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High-level ciprofloxacin resistance among hospital-adapted *Enterococcus faecium* (CC17)

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ABSTRACT

Hospital-adapted Enterococcus faecium differ from their colonising variants in humans and animals by additional genomic content. Molecular typing based on multilocus sequence typing (MLST) allows allocation of isolates to specific clonal complexes (CCs), such as CC17 for hospital-adapted strains. Acquired ampicillin resistance is a specific feature of these hospital isolates, especially in Europe. A few recent reports have described acquired high-level ciprofloxacin resistance as a supposed feature of hospital-adapted E. faecium strains. In the present retrospective analysis, ciprofloxacin minimum inhibitory concentrations (MICs) of 609 clinical isolates from German hospital patients (1997–2007) were determined and a breakpoint for high-level resistance was deduced (>16 mg/L). Acquired high-level ciprofloxacin resistance was distributed among isolates of 26 different MLST types (all CC17), indicating a wide prevalence of this acquired resistance trait among the hospital-adapted E. faecium population. High-level ciprofloxacin resistance was linked to gyrA and parC mutations in 98 investigated isolates. Eleven different allele types or combinations thereof were identified. Their allocation to specific MLST and pulsed-field gel electrophoresis (PFGE) types revealed differences in the emergence and spread of corresponding mutations and strains.

1. Introduction

Isolates of *Enterococcus faecium* are considered facultative pathogens that appear as infectious agents only under specific predispositions and in patients with severe underlying diseases or disorders. Results from recent molecular typing and characterisation experiments, including microarray-based genome comparisons, revealed insights into the population structure within *E. faecium*. Epidemic isolates from hospital patients belong to a specific clonal group [clonal complex (CC) 17]. Among representatives of that group, distinct markers appear to be enriched or exclusively distributed, such as the collagen-adhesin genes *acm* [1–3] and *scm* [4], genes encoding other matrix-binding or pili-associated proteins [4–7], a gene encoding an enterococcal surface protein *esp* [8], a putative hyaluronidase *hyl_{Etm}* [9] and genomic islands of unknown functions [10,11]. Ampicillin resistance appears as a resistance marker for hospital *E. faecium*, at least in Europe [12–16], and high-level ciprofloxacin resistance appears to be increasingly distributed among hospital isolates of *E. faecium* and *Enterococcus faecalis* [17–20].

Isolates of the enterococcal species *E. faecalis* and *E. faecium* show generally low susceptibilities to ciprofloxacin and modern fluoroquinolones. The Clinical and Laboratory Standards Institute defines ciprofloxacin resistance in enterococci as a minimum inhibitory concentration (MIC) of \geq 4 mg/L [21]. Other international bodies such as the European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://www.escmid.org/research_projects/eucast/) do not specify a breakpoint for the combination of fluoroquinolones and enterococci. However, certain epidemic strains of *E. faecium* and *E. faecalis* infecting hospital patients may show increased MICs to ciprofloxacin (acquired 'high-level resistance') [17,18,22–25]. Colonisation often

precedes subsequent infection by facultative pathogenic bacteria such as multiresistant and vancomycin-resistant isolates of *E. faecium* and *E. faecalis*, and treatment of patients with fluoroquinolones, an antibiotic class increasingly used among hospital patients [26,27], appears as an independent risk factor for acquisition of these strains [28–30]. Increased non-susceptibility to ciprofloxacin and modern fluoroquinolones in hospital-adapted enterococcal strains may actively facilitate colonisation and subsequent infection or may simply reflect the antibiotic pressure exerted among the nosocomial setting in general.

The targets of fluoroquinolones are topoisomerases II and IV, and mutational changes among genes encoding mainly subunits A and to a lesser extent also subunits B are associated with increased MICs to ciprofloxacin and other fluoroquinolones [31,32]. Topoisomerase II (DNA gyrase) appears to be the primary target in Gram-negative bacteria and topoisomerase IV is the primary target in Gram-positive bacteria. Corresponding in vitro selection models were also described for enterococci; however, results are somehow conflicting regarding the primary target in *Enterococcus* spp. and the necessity of specific mutations in one or both A subunit genes to confer what is specified as high-level fluoroquinolone resistance [33–37].

Only a few studies exist where ciprofloxacin resistance mutations in *E. faecium* have been investigated to a greater extent and were linked to specific clonal types and strains [17,19]. In these studies, a breakpoint of high-level ciprofloxacin resistance is suggested of >16 mg/L [19] or \geq 64 mg/L (worldwide collection of strains) [17], which was confirmed by molecular analysis of mutations in *gyr* and *par* subunit genes.

In the present study, we investigated a representative collection of 609 clinical strains of *E. faecium* from German hospital patients representing individual infections or outbreaks collected between 1997 and 2007. The breakpoint for high-level ciprofloxacin resistance was specified and the annual changes in the MIC₅₀ and MIC₉₀ values (MICs at which 50% and 90% of all tested isolates were inhibited, respectively) within the test period were determined. In addition, the corresponding resistance phenotype was linked to mutations in *gyrA* and *parC* genes in a subset of 98 invasive isolates. The clonal background of the considered isolates was resolved by multilocus sequence typing (MLST) and partly by pulsed-field gel electrophoresis (PFGE), suggesting different routes of emergence and spread of high-level ciprofloxacin resistance among these hospital-adapted *E. faecium* isolates.

