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Note

Development of a multiplex-PCR to simultaneously detect acquired linezolid resistance genes *cfr, optrA* and *poxtA* in enterococci of clinical origin

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ABSTRACT

Linezolid-resistant *enterococcus* spp. are increasingly recognized by diagnostic laboratories. Resistance can be mediated by the expression of *cfr, optrA* or *poxtA*. We developed a multiplex-PCR to simultaneously detect all three genes. The PCR is suitable for microbiological diagnostics in order to restrict further spread of resistances in enterococci.

Enterococci are considered the 2nd to 3rd most common nosocomial pathogen and are intrinsically resistant to a plethora of antibiotics (ECDC, 2013; Sievert et al., 2013). Last resort treatment options, such as the oxazolidinone antibiotic linezolid, are indispensable to combat infections caused by vancomycin-resistant enterococci (VRE) (Brickner et al., 2008). The prevalence of linezolid-resistant enterococci (LRE) still remains at low levels worldwide (Flamm et al., 2016; Mendes et al., 2016b). Nevertheless, LRE are increasingly recognized by National Reference Centres (NRC) or hospital laboratories (Klare et al., 2015; Bagga et al., 2018). Besides chromosomal mutations affecting the ribosomal structure, resistance can either be mediated by acquisition of resistance determinants such as the methyltransferase Cfr or the ribosomal protection proteins OptrA or PoxtA, respectively (Kloss et al., 1999; Marshall et al., 2002; Mendes et al., 2016a; Long et al., 2006; Wang et al., 2015; Antonelli et al., 2018). These genes are commonly embedded in mobile genetic elements (MGEs) as part of plasmids or present as composite transposons in the bacterial chromosome. Thus, these loci are easily transferable amongst a bacterial population, even between members of one phylum, which has already been reported for *cfr* (Cafini et al., 2016; Shen et al., 2013; He et al., 2016; Antonelli et al., 2018; Bender et al., 2015). With respect to the nature of such MGEs, a rapid distribution of *cfr, optrA* and *poxtA* is to be expected and has been demonstrated to take place, e.g. on a nationwide basis in *enterococcus* spp. clinical isolates (Bender et al., 2018). Therefore, it is of great importance to screen and monitor dissemination of those resistance determinants in order to avoid uncontrolled spread of the respective resistance loci.

We herein developed a multiplex-PCR for rapid and simultaneous detection of *cfr, optrA* and *poxtA* from whole genomic bacterial DNA. For this, enterococci were routinely grown in BHI broth at 37 °C and DNA was extracted from overnight cultures using the DNeasy blood and tissue kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Oligonucleotides used were either published previously (Kehrenberg and Schwarz, 2006; Brenciani et al., 2016) or designed as follows: poxtA_fw2 5′-AAAGCTACCCATAAAATATC-3′, poxtA_rv2 5′-TCATCAAGCTGTTCGAGTTC-3′. Expected fragment sizes range from 422 bp for *optrA*, 533 bp for *poxtA* to 746 bp for *cfr*. Extracted DNA from *E. faecium* strain UW10862 (*optrA*- and *poxtA*-positive) and from *E. faecium* UW10882 (*cfr*-positive) was used as a template for gene amplification. The reaction was set up using 5–10 ng of whole genomic DNA, 0.1 µM of each primer, 12.5 µl DreamTaq Master Mix (2×) and filled up to 25 µl with sterilised water. Amplification was carried out with an initial denaturation step at 96 °C for 2 min, followed by 30 cycles at 96 °C for 30 s, 50 °C annealing for 30 s and elongation at 72 °C for 30 s, respectively. A final elongation step at 72 °C for 5 min was carried out before putting the reaction to hold (8 °C).

