentially be transferred independent of the strain background. Consequently, plasmid and *vanA* transposon typing became a valuable additional typing tool when either analyzing VRE outbreaks or reconstructing potential *vanA* transmission chains. However, the resolution of available typing methods at that time, even in combination, was low and did not allow to specify or confirm the role of horizontal gene transfer in the spread of *vanA*-type resistance reliably enough (Novais et al., 2008; Werner et al., 2011b; Werner et al., 2008c).

Between 2000 and 2009, first cases of resistances to last resort antibiotics in clinical enterococci or VRE were documented in Germany. Resistance against linezolid was either selected by its use or was imported through patient (and strain) transfer from abroad (Halle et al., 2004; Schulte et al., 2008; Schulte et al., 2005). Resistance to quinupristin/dalfopristin, a therapeutic alternative effective against *E. faecium* (VRE) infections, was also described in a small cluster of colonizations in a single hospital in the federal state of Saxony (Werner et al., 2003). Although enterococci and staphylococci generally share a common gene pool, especially regarding resistance genes, back then we learned that genetic determinants encoding for quinupristin/dalfopristin resistance were different in clinical *S. aureus* and *E. faecium* isolates and were not shared between the two genera (Schmitz et al., 2001; Werner et al., 2001).

An improved understanding of the characteristics of hospital-acquired strain types led to the hypothesis that microbiologists might be capable of discriminating between epidemic ("dangerous") and non-epidemic ("less dangerous") VRE strain types (Borgmann et al., 2007; Leavis et al., 2003; Werner et al., 2011a). In this respect, particular emphasis was placed on specific markers enriched in hospital-associated VRE strains such as the esp_{Efm} gene (Leavis et al., 2004; Willems et al., 2001), associated with surface attachment and increased biofilm formation, and the *E. faecium* hyl_{Efm} gene (Rice et al., 2003), a putative hyaluronidase (later on re-classified as a glucuronidase). Other phenotypic traits specific for hospital strain types represented resistance against ampicillin and ciprofloxacin (high-level) (Leavis et al., 2006a; Leavis et al., 2006b; Werner et al., 2010). The hypothesis at that time suggested an increased prevalence of these genetic and phenotypic markers among VRE isolates widely disseminated within the hospital setting (Freitas et al., 2010; Klare et al., 2005; Laverde Gomez et al., 2011; Top et al., 2008). In other words, the more these markers were detectable, the more successfully a corresponding strain could spread. With the first available complete genome sequences and the advent of genome-based strain characterization in the beginning of the early 2010s, a particular assessment of individual genetic markers like esp_{Efm} and hyl_{Efm} became less and less common and the understanding of the *E. faecium* population structure and the success of specific clonal lineages again improved markedly.

4. From 2010 to 2019 – The third decade or "When the shit hits the van"

With the beginning of the new decade, first genome sequences of *E. faecium* (and *E. faecalis*) were published (Lebreton et al., 2013; Palmer et al., 2010; Palmer et al., 2012; Palmer et al., 2014). Lebreton and co-workers were the first to suggest an *E. faecium* population structure divided into three major clades: B for the human commensals; A2 for the animal commensal variants and A1 for the hospital clade strains (Lebreton et al., 2013). The comparably close phylogenetic association of animal commensal and hospital-associated strain types of *E. faecium* was somehow unexpected and odd, but has been confirmed in several subsequent studies using larger strain collections (Gouliouris et al., 2018; Raven et al., 2016; Rios et al., 2020; van Hal et al., 2016). This new model has now completely replaced the previous model of a single clonal complex CC1 (or CC17) combining all *E. faecium* hospital strain types. The specific genomic composition and the specific genetic characteristics of *E. faecium* isolates causing infections and outbreaks in hospital patients were also demonstrated in clinical and epidemiological studies as follows.