2. Materials and methods

2.1. Bacterial strains

Our 'Nosocomial Infections' unit (Robert Koch Institute, Germany) hosts the German National Reference Centre for Staphylococci and serves in addition as a Focal Laboratory for Enterococci. In general, enterococcal isolates sent to us are preselected for being vancomycin-resistant and multiresistant or with the suspicion of clonal spread (outbreak). Our strain collection includes more than 8000 enterococcal isolates from the early 1990s until now, the majority of which are clinical strains. The subset of strains investigated here includes clinical *E. faecium* isolated from 1997 to 2007. They were chosen as being representative by originating from different clinical laboratories or university medical centres from all over Germany. Only one isolate per hospital at a time was allowed (excluding copy isolates). Based on these criteria, approximately 55 *E. faecium* isolates per year were included summing to a total of

609 isolates. A subset of 156 isolates was MLST typed, originating exclusively from invasive infections/blood cultures. Mutations in the genes *gyrA* and *parC* were resolved for 98 of the 156 MLST typed isolates. For the purpose of comparison, altogether 74 commensal *E. faecium* isolates originating from two independent prevalence studies among healthy persons from the community in two German federal states in 2002 and 2004 were included [38,39].

2.2. Antibiotic susceptibility testing

Susceptibility to 19 antibiotics was routinely determined by microbroth dilution using IsoSensitest (until 2006) or Mueller–Hinton broth (from 2007 onwards, according to the German standard DIN58940; data not presented). MICs for ciprofloxacin were determined in a separate dilution (1–1024 mg/L) using Mueller–Hinton broth to determine high-level resistance. MIC_{50} and MIC_{90} values were defined as the MICs at which 50 and 90% of the investigated isolates were inhibited, respectively.

2.3. DNA isolation

Genomic DNA was prepared using a DNA extraction kit (DNeasy Tissue Kit; Qiagen, Hilden, Germany) according to the manufacturer's instructions. An initial cell wall lysis step was added dissolving the cell pellet in TES buffer [10 mM Tris, 0.5 mM ethylene diamine tetra-acetic acid (EDTA), 10% sucrose (pH 8.0)] plus 10 mg/mL lysozyme (Roche Applied Science, Mannheim, Germany) followed by incubation at 37 °C for 30 min.

2.4. Polymerase chain reaction (PCR)

PCR was performed with Ready-To-Go[™] PCR Beads (GE Healthcare, Freiburg, Germany) according to the manufacturer's instructions. Approximately 0.5 µL of isolated genomic DNA (ca. 10 ng) and primers (200 nM each) were added. The presence of the resistance genes *vanA* and *vanB* was tested in a multiplex PCR [40]. Primers and PCR conditions to amplify fragments of the corresponding subunits A (*gyrA*, *parC*) and B (*gyrB*, *parE*) were chosen as described recently [17] and were as follows: gyrA-F, 5'-CGGGATGAACGAATTGGGTGTGA; gyrA-R, 5'-AATTTTACTCATACGTGCTTCGG; gyrB-F, 5'-TGAAATTCTTGCTGGAAAAC; gyrB-R: 5'-CAACAATAGGACGCATGTAAC; parC-F, 5'-TTCCCGTGCATTTCGATCAGTACTTC; parC-R, 5'-CGTATGACAAAGGATTCSGTAAATC; parE-F, 5'-GTCCGTAAAGCAATCAAAG; and parE-R, 5'-CTTTATATAAAGGCGGTAACG. These primers cover the so-called

'quinolone resistance-determining region' (QRDR).

2.5. Pulsed-field gel electrophoresis typing

Genomic DNA for PFGE analysis was isolated, digested with restriction endonuclease *Sma*l and treated as described recently [40]. A 1% agarose gel concentration and a CHEF-DR III apparatus (Bio-Rad Laboratories, Hercules, CA) were used for PFGE. The ramped pulsed times were as follows: 1–11 s for 15 h and 11–30 s for 14 h at 14 °C. *Sma*l-digested *Staphylococcus aureus* NCTC 8325 was used as a size marker on all PFGE gels. Composite trees were generated using a band-based similarity coefficient (Dice) and unweighted pair group method with arithmetic mean (UPGMA) clustering (BioNumerics v. 5.1; Applied Maths, Sint-

Martens-Latem, Belgium). Definition of clusters and subgroups was according to Morrison et al. [41], based on an 82% similarity of pattern.

2.6. DNA sequencing

Sequencing reactions were performed according to the manufacturer's recommendations for cycle sequencing of PCR products (Applied Biosystems, Darmstadt, Germany). Sequence files were read, evaluated, aligned and compared with the reference set of alleles using DNASTAR's Lasergene 8.0 software (SeqMan 8.0; MegAlign 8.0, EditSeq 8.0).

2.7. Multilocus sequence typing

PCRs amplifying the seven loci used for MLST were done according to the reference MLST database (http://efaecium.mlst.net/). Sequencing reactions were performed according to the manufacturer's recommendations for cycle sequencing of PCR products (Applied Biosystems). Sequence files were read, evaluated, aligned and compared with the reference set of alleles using sequencing software Lasergene 8.0 from DNASTAR (SeqMan 8.0; EditSeq 8.0), TraceEditPro v. 1.1.1 from Ridom (http://www.ridom.de) and via the official MLST webpage (http://efaecium.mlst.net/).

