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Short Communication

Increased frequency of linezolid resistance among clinical *Enterococcus faecium* isolates from German hospital patients

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ABSTRACT

Linezolid is an antibiotic of last resort for the treatment of infections with vancomycin-resistant enterococci (VRE). Here we report the increasing prevalence of linezolid resistance among clinical *Enterococcus faecium* strains from German hospital patients. Linezolid minimum inhibitory concentrations (MICs) were determined for 4461 clinical *E. faecium* strains isolated between 2008 and 2014. Isolates originated from the network of diagnostic laboratories collaborating with the National Reference Centre (NRC) for Staphylococci and Enterococci covering all German federal states. All linezolid-resistant isolates were determined by broth microdilution and confirmed by Etest as well as by analysing the 23S rDNA for putative mutations. Marker genes were determined by PCR. Genotyping was performed by *Sma*I macrorestriction analysis in pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) for selected isolates. An increase in linezolid resistance was observed, from <1% in 2008 to >9% in 2014. Occasionally, outbreaks with linezolid-resistant VRE (ST117) were observed. In total, 232 (92.4%) of 251 linezolid-resistant *E. faecium* isolates (including 61 *vanA* and 29 *vanB*) contained the G2576T 23S rDNA mutation and showed a varying mixture of wild-type and mutated alleles per genome sufficient to confer linezolid resistance. In vitro growth experiments revealed a stable linezolid MIC. Of the 251 linezolid-resistant isolates, 5 were *cfr*-positive. In conclusion, these NRC data identified a country-wide ongoing trend of increasing linezolid resistance among clinical *E. faecium* isolates within the last 5 years.

1. Introduction

Multidrug-resistant enterococci occur at an increasing prevalence as a causative agent of healthcare-associated infections, especially among immunocompromised and seriously ill patients. Only a few therapeutic options remain to treat infections with multidrug- and vancomycin-resistant enterococci (VRE). Linezolid, a synthetic oxazolidinone antibiotic, represents one last-resort alternative. Resistance to linezolid occurs infrequently in *Enterococcus faecium* and *Enterococcus faecalis*; outbreaks with linezolid-resistant VRE have been reported but are still rare [1,2]. Resistance in enterococci is mediated via G2576T mutations in the 23S rDNA (*Escherichia coli* numbering). *E. faecalis* and *E. faecium* possess four and six 23S rDNA alleles per genome, respectively. It has been reported that the number of mutated versus wild-type alleles per genome correlates with the minimum inhibitory concentration (MIC) of the respective isolate [3]. The initial point mutation of a single 23S allele is the key step in resistance development, and subsequent recombination between wild-type and mutated allele variants appears frequently under selective pressure [4,5]. However, it has also been reported that under non-selective conditions linezolid-resistant strains may revert to susceptible progenitors [6,7]. Mutational changes in other ribosomal determinants such as ribosomal proteins L3, L4 and L22, which are known from other Gram-positive bacteria such as *Staphylococcus aureus*, have been very rarely described in linezolid-resistant enterococci [8]. In

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addition, plasmid-mediated linezolid resistance by a transferable methyltransferase gene *cfr* has recently been demonstrated in *Enterococcus* isolates [9].

The German Antibiotic Resistance Surveillance System, provided by the Robert Koch Institute (https://ars.rki.de), has collected antimicrobial susceptibility data for important bacterial pathogens from German patients since 2008 (28 participating laboratories and laboratory service providers, allowing country-wide coverage). Frequencies of linezolid resistance did not change over the years and remained constant at ≤1% (Table 1a). In the present study, we report the linezolid resistance frequencies and linezolid MIC distribution of all *E. faecium* isolates ($n = 4461$) that were isolated from German hospital patients and sent to the National Reference Centre (NRC) for Staphylococci and Enterococci (Wernigerode, Germany) between 2008 and 2014. In addition, the molecular causes of linezolid resistance were determined.

2. Materials and methods

2.1. Strain collection

E. faecium isolates from German hospital patients were submitted to the reference laboratory from collaborating laboratories all over Germany. The network of the NRC consists of >250 collaborating laboratories sending samples for analysis on their own request (a diagnostic laboratory generally serves several hospitals, hence the exact number of participating hospitals cannot be extracted). Between 2008 and 2014, 4461 *E. faecium* isolates were received by the NRC, for which the linezolid MICs were determined by microdilution in cation-adjusted Mueller–Hinton broth (MHB). Categorisation as susceptible and resistant was done according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (http://www.eucast.org) as follows: susceptible, ≤4 mg/L; resistant, >4 mg/L. All linezolid-resistant *E. faecium* isolates were confirmed by linezolid Etest (bioMérieux, Nürtingen, Germany). The maximum linezolid concentrations were 32 mg/L for MHB microdilution and 256 mg/L for Etest. Susceptibilities to other antibiotics, including vancomycin and teicoplanin, were determined by microdilution in MHB; categorisation as susceptible and resistant was done using EUCAST clinical breakpoints or epidemiological cut-off values. Inclusion criteria for considering isolates as 'linezolid-resistant' were based on the MHB microdilution MIC result.