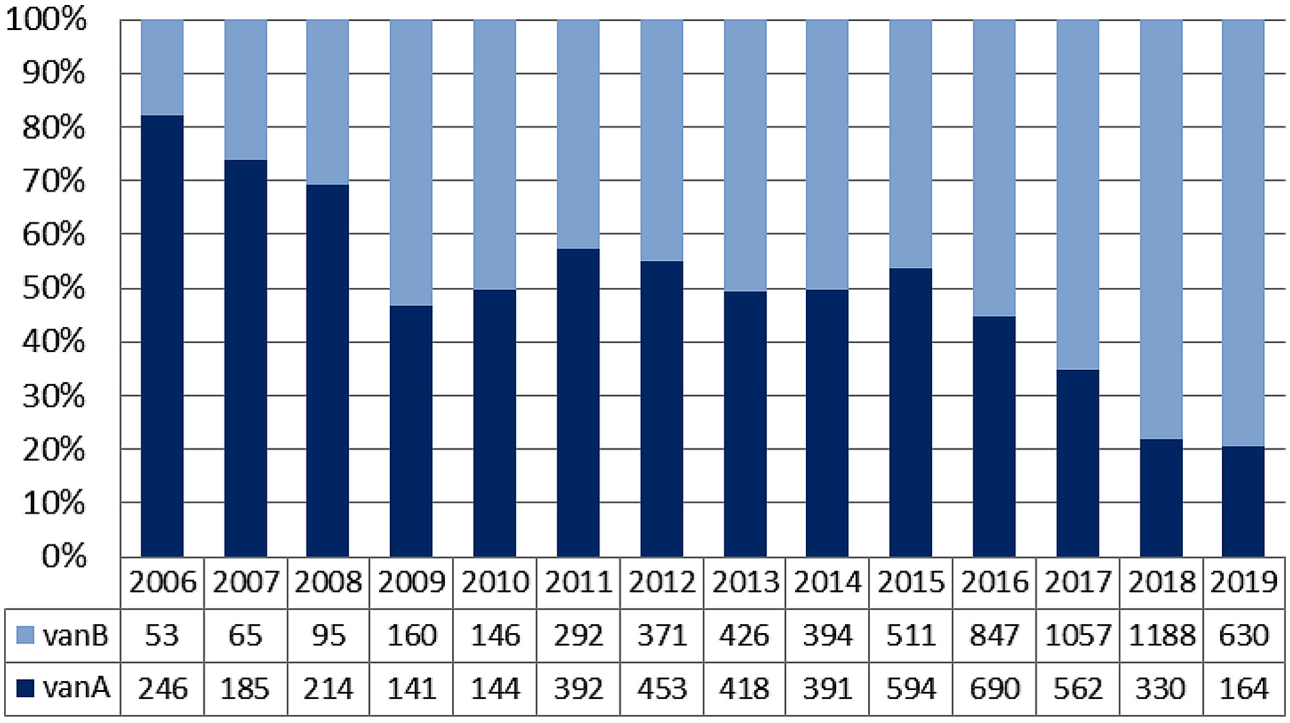
In 2018, the NRC for Surveillance of Nosocomial Infections published a study about the burden of enterococcal infections using data from three German tertiary care hospitals (Kramer et al., 2018). This retrospective cohort study on patients with bloodstream infections (BSI) caused by *E. faecium* and *E. faecalis* was performed between 2008 and 2015. The study revealed that the distribution between *E. faecium* and *E. faecalis* BSI was almost equal with n = 596 (51.4%) cases and 564 (48.6%) cases, respectively. When adjusting for the species, *E. faecium* was an independent risk factor for in-hospital mortality and prolonged hospital stay, and vancomycin resistance did not further increase this risk. These findings are in line with genome-based and pathophysiological studies demonstrating that currently circulating hospital-associated *E. faecium*/VRE strains are different from colonizing *E. faecium* variants and from the first VRE strain variants found in

hospitals 15 to 20 years ago (see chapter 2).