A single PCR for the novel *poxtA* primers resulted in a product at the expected size of 533 bp (data not shown). In order to adjust the ratio of *poxtA* to *cfr* and *optrA* oligonucleotides, we varied the molarity of *poxtA* primer in subsequent multiplex-PCR reactions from 1- to 4-fold. As could be deduced from Fig. 1, equal molarities of each primer pair yielded a uniform amplification of all three gene products (Fig. 1, lanes 2 and 3). Increasing the amount of *poxtA* primer sequentially impairs amplification of *cfr* and *optrA* (Fig. 1, lanes 4 to 7). Amplification of

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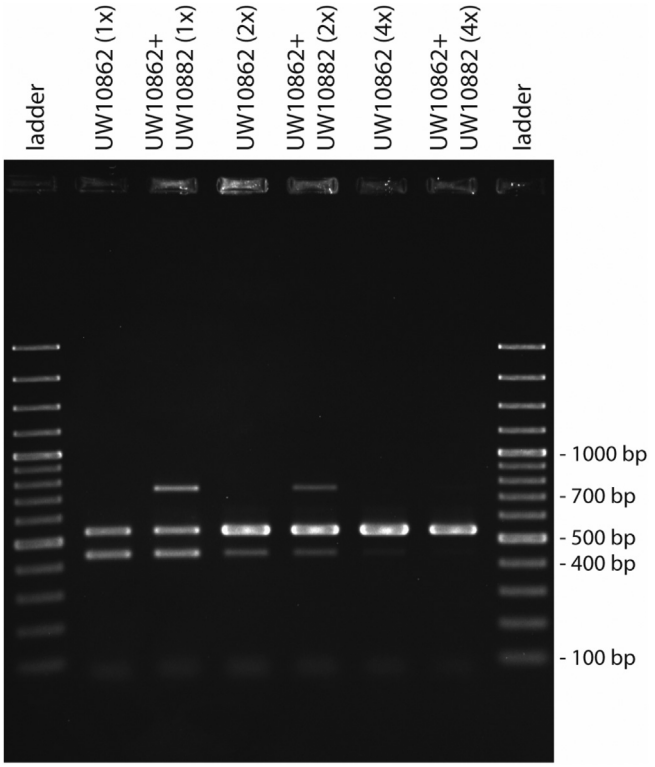

Fig. 1. Multiplex-PCR to detect *cfr, optrA* and *poxtA* from *E. faecium* clinical isolates. The intensity of the *poxtA* amplification product (middle band) gradually increases with increasing amount of *poxtA* primer (1- to 4-fold). Simultaneously, *optrA* (lower band) and *cfr* (upper band) amplification decreases. Ladder: Generuler 100 bp Plus DNA ladder (Thermo Fisher Scientific; Henningsdorf, Germany).

desired target genes was verified by Sanger sequencing (data not shown).

The multiplex-PCR was subsequently implemented to screen linezolid-resistant *enterococcus* spp. sent to the NRC for Staphylococci and Enterococci in Germany. For those isolates, where a duplex-PCR on *cfr* and *optrA* has already been performed, the single *poxtA*-PCR was carried out. We thereby retrospectively screened our entire strain collection of LRE received from 2007 up until 2018 (n = 935; including isolates from abroad). In summary, the number of LRE received by the NRC increased annually from two isolates in 2007 to 190 isolates in 2018. Linezolid-resistant *E. faecalis* remained on a constantly low level when compared to the amount of *E. faecium* isolates investigated (Fig. 2). With respect to the distribution of acquired linezolid resistance determinants, a clear association could be deduced for both *enterococcus* species. Unequivocally, *optrA* dominates in *E. faecalis* whereas *cfr* and *poxtA* seemed to associate solely and primarily with *E. faecium* clinical isolates, respectively (Fig. 2). This is congruent with reports from previous studies where *optrA* is predominantly present in *E. faecalis* (Wang et al., 2015; Cai et al., 2016; Mendes et al., 2016a; Cui et al., 2016; Lee et al., 2017). On the contrary, linezolid-resistant *E. faecium* mostly acquire mutations of 23S rDNA alleles, which has been demonstrated for our strain collection in a previous study (2007 until 2015) (Mendes et al., 2016a) (Streit et al., 2015; Klare et al., 2015). It is worth mentioning that, although chromosomal mutations were observed in *E. faecalis*, none of the *optrA*- or *poxtA*-positive *E. faecalis* isolates investigated herein displayed alterations of any of the 23 rDNA alleles (data not shown). Simultaneous occurrence of the different resistance loci within the strain collection was noticed for *optrA* and *poxtA* in six *E. faecium* isolates (2009–2017) and for *optrA* and *cfr* in a single *E. faecium* from 2017 (data not shown). On a global scale, double-carriage is still rare and, up until today, has been reported for *optrA* and *cfr* only (Brenciani et al., 2016; Li et al., 2016). The resistance genes *cfr* and *optrA* have been demonstrated to appear in multiple variants (Long et al., 2006; Bender et al., 2016; Bender et al., 2018; Cai et al., 2016) and, as part of conjugative plasmids or MGEs, are globally distributed amongst enterococci of human or animal origin (He et al., 2016; Shen