2.8. Multiple-locus variable-number tandem repeat (VNTR) analysis (MLVA)

VNTRs were amplified according to Top et al. [42] using modifications given at the corresponding website of the University of Utrecht, The Netherlands (http://www.mlva.umcutrecht.nl). MLVA profiles were assigned using the corresponding tool at the webpage (see above).

2.9. Statistics

Mathematical calculations and basic statistical analyses were performed with software package EpiCompare 1.0 (Ridom).

3. Results

3.1. Distribution of ciprofloxacin minimum inhibitory concentrations

The strain collection of *E. faecium* sampled between 1997 and 2007 consisted of 3303 mostly hospital isolates from 179 clinical or university microbiological laboratories from all over Germany. A representative subset of 609 E. faecium isolates from this collection (median 53/year, excluding copy isolates) was chosen for further analysis to determine MICs for ciprofloxacin. After plotting MIC values against the number of tested strains at that MIC, a bimodal distribution was observed, suggesting a breakpoint of >16 mg/L for defining high-level ciprofloxacin resistance (Fig. 1). When MIC₅₀ and MIC₉₀ data were displayed, an almost constant annual increase was observed (Table 1), with the number of isolates belonging to the group of supposed high-level ciprofloxacin-resistant isolates also increasing annually (Table 1). For the collection of isolates from 2003 onwards, the MIC₅₀ and MIC₉₀ values were constantly above the suggested breakpoint for high-level ciprofloxacin resistance, illustrating the high prevalence of this feature among clinical isolates of E. faecium from this time onwards. A subset of 156 blood culture isolates was typed using MLST. The data showed the distribution of high-level ciprofloxacin resistance (MIC > 16 mg/L) among 26 MLST types (Table 2), all of which were allocated to CC17 using eBURST (not shown). This finding indicates that high-level ciprofloxacin

resistance did not emerge and disseminate among a limited number of a few MLST types but is rather distributed among the general hospital-adapted *E. faecium* population (CC17). In particular, early representatives (isolated before 2002) of major MLST types such as sequence type ST17 were not high-level ciprofloxacin resistant, whereas later isolates expressed higher MICs (Fig. 2a). In line with that, isolates of ST192 that were highly prevalent among German hospitals only during recent years were all high-level ciprofloxacin-resistant (n = 27, from 10 different diagnostic laboratories, isolated 2004–2007; see below). For the purpose of comparison, altogether 74 *E. faecium* isolates from two prevalence studies among non-hospitalised persons were investigated. All commensal isolates displayed MICs below the suggested breakpoint for acquired high-level ciprofloxacin resistance (MICs \leq 8 mg/L; see also Table 1).

3.2. Screening for gyrA/B and parC/E mutations

A subset of 98 MLST-typed invasive *E. faecium* isolates was selected for molecular analysis of *gyrA/B* and *parC/E* mutations. Tested isolates spanned a range of ciprofloxacin MICs from \leq 1 mg/L to 512 mg/L, with the vast majority of isolates considered as high-level ciprofloxacin-resistant (*n* = 77 with MICs of >16 mg/L). Supposed mutations in the QRDR of *gyrA* and *parC* alleles were determined (Table 3). Isolates possessing MICs of <16 mg/L did not possess any mutational changes in *gyrA* or *parC*. The most frequent mutations in isolates with ciprofloxacin MICs of ≥16 mg/L appeared at a position in *gyrA* encoding for Ser-83 and a position in *parC* encoding for Ser-80 (*E. coli* numbering) (Table 3). Occasionally, a position in *gyrA* encoding for Glu-87 was modified (4 of 98 isolates). Mutations at a position in *parC* encoding Glu-84 or any of the other described modifications [17,19,20,33] were not

identified in the present set of strains. If mutations appeared, both targets *gyrA* and *parC* were always affected; no clinical isolate possessed single mutations in either *gyrA* or *parC*. Corresponding fragments of subunits B (*parE/gyrB*) were investigated from a subset of 33 isolates, including 26 isolates with ciprofloxacin MICs of \geq 16 mg/L. It was known from previous studies in enterococci that both targets were not affected; we also did not identify any relevant mutation in *gyrB* or *parE* genes.

3.3. Pulsed-field gel electrophoresis

The most prevalent MLST types in our collection of 98 strains were ST17 (n = 28; 29%), ST192 (n = 27; 28%) and ST18 (n = 20; 20%). A different prevalence of ciprofloxacin MICs and QRDR mutations among isolates of identical MLST types prompted us to type isolates from selected STs with a highly discriminative method such as Smal macrorestriction PFGE. Isolates of ST17 and ST192 were compared. The collection of 28 ST17 isolates consisted of strains isolated between 1999 and 2007 with ciprofloxacin MICs between ≤1 mg/L and 512 mg/L (Fig. 2a). High-level ciprofloxacin-resistant isolates harboured various mutations at positions encoding Ser-83 in gyrA or Ser-80 in parC (Fig. 2a; Table 3). Individual mutations as well as combinations of gyrA and parC allele types were very diverse, suggesting an independent development of high-level ciprofloxacin resistance among various ST17 clones. Isolates of ST192 were only prevalent during recent years. All the 27 highlevel ciprofloxacin-resistant ST192 E. faecium isolates were epidemiologically unlinked and originated from 10 microbiological laboratories; however, all showed similar characteristics such as a combination of identical gyrA (Ser-83 [AGT] to IIe-83 [ATT]) and parC (Ser-80 [AGC] to Arg-80 [CGC]) mutations (Fig. 2b). In addition, all ST192 isolates possessed a silent/synonymous mutation in parC encoding His-75

(CAT to CAC), which appeared almost exclusively among isolates of that MLST type and was found only once in a non-ST192 isolate in our collection (isolate UW6918, ST17). PFGE typing and subsequent fragment analysis using the criteria of Morrison et al. [41] (82% similarity cut-off) revealed 17 clusters among the 28 ST17 isolates [discriminatory index (DI) 0.952; 95% confidence interval (CI) 0.914–0.99] and 11 clusters among the 27 ST192 isolates (DI 0.815; 95% CI 0.689–0.94).