In Germany, the antibiotic resistance surveillance system ARS (Antibiotic Resistance Surveillance), hosted by the Robert Koch Institute (RKI), was initiated in 2008. Nowadays, it collects antimicrobial susceptibility data of university and hospital laboratories, commercial diagnostic companies and private laboratories reaching an approximately 50% coverage of the German population including ambulatory and hospital patients (https://ars.rki.de/Default.aspx). Additional data were provided by the aforementioned studies of the Paul Ehrlich Society, KISS and SARI; the latter two hosted and coordinated by the Charité clinics (NRC for Surveillance of Nosocomial Infections). KISS assesses nosocomial infections at the national level and focusses on specific hospital departments which are grouped in programs such as ICU-KISS (focusing on ICUs) or NEO-KISS (focusing on neonatology wards). SARI determines antibiotic consumption and nosocomial infections in German intensive care units, thus enabling joint and comprehensive data analyses of antibiotic consumption and resistance development. Although the focus of these two tools was primarily on assessing MRSA dynamics, assessing the situation of VRE (and multidrug-resistant Enterobacteriaceae) became also prominent during the reporting period.

All studies and schemes mentioned are based on different resources, but the overall trends for VRE prevalence extracted from the collected and evaluated data point in the same direction. An analysis of ARS data revealed that the amount of *E. faecium* isolates with resistance to vancomycin increased from 11.2% in 2014 to 26.1% in 2017 (Markwart et al., 2019). This increase of VRE rates was primarily observed in the Southern regions of Germany, where vancomycin resistance rates were as high as 37%. Data from ICU-KISS contributed to a comparative study using data from 645 ICUs and 681 surgical departments collected between 2007 and 2012 (Gastmeier et al., 2014). German ICUs reported a 282% increase in VRE cases. Most cases and increased prevalence were based on data provided by four German federal states ("the VRE belt"). Subsequent analysis of the KISS data from the following years (857 ICUs and 1.119 surgical departments) documented an ongoing and still increasing VRE prevalence; however, they were no longer restricted to distinct federal states. When using data from contributing ICUs (the "core group") it became evident that, with the exclusion of any selection bias, the increase in VRE from bloodstream infections was substantial (5.5% to 21.6%) (Remschmidt et al., 2018). Likewise, data from the resistance studies of the Paul Ehrlich Society performed in 2010, 2013 and 2016 revealed a further and sustained increase in vancomycin resistance rates among clinical *E. faecium* isolates from 12.6 % (n = 301), 16,6% (n = 320) to 24,4% (n = 316). The mere increase in vancomycin resistance rates among hospital *E. faecium* isolates was only one side of the coin. A retrospective analysis of all VRE genomes collected by the Paul Ehrlich Society in 2010, 2013 and 2016 revealed a shift from *vanA*-type resistance to *vanB*-type resistance and a prevalence of specific strain types such as ST117 (Neumann et al., 2020b); both findings are indicative of unpredictable dynamics when only resistance rates are taken into account (see next paragraph). Consistent with these results, an analysis of all VRE strain samples received by the NRC for Enterococci over the last 15 years showed a clear shift of the ratio of VRE *vanA*:*vanB* from 4:1 to 1:4 (Fig. 1). These findings additionally reflect *van* genotype dynamics extractable from resistance surveillance studies and schemes when assessing differences in teicoplanin susceptibility (*vanB*-genotype) and teicoplanin resistance (*vanA*-genotype).

Expression of vancomycin resistance in *vanB*-type *E. faecium* isolates can be low and thus may complicate a proper resistance prediction and strain classification, especially when using automated antimicrobial susceptibility testing. Given the sharp increase in *vanB*-type vancomycin resistance in Germany over the last decade, the question was raised of how reliable VanB diagnostics really are and whether a certain amount of VRE has been, and is still, overlooked due to improper susceptibility testing. During the last decade, two comparative studies were conducted with a comprehensive, representative and carefully balanced collection of *vanB* carrying strains to estimate the burden of this low level expression and to suggest diagnostic refinements in order to circumvent potential diagnostic complications (Klare et al., 2019; Klare et al., 2012a). As a result, EUCAST adjusted and clarified its corresponding warning for confirmatory diagnostic tests based on gradient strip assays