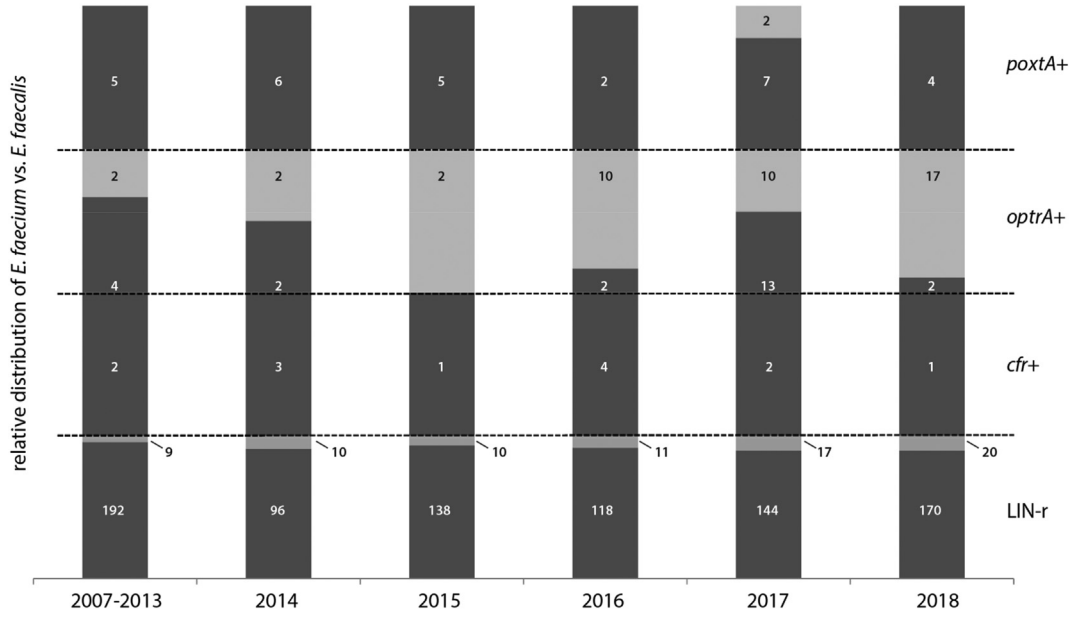

Fig. 2. Distribution of *cfr, optrA* and *poxtA* amongst *E. faecium* and *E. faecalis* clinical isolates received by the NRC for Staphylococci and Enterococci in Germany from 2007 until 2018. *E. faecalis* are represented by light grey, *E. faecium* by dark grey bars. The lower quarter of each bar displays linezolid-resistant (LIN-r) *E. faecium* and *E. faecalis* isolates per year (expressed as relative distribution of each species compared to the total number of LIN-r strains received). The upper three quarter of each bar demonstrate the species distribution for the distinct LIN-r resistance genes (expressed as relative distribution of each species for each gene). Numbers within or beside bars depict the total number (n) of isolates of each section. The majority of LIN-r *E. faecalis* harbor *optrA*, while *poxtA* was more frequently and *cfr* exclusively detected in *E. faecium* clinical isolates.

et al., 2013). As *poxtA* was discovered just recently, a similar scenario could be assumed.

The herein developed multiplex-PCR is suitable to simultaneously screen linezolid-resistant enterococci for *cfr, optrA* and *poxtA* and thus should provide a valuable tool to identify and subsequently intensify measures in order to restrict rapid dissemination of transferable resistance to the last resort antibiotic linezolid in *enterococcus* spp.

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