4. Discussion

High-level ciprofloxacin resistance in isolates of E. faecium and/or E. faecalis was analysed and described in a few recent papers. However, the respective strain collections were mostly limited or restricted in terms of time, geographical coverage or any homogeneity, thus not allowing any specific epidemiological conclusions [20,33,34]. Here, based on a comprehensive collection of clinical *E. faecium* isolated from German hospital patients between 1997 and 2007, we showed a permanent increase in the MICs for ciprofloxacin (Table 1). Based on a bimodal distribution of ciprofloxacin MICs of the 609 representative isolates from that time span, an MIC of >16 mg/L could be deduced as the microbiological breakpoint (Fig. 1) for defining high-level ciprofloxacin resistance in *E. faecium*. This is in agreement with previous reports suggesting a breakpoint of >16 mg/L [19] or ≥64 mg/L [17] and is also confirmed here by sequence determination of the QRDR in gyrA and parC. All (n =77) but three isolates possessing MICs of >16 mg/L harboured modifications within the genes gyrA and parC that have been shown to be linked with ciprofloxacin resistance. Of the eight isolates having an MIC of 16 mg/L, three showed wild-type alleles and five had acquired different mutations in gyrA and parC.

In our collection of clinical strains, wild-type alleles of gyrA/B and parC/E were detectable in isolates of different MLST types and with MICs ranging from ≤ 1 mg/L to 16 mg/L and also exceptionally with higher MICs of 32 mg/L or 64 mg/L (n = 3; Table Isolates with acquired high-level ciprofloxacin resistance possessed mutations in both gyrA and parC, but MICs differed by several dilutions in isolates harbouring identical gyr/par mutations (Table 3). This indicates alternative resistance mechanisms or upregulation and downregulation of genes other than those investigated here but involved in higher expression of fluoroquinolone nonsusceptibility, such as efflux pumps or topoisomerase protection genes [36,43–45]. Several authors have also reported double mutations in *parC* and *gyrA* prevalent among high-level ciprofloxacin-resistant clinical isolates of *E. faecium* [19,20,34], whereas other reports demonstrated single parC mutations as sufficient to confer elevated ciprofloxacin MICs in natural isolates [17,33]. In some of the previous studies, sequential mutations were introduced by challenging test strains with growing concentrations of fluoroquinolones in vitro [36,37]. Differences in the in vitro selective potential were discussed as being drug-related (due to different fluoroquinolones used), or species- and even strain-dependent [35-37]. Based on our collection of isolates, we could not confirm special features associated with ciprofloxacin resistance in Gram-positive bacteria in general, such as a more susceptible target in genes encoding topoisomerase IV (parC/E), and combined with that a sequential accumulation of specific mutations in different targets increasing step by step the MICs for ciprofloxacin [31,36,37].

Taking the data already published and that from the present study together, it appears unambiguous that acquired high-level ciprofloxacin resistance is, in addition to acquired ampicillin resistance, increasingly prevalent among hospital strains of *E*.

faecium. The absence of acquired high-level ciprofloxacin resistance among commensal *E. faecium* isolates from two outpatient prevalence studies performed during the study period supports this model (Table 1). Screening for high-level ciprofloxacin resistance for all of the enterococcal strains sent to the German Focal Laboratory for Enterococci was introduced in October 2007. From this time until December 2008, in total 376 clinical *E. faecium* isolates were received (from 45 microbiological laboratories representing more than 60 different German hospitals), 364 (97%) of which expressed MICs of >16 mg/L, showing that high-level ciprofloxacin resistance is highly prevalent among clinical *E. faecium* among German hospital patients (>99% were ampicillin-resistant).

The prevalence of QRDR mutations among isolates from various MLST types differed. The supposed clonal divergence based on different QRDR mutations in *gyrA* and *parC*, for instance, among ST17 isolates could be confirmed by PFGE and subsequent cluster analysis (Fig. 2a). In contrast, all ST192 isolates possessed identical *gyrA* and *parC* mutations and results of PFGE analysis revealed a recognisable fragment pattern and a distinct level of clonal relatedness according to the set criteria (Fig. 2b) [41]. Combination of all data for the ST192 isolates suggests a model where high-level ciprofloxacin resistance once emerged among a specific ST192 clone that later on disseminated among several hospitals in different cities and federal states in Germany, before the occasional horizontal acquisition of vancomycin resistance genes (*vanA/B*) (Fig. 2b).