	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
vanB	53	65	95	160	146	292	371	426	394	511	847	1057	1188	630
vanA	246	185	214	141	144	392	453	418	391	594	690	562	330	164

Fig. 1. Ratio of *vanA*-type to *vanB*-type *E. faecium* submitted to the National Reference Centre for Staphylococci and Enterococci between 2006 and 2019. The ratio as percentage is given by the color code (light blue = *vanB*; dark blue = *vanA*); the exact numbers of corresponding isolates are shown below. *vanA* and *vanB* positive (n<5/year) *E. faecium* are not included.

(http://www.eucast.org/ast_of_bacteria/warnings/).

At the beginning of the 2010s, the increase in *vanB*-type resistance in Germany was linked to a wider dissemination of ST192 isolates, both locally and country-wide (Bender et al., 2016b; Werner et al., 2012). By using genome-based approaches we showed the *vanB*-type resistance spread clonally; however, and in addition, our studies were indicative of a horizontal transfer of *vanB* type resistance within ST192 isolates and other strain/sequence types (Bender et al., 2016b). Despite the former dominance of ST192 isolates in clinical settings, the number of corresponding isolates dropped tremendously until the end of the decade. None of the 169 BSI *E. faecium* isolates analyzed in 2019 by the NRC was typed as ST192 (see also next paragraph).

All *E. faecium* isolates associated with BSI that were sent to the NRC had been analyzed by molecular typing methods, first by MLST and since 2015 onwards by next generation sequencing (NGS)-based strain typing. For the purpose of *E. faecium* strain comparisons, we routinely perform core genome MLST (cgMLST) typing by using a set of 1423 genes and the allocation of so-called Complex Types (CT). Based on MLST analysis of BSI isolates collected in Germany over the last 10 years, an increasing prevalence of ST117 isolates was observed, followed by ST80 and ST78 (Fig. 2). Strain types that were most prominent during the previous decade (2000–2009) such as ST18, ST203 and ST17, almost disappeared by the end of 2019 (at least as a causative agent of BSI). Certain MLST types were mainly associated with a distinct *van* genotype such as ST203 that almost always carried *vanA*. The decrease in *vanA*-type strains also applies to a reduction of ST203 isolates or *vice versa*. On the contrary, the sharp increase in ST80 isolates is directly linked to an increasing *vanB* type resistance (or *vice versa*). The much higher discriminatory power of cgMLST in relation to traditional MLST analysis allows for much more detailed analyses and more precise assumptions. As an example, we analyzed a dataset of 169 BSI *E. faecium* isolates from the year 2019 by cgMLST. The most frequent isolates correspond to CT71 (ST117, *vanB*) and CT1065 (ST80, *vanB*) with 44% and 8%, respectively. All other CTs were less frequent (n<6; <3%; unpublished data). A substantial amount of *vanB*-type ST117 BSI isolates collected between 2015 and 2019 was classified as CT71 and originated from hospital patients from 11 of the 16 German federal states, thus demonstrating a country-wide spread of an epidemic and invasive VRE strain type. Within ST80, only isolates of CT1065 appeared to be more widely disseminated (unpublished data). Recently published genome-based VRE surveillance studies revealed the ST117 (CT71) and ST80 (CT1065) strain variants to be prevalent in specific hospitals, cities and regions of Germany, including reports from Bavaria, Hesse, North-Rhine Westphalia, Baden-Wuerttemberg and Berlin, perfectly supporting our findings (Eichel et al., 2020; Eisenberger et al., 2020; Falgenhauer et al., 2019a; Liese et al., 2019; Weber et al., 2020).