2.2. Molecular characterisation and typing

PCR was performed according to validated in-house multiplex and standard PCR protocols. Multiplex 1 PCR was designed to amplify the *vanA* and *vanB* genes (including a 23S rDNA control), whereas multiplex 2 PCR was designed to amplify the *esp*, hyl_{Efm} and IS*16* genes [10]. Part of the *cfr* gene was amplified using primers as described recently [11]. The 23S rDNA wild-type and mutated alleles were amplified individually as described, followed by fragment analysis of digested PCR products on a microfluidic chip using an Agilent Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA). The newly generated *Nhe*I restriction site served to discriminate between wild-type and mutated alleles [12]. Subsequent densitometric analysis allows a qualitative assessment of wild-type and mutated alleles as well as quantification of both allele types per isolate. *Sma*I macrorestriction analysis in pulsed-field gel electrophoresis (PFGE) was done as described recently and was performed if a cluster of infections and colonisations (outbreak) was suspected [10]. Multilocus sequence typing (MLST) was done according to a standardised scheme (http://efaecium.mlst.net/) for isolates from invasive infections, individual isolates representing supposed outbreaks, and for all five *cfr*-positive strains ($n = 28$).

2.3. Serial passages to determine the stability of linezolid MICs

The stability of the linezolid resistance genotype was assessed by in vitro growth experiments of five linezolid-resistant isolates under non-selective conditions and including daily passages in MHB for 7 weeks. All isolates had initial MICs of 32 mg/L but different ratios of wild-type:mutated alleles (2:4, 3:3 and 4:2, determined as described above [12]). After each week, samples were streaked on non-selective agar plates and each time three colonies were kept for further analysis. Derived isolates were analysed phenotypically and genotypically by determining the linezolid MIC and the ratio of wild-type:mutated alleles as described above. Generation times were determined as described recently [13].

3. Results and discussion

3.1. Determination of linezolid MICs

Determining the linezolid MICs of 4461 *E. faecium* isolates sent to the reference laboratory between 2008 and 2014 revealed a permanent increase in resistance frequencies to linezolid from <1% in 2008 and 2009 to >9% in 2014 (Table 1b). The corresponding linezolid MICs in the linezolid-resistant *E. faecium* isolates available for further analyses ($n = 251$) revealed the following distribution: 63 isolates (25.1%) at 8 mg/L; 131 (52.2%) at 16 mg/L; 55 (21.9%) at 32 mg/L; and 2 (0.8%) at >32 mg/L. Linezolid resistance determined by MHB microdilution was confirmed for all 251 isolates by Etest (>4 mg/L); the linezolid Etest MIC result was, in general, slightly higher than the MHB microdilution MIC (median Etest MIC, 24 mg/L versus median MHB microdilution MIC, 16 mg/L). In total, 61 (24.3%) of the 251 isolates

Table 1a
Resistance frequencies to linezolid of clinical *Enterococcus faecium* in the German Antimicrobial Resistance Surveillance System (https://ars.rki.de).

Year	Resistant *n* (%)	Intermediate *n* (%)	Susceptible *n* (%)	Total (*N*)
2012	65 (0.9)	8 (0.1)	6888 (99.0)	6961
2011	27 (0.6)	22 (0.5)	4517 (98.9)	4566
2010	32 (0.9)	29 (0.8)	3475 (98.3)	3536
2009	27 (1.0)	25 (1.0)	2566 (98.0)	2618
2008	14 (1.0)	12 (0.8)	1401 (98.2)	1427

Categorisation was according to Clinical and Laboratory Standards Institute (CLSI) criteria, as follows: susceptible, ≤2 mg/L; intermediate, 4 mg/L; and resistant, ≥8 mg/L.

Table 1b
Resistance frequencies to linezolid of German clinical *Enterococcus faecium* sent to the National Reference Centre for Staphylococci and Enterococci.

Year	Resistant *n* (%)	Susceptible *n* (%)	Total (*N*)
2014	74 (9.4)	714 (90.6)	788
2013	78 (8.7)	823 (91.3)	901
2012	39 (4.0)	933 (96.0)	972
2011	45 (5.7)	740 (94.3)	785
2010	10 (3.0)	327 (97.0)	337
2009	3 (0.8)	352 (99.2)	355
2008	2 (0.6)	321 (99.4)	323

Categorisation was according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria, as follows: susceptible, ≤4 mg/L; and resistant, >4 mg/L.
The linezolid-resistant isolates originated from 60 diagnostic or hospital laboratories from all over Germany.