Between 1995 and 1999, a specific *vanA*-type *E. faecium* clone spread country-wide [46,47]. These isolates possessed a specific PFGE and plasmid pattern, were MLST type ST117 and MLVA type MT12, and demonstrated a characteristic marker gene

pattern (*esp*+, *hyl_{Efm}*-). None of the ST117 isolates investigated (n = 5) showed any *gyrA* or *parC* mutation despite reaching MICs of up to 64 mg/L (Table 3). Therefore, if and to what extend acquired high-level resistance to ciprofloxacin indeed supports the successful dissemination of isolates among the nosocomial setting is still debatable and must be resolved in further studies. The rapid spread of this resistance trait within recent years and among various clonal types and only among hospital-adapted isolates (CC17) suggests an obvious benefit for the bacterium in this specific setting. The increased prevalence of high-level ciprofloxacin resistance in clinical *E. faecium* isolates in recent years coincides with an increasing prevalence of vancomycin resistance in *E. faecium* since 2003 in Germany, accumulating in a wider prevalence of a various number of multiresistant and vancomycin-resistant clinical *E. faecium* clones [15,48,49].

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Competing interests

None declared.

Ethical approval

Not required.

References

- [1] Nallapareddy SR, Weinstock GM, Murray BE. Clinical isolates of *Enterococcus faecium* exhibit strain-specific collagen binding mediated by Acm, a new member of the MSCRAMM family. Mol Microbiol 2003;47:1733–47.
- [2] Nallapareddy SR, Singh KV, Okhuysen PC, Murray BE. A functional collagen adhesin gene, *acm*, in clinical isolates of *Enterococcus faecium* correlates with the recent success of this emerging nosocomial pathogen. Infect Immun 2008;76:4110–9.
- [3] Nallapareddy SR, Singh KV, Murray BE. Contribution of the collagen adhesin Acm to pathogenesis of *Enterococcus faecium* in experimental endocarditis. Infect Immun 2008;76:4120–8.
- [4] Sillanpaa J, Nallapareddy SR, Prakash VP, Qin X, Hook M, Weinstock GM, et al. Identification and phenotypic characterization of a second collagen adhesin, Scm, and genome-based identification and analysis of 13 other predicted MSCRAMMs, including four distinct pilus loci, in *Enterococcus faecium*. Microbiology 2008;154:3199–211.
- [5] Hendrickx APA, Van Wamel WJB, Posthuma G, Bonten MJM, Willems RJL. Five genes encoding surface exposed LPXTG proteins are enriched in hospitaladapted *Enterococcus faecium* clonal complex-17 isolates. J Bacteriol 2007;189:8321–32.
- [6] Hendrickx APA, Bonten MJM, van Luit-Asbroek M, Schapendonk CME, Kragten AHM, Willems RJL. Expression of two distinct types of pili by a hospital-acquired *Enterococcus faecium* isolate. Microbiology 2008;154:3212–23.

- [7] Sillanpaa J, Prakash VP, Nallapareddy SR, Murray BE. Distribution of genes encoding MSCRAMMs and pili in clinical and natural populations of *Enterococcus faecium*. J Clin Microbiol 2009;47:896–901.
- [8] Willems RJ, Homan W, Top J, van Santen-Verheuvel M, Tribe D, Manzioros X, et al. Variant *esp* gene as a marker of a distinct genetic lineage of vancomycinresistant *Enterococcus faecium* spreading in hospitals. Lancet 2001;357:853–5.
- [9] Rice LB, Carias L, Rudin S, Vael C, Goossens H, Konstabel C, et al. A potential virulence gene, *hyl*_{Efm}, predominates in *Enterococcus faecium* of clinical origin. J Infect Dis 2003;187:508–12.
- [10] Heikens E, van Schaik W, Leavis HL, Bonten MJM, Willems RJL. Identification of a novel genomic island specific to hospital-acquired clonal complex 17 *Enterococcus faecium* isolates. Appl Environ Microbiol 2008;74:7094–7.
- [11] Leavis HL, Willems RJ, van Wamel WJ, Schuren FH, Caspers MP, Bonten MJ. Insertion sequence-driven diversification creates a globally dispersed emerging multiresistant subspecies of *E. faecium*. PLoS Pathog 2007;3:e7.
- [12] Lester CH, Sandvang D, Olsen SS, Schonheyder HC, Jarlov JO, Bangsborg J, et al. Emergence of ampicillin-resistant *Enterococcus faecium* in Danish hospitals. J Antimicrob Chemother 2008;62:1203–6.
- [13] Oteo J, Cuevas O, Navarro C, Aracil B, Campos J; Spanish Group of The European Antimicrobial Resistance Surveillance System (EARSS). Trends in antimicrobial resistance in 3469 enterococci isolated from blood (EARSS experience 2001–06, Spain): increasing ampicillin resistance in *Enterococcus faecium*. J Antimicrob Chemother 2007;59:1044–5.
- [14] Top J, Willems R, van der Velden S, Asbroek M, Bonten M. Emergence of clonal complex 17 *Enterococcus faecium* in The Netherlands. J Clin Microbiol 2008;46:214–9.