In 2017, the RKI was requested to support a VRE outbreak investigation affecting two hospitals in southwest Germany. By the end of 2015, a rapid increase in VRE cases was noted and by 2019, when the outbreak was officially declared over, more than 2,900 patients tested positive for VRE. Approximately 400 isolates were examined at the NRC by means of whole genome sequencing and cgMLST typing. The initial analyses of the isolates from 2015-2017 revealed the presence of a dominant clone of type ST80/CT1013. This specific strain type has not been reported from any hospital at that time nor was it detected in BSI isolates that we had analyzed at the NRC back then. Further, smaller clusters characterized by other VRE strain types were detected in parallel during that time. These clusters consisted of ST80/CT1065, ST80/CT1066 and ST117/CT469 isolates, strain types that were already known to the NRC and nowadays represent most prominent and disseminated lineages, especially ST80/CT1065 (see above). Of note, almost all isolates that were analyzed were *vanB*-positive. Combining the genotypic data with epidemiological information on acquisition of the respective VRE revealed that ST80/CT1013 was mostly transmitted inside the hospital, whereas the secondary clusters were composed of isolates that were more often introduced into the hospital from somewhere outside. In order to further describe strain dynamics over time, we additionally analyzed 95 isolates that were obtained between December 2018 and March 2019. We compared the 2018/2019 isolates to the ones from earlier periods and observed a change in the composition of genotypes. Although the main clone ST80/CT1013 was still detected, it was no longer the most prominent genotype but had been replaced by ST80/CT1065. This again underlines the highly dynamic population structure of VRE, even of “single” VRE outbreaks. Nevertheless, manifestation of certain lineages seems obvious for which the reasons are fairly unknown.

The NRC fulfils an important role in the early detection of antibiotic resistance developments, even before such developments become

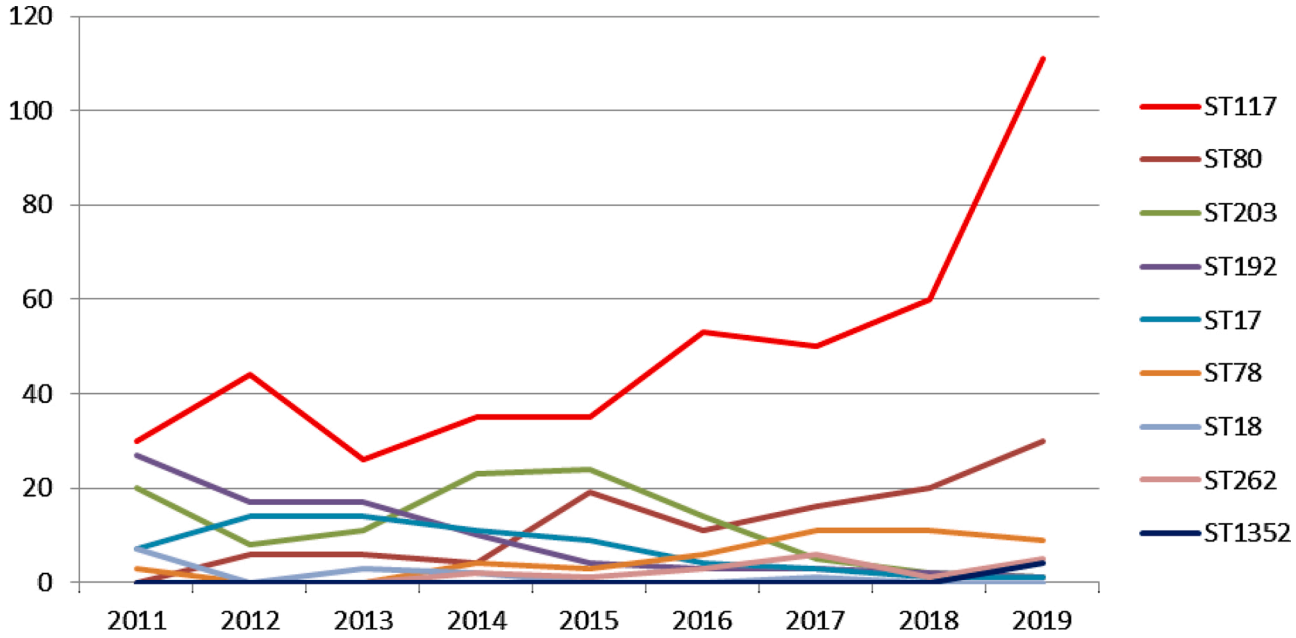


Fig. 2. Most common sequence types (MLST) of invasive *E. faecium* (mostly VRE) isolates received between 2011 and 2019 (n = 757) by the National Reference Centre. Legend: X-axis: data by year; Y-axis: data in number.

noticeable in resistance surveillance systems and studies. First reports about VRE with resistances to last resort antibiotics in Germany have been published much before 2010 (Schulte et al., 2008; Schulte et al., 2005; Werner et al., 2008b; Werner et al., 2003). However, until the beginning of this century, those cases remained rare. In the beginning of this decade, the number of linezolid-resistant enterococcal isolates submitted to the enterococcal NRC increased steadily (Klare et al., 2015). Although the majority of linezolid-resistant enterococci (LRE) were vancomycin-susceptible in the early years (for instance, in 2012 4% of all *E. faecium* submissions were LRE and 1% LVRE revealing a ratio of "LVRE/LRE" of 25%), there has been a trend towards vancomycin-resistant LRE (LVRE) in recent years (Table 1). About 90% of the submitted LRE isolates (*E. faecium*) demonstrated 23S rDNA mutations that are known to be associated with linezolid resistance. LRE were retrospectively screened for the presence of transferable linezolid resistance determinants *cfr*(B), *optrA* and *poxtA*. We were able to identify *cfr*(B) in a LRE isolate from 2011 and *optrA* and *poxtA* back to LRE isolates from 2012, which is long before these determinants have been described the first time. During the last decade *cfr*(B), *optrA* and *poxtA* can be increasingly observed in clinical *E. faecium* and *E. faecalis* isolates in Germany (Bender et al., 2016a; Bender et al., 2019; Bender et al., 2018b); most of the latter were plasmid-located and transferable, potentially also across strain-, species- and genus-barriers (Egan et al., 2020).

Since the NRC receives staphylococcal isolates as well, mainly *S. aureus* and MRSA, we have been able to compare the dynamics of resistance development between *S. aureus* (MRSA) and *E. faecium* (VRE), especially with regard to antibiotics of last resort. In the clinical setting, we assume a similar situation that is due to the use of corresponding substances and the resulting selective pressure. On the one hand, daptomycin resistance is a growing problem among clinical MRSA isolates, whereas it is not for *E. faecium*/VRE (Bender et al., 2018a). On the other hand, and in contrast to enterococci, we have only occasionally recognized linezolid resistance in clinical *S. aureus* and MRSA isolates so far. Nevertheless, we noticed a growing number of cases of linezolid resistance among coagulase-negative staphylococci, especially among *S. epidermidis* (Bender et al., 2015; Layer et al., 2018; Wessels et al., 2018). Tigecycline resistance in clinical enterococci and staphylococci from Germany still remains rare (Bender et al., 2018a; Fiedler et al., 2016), but an outbreak with tigecycline- and vancomycin-resistant *E. faecium* isolates has already been described in a Northern German university hospital (Bender et al., 2020). As part of a collaborative approach, a review article has been published recently describing the situation of VRE/enterococci with resistances to last resort antibiotics in Europe and in addition has also suggested a common nomenclature for corresponding isolates (Bender et al., 2018a).