- [15] Werner G, Coque TM, Hammerum AM, Hope R, Hryniewicz W, Johnson A, et al. Emergence and spread of vancomycin resistance among enterococci in Europe. Euro Surveill 2008;13:pii: 19046.
- [16] Freitas AR, Novais C, Ruiz-Garbajosa P, Coque TM, Peixe L. Dispersion of multidrug-resistant *Enterococcus faecium* isolates belonging to major clonal complexes in different Portuguese settings. Appl Environ Microbiol 2009;75:4904– 8.
- [17] Leavis HL, Willems RJL, Top J, Bonten MJM. High-Level ciprofloxacin resistance from point mutations in *gyrA* and *parC* confined to global hospitaladapted clonal lineage CC17 of *Enterococcus faecium*. J Clin Microbiol 2006;44:1059–64.
- [18] Woodford N, Reynolds R, Turton J, Scott F, Sinclair A, Williams A, et al. Two widely disseminated strains of *Enterococcus faecalis* highly resistant to gentamicin and ciprofloxacin from bacteraemias in the UK and Ireland. J Antimicrob Chemother 2003;52:711–4.
- [19] Torell E, Kuhn I, Olsson-Liljequist B, Haeggman S, Hoffman BM, Lindahl C, et al. Clonality among ampicillin-resistant *Enterococcus faecium* isolates in Sweden and relationship with ciprofloxacin resistance. Clin Microbiol Infect 2003;9:1011–9.
- [20] Valdezate S, Labayru C, Navarro A, Mantecon MA, Ortega M, Coque TM, et al. Large clonal outbreak of multidrug-resistant CC17 ST17 *Enterococcus faecium* containing Tn*5382* in a Spanish hospital. J Antimicrob Chemother 2009;63:17–20.
- [21] Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing*. Sixteenth informational supplement. Document M100-S16. Wayne, PA: CLSI; 2006.

- [22] Leavis HL, Bonten MJ, Willems RJ. Identification of high-risk enterococcal clonal complexes: global dispersion and antibiotic resistance. Curr Opin Microbiol 2006;9:454–60.
- [23] Top J, Willems R, Bonten M. Emergence of CC17 *Enterococcus faecium*: from commensal to hospital-adapted pathogen. FEMS Immunol Med Microbiol 2008;52:297–308.
- [24] Jureen R, Top J, Mohn SC, Harthug S, Langeland N, Willems RJL. Molecular characterization of ampicillin-resistant *Enterococcus faecium* isolates from hospitalized patients in Norway. J Clin Microbiol 2003;41:2330–6.
- [25] Nichol KA, Sill M, Laing NM, Johnson JL, Hoban DJ, Zhanel GG. Molecular epidemiology of urinary tract isolates of vancomycin-resistant *Enterococcus faecium* from North America. Int J Antimicrob Agents 2006;27:392–6.
- [26] van de Sande-Bruinsma N, Grundmann H, Verloo D, Tiemersma E, Monen J, Goossens H, et al. Antimicrobial drug use and resistance in Europe. Emerg Infect Dis 2008;14:1722–30.
- [27] Deshpande LM, Fritsche TR, Moet GJ, Biedenbach DJ, Jones RN. Antimicrobial resistance and molecular epidemiology of vancomycin-resistant enterococci from North America and Europe: a report from the SENTRY antimicrobial surveillance program. Diagn Microbiol Infect Dis 2007;58:163–70.
- [28] Donskey CJ, Helfand MS, Pultz NJ, Rice LB. Effect of parenteral fluoroquinolone administration on persistence of vancomycin-resistant *Enterococcus faecium* in the mouse gastrointestinal tract. Antimicrob Agents Chemother 2004;48:326–8.
- [29] Donskey CJ. The role of the intestinal tract as a reservoir and source for transmission of nosocomial pathogens. Clin Infect Dis 2004;39:219–26.

- [30] Bhalla A, Pultz NJ, Ray AJ, Hoyen CK, Eckstein EC, Donskey CJ. Antianaerobic antibiotic therapy promotes overgrowth of antibiotic-resistant, Gram-negative bacilli and vancomycin-resistant enterococci in the stool of colonized patients. Infect Control Hosp Epidemiol 2003;24:644–9.
- [31] Jacoby GA. Mechanisms of resistance to quinolones. Clin Infect Dis 2005;41(Suppl 2):S120–6.
- [32] Hooper DC. Fluoroquinolone resistance among Gram-positive cocci. Lancet Infect Dis 2002;2:530–8.
- [33] Brisse S, Fluit AC, Wagner U, Heisig P, Milatovic D, Verhoef J, et al. Association of alterations in ParC and GyrA proteins with resistance of clinical isolates of *Enterococcus faecium* to nine different fluoroquinolones. Antimicrob Agents Chemother 1999;43:2513–6.
- [34] el Amin N, Jalal S, Wretlind B. Alterations in *gyrA* and *parC* associated with fluoroquinolone resistance in *Enterococcus faecium*. Antimicrob Agents Chemother 1999;43:947–9.
- [35] Onodera Y, Okuda J, Tanaka M, Sato K. Inhibitory activities of quinolones against DNA gyrase and topoisomerase IV of *Enterococcus faecalis*. Antimicrob Agents Chemother 2002;46:1800–4.
- [36] Oyamada Y, Ito H, Inoue M, Yamagishi J. Topoisomerase mutations and efflux are associated with fluoroquinolone resistance in *Enterococcus faecalis*. J Med Microbiol 2006;55:1395–401.
- [37] Oyamada Y, Ito H, Fujimoto K, Asada R, Niga T, Okamoto R, et al. Combination of known and unknown mechanisms confers high-level resistance to fluoroquinolones in *Enterococcus faecium*. J Med Microbiol 2006;55:729–36.
- [38] Lietzau S, Hoewner M, von Baum H, Marre R, Brenner H. Antibiotic resistant fecal isolates of enterococci among unselected patients outside the clinical sector:

an epidemiological study from Southern Germany. Pharmacoepidemiol Drug Saf 2006;15:275–7.