In Germany, there is a permanent and ongoing debate about the general burden of VRE infections and the population at risk. In this context, discussions are held about how strict measures should be applied and how effective individual measures are to control and prevent a further spread of VRE within the hospital setting (Mischnik et al., 2019; Mutters et al., 2013a; Mutters et al., 2013b; Vehreschild et al., 2019). In 2018, the Commission for Hospital Hygiene and Infection Prevention (KRINKO) published a guideline that is based on the consolidated opinion of dozens of professional medical societies in Germany (Anon, 2018). In general, KRINKO recommends specific measures only when there is sufficient evidence available from scientific studies (and provides a grading for the evidence for each measure) – a situation that is mainly impossible regarding an assessment of individual measures mostly provided as a bundle to control and prevent VRE transmission. In Germany, where VRE are highly endemic in many regions, a prevention of further VRE colonization did not appear to be an achievable goal and was therefore not further pursued. As a consequence, all measures described in this document concentrate on the prevention of infections in high-risk patients (Mischnik et al., 2019). It is known that specialists, experts and stakeholders in other European countries recommend much stricter guidelines and criticize German authorities for their special risk assessment and management regarding VRE (Vuichard-Gysin et al., 2018). Apart from VRE, that guideline paper additionally focuses on enterococci with resistances to last resort antibiotics independent of its vancomycin status for the first time. It recommends screening for LRE, also considering vancomycin-susceptible variants, when more than one isolate is notified at a single hospital ward and within a time frame of three months. Until recently, no commercial or validated medium for LRE-screening was available. Meanwhile, two possible approaches have been suggested for LRE screening, either by using a non-selective agar medium supplemented with various antibiotics including linezolid (Nordmann et al., 2019) or using an enterococcal selective agar supplemented with linezolid only (Werner et al., 2019). Additionally, in 2020, commercial LRE screening agar plates will be introduced (G.W., personal communication). However, it remains to be elucidated how well all these new screening agar plate assays will perform under routine conditions.

Table 1
Numbers of linezolid-resistant *E. faecium* isolates submitted to the National Reference Centre in Germany, 2015 to 2019.

	Numbers (all)	LIN resistant	LIN resistant %	% LVRE/LRE
2015	1163	135	11,6	60,0
2016	1666	119	7,1	62,2
2017	1777	143	8,0	61,5
2018	1652	166	10,0	71,7
2019	937	206	22,0	69,9

5. Conclusions

"Expect the unexpected" – this rather affirmative and philosophical phrase is highly true for the VRE situation in Germany during the past 30 years. VRE first appeared in Germany in 1990 and since then has remained with us. The entire period of exactly 30 years can be divided into three major VRE periods that have been specified by certain events and circumstances. The first period is marked by a reservoir of *vanA*-type vancomycin resistance in enterococci from livestock and healthy humans but comparably minor infections among hospital patients and only a few VRE outbreaks in hospitals. Although there have been episodes of relative "silence" in following years (second decade), meaning that only a few VRE outbreaks were noticed and VRE rather spread only locally or regionally, we have witnessed a constant increase in the rates and frequencies of infections with vancomycin-resistant *E. faecium* all over Germany (mainly *vanA*-type). As the surveillance schemes and systems became more comprehensive during the last ten years (third decade) and the methods for strain characterization and typing became more precise and discriminatory, the understanding of how VRE emerged and spread substantially improved. This last period is characterized by a sharp increase of *vanB*-type vancomycin resistance and a dominance of a few strain types, first ST192 and later on ST117 (CT71, CT469) and ST80 (CT1065). Between 2016 and 2019 we have seen the largest VRE outbreak involving about 2,900 patients and lasting for more than three years in a single German hospital; this outbreak was mainly caused by a novel and until that time unknown strain type of ST80/CT1013 (*vanB*) that later on was replaced by other strain types. VRE spread demonstrated a number of peculiarities: (i) prevalence rates among neighboring countries were and still are very different (Piezzi et al., 2020; Zhou et al., 2017); (ii) the increase in *vanB*-type resistance in Germany in the last ten years has not been mirrored in neighboring countries, and (iii) widely spread VRE variants like vancomycin-variable enterococci highly prevalent in Denmark (Hammerum et al., 2019a; Hansen et al., 2018; Kohler et al., 2018) and ST796 VRE widely disseminated in Australia and Switzerland have never been detected so far in Germany (Buultjens et al., 2017; Wassilew et al., 2018).

6. Future perspectives

"Prediction is very difficult, especially about the future" (Niels Bohr, 1885–1962). A review of the last 30 years of VRE in Germany suggests that the dynamics in the emergence and spread of certain strain variants will continue in the near future and the overall trend might be hardly predictable. Intensified hand hygiene practice, antibiotic stewardship programs, legal requirements and employment of more infection prevention specialists in hospitals and advances in fast and molecular diagnostics were correlated with success in reducing MRSA rates and frequencies in Germany. However, all these measures did not reduce the burden of VRE in German hospital patients suggesting other, yet unknown factors, e.g. microbiological factors associated with these developments.

It appears as if VRE respect political borders, but a stronger cross-border exchange of VRE strain variants could be expected; (i) either as an export from Germany as a high VRE prevalence country into neighboring countries showing lower VRE prevalence rates like France, The Netherlands, Switzerland, Austria and Poland and/or (ii) as an import from neighboring countries into Germany. The NRC for Enterococci will closely monitor and survey the occurrence and dissemination of strain variants that are widely spread in European countries other than Germany, like the aforementioned ST796 strain types in Switzerland. Some Scandinavian countries experienced VRE with variable expression of vancomycin resistance (VVE; similar to "stealthy vancomycin resistance") making a proper diagnosis challenging. The VVE phenomenon can also take on larger dimensions, as has been demonstrated by the expansion of a VVE strain type ST1421/CT1134 in the greater Copenhagen area in recent years (Hammerum et al., 2019b). Therefore, we need to be aware of these diagnostically challenging VVE isolates and monitor them carefully to allow for proper patient treatment.

In the near future, the endemic prevalence of certain VRE strain types will require more sophisticated, multidisciplinary and higher-resolution analyses to accurately resolve hospital outbreaks and the suspected spread of VRE across federal states and the whole country (Eichel et al., 2020; Falgenhauer et al., 2019b; Liese et al., 2019; Neumann et al., 2020a). This approach will undoubtedly be accompanied by an increased use of WGS and supporting bioinformatics' modelling as the new standard for outbreak analyses and molecular surveillance.

It is well known that *vanA*-encoded resistance is mainly plasmid-harbored, whereas *vanB* mainly resides on mobile chromosomal elements; both variants thus allow horizontal transfer of resistance properties. The first studies have now been published, which use genome comparisons to investigate the role of horizontal gene transfer in the transmission of vancomycin resistance of *vanA* and *vanB* types in outbreak settings and at population level (Arredondo-Alonso et al., 2020; Gouliouris et al., 2018; Hashimoto et al., 2020; Pinholt et al., 2019). It is important to pay more attention to this aspect in future analyses to better understand the role of the horizontal spread of vancomycin resistance in general.

In the last 10 to 15 years, Germany has experienced a strong increase and a permanently high rate of colonization and infection with VanB-type enterococci. Therefore, a thorough search for potential reservoirs of transmissible *vanB*-type resistance or resistant strains outside the hospital environment is highly desirable. To date, no sources of VanB-type VRE other than hospitals are known and hypotheses about a reservoir in the environment, livestock, food and the community emerged and have yet to be disproved or confirmed. At present, it is largely unknown whether *vanB* gene clusters that are known to predominate in anaerobic intestinal colonizers, can serve as a source for the acquisition of vancomycin resistance by hospital-associated *E. faecium* strains (Knight et al., 2016; Marvaud et al., 2011).

Last but not least, resistance to last resort antibiotics appears to be on the rise in recent years. Consequently, an intensified monitoring will be required in the near future to keep treatment alternatives available as long as possible. In particular, an increase in linezolid resistance rates among enterococci has been noticed at the NRC for several years and is now also being observed in resistance surveillance studies. Here, the mobile and thus transferable linezolid resistance determinants *cfr*, *optrA* and *poxtA* provide for another possibility of inter- and intraspecies transfer of linezolid resistance even without direct selective pressure. Many of these determinants originate from livestock, thus illustrating the close interdependence of the different origins and the need for cross-sectoral surveillance activities including high-resolution molecular analyses.

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