- [39] Witte W, Werner G, Tietze E, Reissbrodt R, Klare I. Routes of transmission of antibiotic-resistant bacteria and their resistance determinants to humans via various environmental sources like crop food or bathing freshwater. Final report project UFOPLAN 20162211 (2001–2004); 2005. p. 1–95.
- [40] Klare I, Konstabel C, Mueller-Bertling S, Werner G, Strommenger B, Kettlitz C, et al. Spread of ampicillin/vancomycin-resistant *Enterococcus faecium* of the epidemic-virulent clonal complex-17 carrying the genes *esp* and *hyl* in German hospitals. Eur J Clin Microbiol Infect Dis 2005;24:815–25.
- [41] Morrison D, Woodford N, Barrett SP, Sisson P, Cookson BD. DNA banding pattern polymorphism in vancomycin-resistant *Enterococcus faecium* and criteria for defining strains. J Clin Microbiol 1999;37:1084–91.
- [42] Top J, Schouls LM, Bonten MJM, Willems RJL. Multiple-locus variable-number tandem repeat analysis, a novel typing scheme to study the genetic relatedness and epidemiology of *Enterococcus faecium* isolates. J Clin Microbiol 2004;42:4503–11.
- [43] Arsene S, Leclercq R. Role of a *qnr*-like gene in the intrinsic resistance of *Enterococcus faecalis* to fluoroquinolones. Antimicrob Agents Chemother 2007;51:3254–8.
- [44] Lee EW, Huda MN, Kuroda T, Mizushima T, Tsuchiya T. EfrAB, an ABC multidrug efflux pump in *Enterococcus faecalis*. Antimicrob Agents Chemother 2003;47:3733–8.
- [45] Jonas BM, Murray BE, Weinstock GM. Characterization of *emeA*, a NorA homolog and multidrug resistance efflux pump, in *Enterococcus faecalis*.
 Antimicrob Agents Chemother 2001;45:3574–9.

- [46] Klare I, Konstabel C, Badstübner D, Werner G, Witte W. Occurrence and spread of antibiotic resistances in *Enterococcus faecium*. Int J Food Microbiol 2003;88:269–90.
- [47] Reinert RR, Conrads G, Schlaeger JJ, Werner G, Witte W, Lutticken R, et al. Survey of antibiotic resistance among enterococci in North Rhine-Westphalia, Germany. J Clin Microbiol 1999;37:1638–41.
- [48] European Antimicrobial Resistance Surveillance System. EARSS annual report 2007. Bilthoven, The Netherlands: National Institute for Public Health and the Environment; 2008. p. 53–8.
- [49] Kresken M, Wallmann J, Kern WV. GERMAP 2008—antibiotic resistance and usage. Rheinbach, Germany: Antiinfectives Intelligence; 2009. p. 41–6.

Fig. 1. Bimodal distribution of ciprofloxacin minimum inhibitory concentrations (MICs) for the 609 *Enterococcus faecium* clinical isolates from German hospital patients (1997–2007).

Fig. 2. Similarity dendrogram [unweighted pair group method with arithmetic mean (UPGMA)] of *Sma*l macrorestriction analyses resolved by pulsed-field gel electrophoresis (PFGE) of epidemic *Enterococcus faecium* from multilocus sequence type (MLST) (a) ST17 and (b) ST192. An 82% similarity cut-off value has been delineated for reasons of comparison based on the criteria of Morrison et al. [41]. Origin, town from which the isolate originated (please consider that an identical 'town' could represent isolates from different hospitals); year, year of isolation; MIC CIP, minimum inhibitory concentration of ciprofloxacin (mg/L); esp and hyl, results of polymerase chain reaction (PCR) screenings for fragments of the *esp* or *hyl_{Efm}* genes; *vanA/B*, presence of *vanA* or *vanB* by PCR. * Ciprofloxacin MIC of UW6930 (ST192) was 4 mg/L in Mueller–Hinton broth owing to weak growth in Mueller–Hinton in general; the MIC was 32 mg/L in brain–heart infusion broth.

Table 1

Annual development of minimum inhibitory concentrations (MICs) for ciprofloxacin among 609 clinical *Enterococcus faecium* isolates from 1997–2007 (I) and 74 commensal *E. faecium* isolates from two outpatient prevalence studies performed in 2002 and 2004 (II)

Collection	Year	No. of	MIC ₅₀	MIC ₉₀	% of isolates	% of isolates
		isolates	(mg/L)	(mg/L)	with MIC ≤ 16	with MIC > 16
					mg/L	mg/L
	1997	51	2	4	100	0
	1998	52	2	16	94	6
	1999	61	4	64	85	15
	2000	51	4	256	61	39
	2001	55	32	256	47	53
	2002	35	4	256	54	46
	2003	51	64	256	33	67
	2004	58	128	256	16	84
	2005	53	256	256	4	96
	2006	72	256	256	8	92
	2007	70	128	256	13	87
II	2002	20	1	4	100	0
	2004	54	2	4	100	0

 $MIC_{50/90}$, MICs at which 50% and 90% of all tested isolates were inhibited,

respectively.

Table 2

High-level ciprofloxacin resistance (MIC > 16 mg/L) according to multilocus sequence

type (MLST)

MLST	No. of isolates	Town ^a	MIC (mg/L)
16	2	2	32/256
17	28 ^b	16	≤1–512
18	20 ^b	14	≤1–512
65	2	1	128/256
78	11 ^b	11	16–256
80	2	1	32
81	4 ^b	2	≤1–256
117	14 ^b	9	4–256
125	1	1	256
132	1	1	128
192	27 ^b	12	16–512 °
202	8 ^b	5	≤1–256
203	5	5	64–128
208	1	1	128
252	2	2	128/256
275	1	1	256
279	1	1	64
282	1	1	128
294	1	1	256
306	1	1	256
371	1	1	128
375	1	1	256
376	1	1	256
377	1	1	256
378	3	2	256
485	1	1	128

MIC, minimum inhibitory concentration.

^a Town from where isolates originated (hospital laboratory/microbiological laboratory).

^b Also contains isolates with ciprofloxacin MICs of \leq 16 mg/L.

^c The ciprofloxacin MIC of UW6930 (ST192) was 4 mg/L in Mueller–Hinton broth owing to weak growth in Mueller–Hinton in general; the MIC was 32 mg/L in brain– heart infusion broth.

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Table 3

Mutations leading to nucleotide substitutions in the genes gyrA and parC correlated with their corresponding ciprofloxacin minimum

inhibitory concentrations (MICs)

gyrA parC			Total (n MLST			Ciprofloxacin MIC (mg/L)										
Ser-83	Glu-87	His-75	Ser-80	Glu-84	= 98)		≤1	2	4	8	16	32	64	128	256	512
(AGT)	(GAG)	(CAT)	(AGC)	(GAA)												
+	+	+	+	+	19	16,17,18,19,81,117,202	6	2	4	1	3	2 ^a	1			
													а			
+	Lys	+	lle	+	3	16,81,252					2				1	
	(AAG)		(ATC)													
Arg	+	+	Arg	+	1	282								1		
(AGA)			(CGC)													
Arg	+	+	lle	+	2	17									2	
(AGG)			(ATC)													
Arg	+	+	Arg	+	4	17						2			2	
(AGG)			(AGA)													
Arg	+	+	lle	+	10	17,18,371					1	1	1	3	4	
(CGT)			(ATC)													
lle (ATT)	+		Arg	+	1	78									1	
			(AGA)													

lle (ATT)	+	+	Arg	+	1	17	\mathcal{O}^{*}	1			
			(CGC)			C C					
lle (ATT)	+	His	Arg	+	28	17,192 °		1	13 8 1	5	
		(CAC) ^b	(CGC)						d		
lle (ATT)	+	+	lle	+	4	17,202				4	
			(ATC)								
Tyr	+	+	lle	+	24	17,18,80,11	7		2	19	3
(TAT)			(ATC)								
Tyr	Gly	+	lle	+	1	78				1	
(TAT)	(GGG)		(ATC)								

MLST, multilocus sequence type.

+, wild-type allele.

^a MIC results were confirmed in repeated experiments.

^b Synonymous/silent mutation.

^c Altogether one ST17 and 27 ST192 isolates.

^d One isolate (UW6930, ST192) grew weakly in general in Mueller–Hinton broth with a ciprofloxacin MIC of 4 mg/L; in brain–heart infusion broth its ciprofloxacin MIC was 32 mg/L.



Fig. 1.

Dice (Opt:0.50%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]

	strain	origin	year	MIC	gyrA	parC	esp	hyl	vanA/B
				CIP	Ser83	Ser80			
	UW2493	Munich	1999	4	+	+	-	-	-
	UW6991	Karlsruhe	2006	256	Arg	Ile	+	-	-
	UW6942	Greifswald	2006	256	Ile	Ile	+	-	-
	UW3698	Berlin	2002	256	Arg	Ile	+	-	-
	UW6713	Berlin	2006	128	Arg	Ile	+	-	-
	UW6203	Schwerin	2005	256	Arg	Ile	+	-	vanA
	UW6881	Berlin	2006	256	Arg	Ile	+	-	-
	UW6449	Wolfsburg	2006	128	Arg	Ile	+	-	vanB
	UW5662	Berlin	2004	256	Arg	Ile	+	-	vanB
	UW6947	Greifswald	2006	256	Ile	Ile	+	+	-
	UW2806	Essen	1999	32	+	+	-	-	vanB
	UW6918	Berlin	2007	64	Ile	Arg	-	+	vanB
	UW2687	Berlin	1999	4	+	+	-	-	vanA
	UW3311	Leipzig	2001	32	Arg	Arg	+	-	vanA
	UW4194	Leipzig	2003	32	Arg	Arg	+	-	vanB
	UW4672	Leipzig	2003	256	Arg	Arg	+	-	vanA
	UW6695	Mönchengladbach	2006	256	Ile	Ile	-	-	vanA
	UW3132	Schwerin	2000	32	Arg	Ile	+	-	vanB
	UW5274	Göttingen	2004	256	Arg	Arg	+	-	-
	UW6151	Cologne	2005	256	Tyr	Ile	-	+	vanA
	UW6917	Berlin	2007	512	Tyr	Ile	-	+	-
	UW3544	Kiel	2002	2	+	+	-	-	vanA
	UW2490	Heidelberg	1999	≤1	+	+	-	-	vanA
	UW6589	Würzburg	2004	4	+	+	-	-	vanA
	UW2460	Köln	1999	16	Arg	Ile	+	+	vanA
	UW5352	Hanau	2004	16	Ile	Arg	+	+	vanB
	UW6900	Hanover	2006	256	Tyr	Ile	-	-	vanA
	UW6950	Kiel	2006	512	Tyr	Ile	-	-	-
I									

Dice (Opt:0.50%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]

