



Technical Note

Excellent performance of CHROMagar™ LIN-R to selectively screen for linezolid-resistant enterococci and staphylococci

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ABSTRACT

The increasing number of nosocomial pathogens with resistances against last resort antibiotics like linezolid leads to a pressing need for the reliable detection of these drug-resistant bacteria. National guidelines on infection prevention, e.g., in Germany, have already recommend screening for linezolid-resistant bacteria, although a corresponding screening agar medium has not been provided. In this study we analyzed the performance and reliability of a commercial, chromogenic linezolid screening agar. The medium was capable to predict more than a hundred linezolid-resistant isolates of *E. faecium*, *E. faecalis*, *S. aureus*, *S. epidermidis*, and *S. hominis* with excellent sensitivity and specificity. All isolates were collected at the National Reference Centre between 2010 and 2020.

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1. Introduction

Last resort antibiotics such as linezolid, tigecycline, and daptomycin are therapeutic alternatives for the treatment of infections with multidrug-resistant staphylococci and enterococci including methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis* (MRSE), and vancomycin-resistant enterococci (VRE). Although an increased use of these substances has already led to increasing rates of resistance, corresponding studies that show a clear association between use and resistance progression are limited to single institutions (Bai et al., 2019; Jian et al., 2020; Wessels et al., 2018; Zou and Xia, 2020).

Linezolid is a synthetic oxazolidinone antibiotic that is active against most multidrug-resistant, clinical isolates of staphylococci, and enterococci such as MRSA, MRSE and VRE. Although linezolid was already approved for commercial use in 2000, resistance surveillance schemes and systems did not report increasing linezolid resistance in clinical isolates over time ((ECDC 2020; Heining et al., 2020; Markwart et al., 2019; Pfaller et al., 2019; Walter et al., 2015; Walter et al., 2017; Zou and Xia 2020). However, in recent years a trend toward an increased prevalence of linezolid resistance in clinical *E. faecium* and *S. epidermidis* isolates has been recognized at the German National Reference Centre for Staphylococci and Enterococci, while the numbers of linezolid-resistant *S. aureus* remained stable

(Klare et al., 2015; Klare et al., 2019; Layer et al., 2019). Therefore, reference laboratories may play an important role in the early recognition of upcoming resistance trends before they might get noticed in surveillance schemes (Klare et al., 2015; Klare et al., 2019; Layer et al., 2019).

In contrast to most protein synthesis inhibitors, linezolid targets the 23S ribosomal DNA of the 50S subunit, thus preventing the formation of a functional 70S-initiation complex. In staphylococci and enterococci, development of oxazolidinone resistance has been associated with mutations in 23S rDNA alleles (Bender et al., 2018; Ruiz-Ripa et al., 2020; Sadowy, 2018). Additionally, variations in ribosomal protein genes may occur (Bender et al., 2018; Rouard et al., 2017). A reservoir of transferable linezolid resistance genes was selected in animal farming and reported for nonclinical isolates of *E. faecium* and *E. faecalis* and coagulase-negative staphylococci, coded by *cfr* (including *cfr*(B)) and/or *optrA* (Cuny et al., 2017; Wang et al., 2015). Corresponding resistance determinants were also reported in enterococcal and staphylococcal isolates from healthy humans and hospitalized patients (Bender et al., 2018; Chen et al., 2019; Cai et al., 2019; Deshpande et al. 2018; Elghaieb et al., 2020; Gawryszewska et al., 2017; Zhou et al., 2019). In 2018, the novel linezolid resistance gene *poxxA* was first described from a clinical MRSA in Italy (Antonelli et al., 2018) and later demonstrated to be wider distributed among enterococci of various origins (Bender et al., 2019; Egan et al., 2020; Elghaieb et al., 2019; Freitas et al., 2020). However, it is not entirely clear whether these transferable resistance determinants can mediate linezolid resistance in various strain backgrounds,

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as e.g., experimental data question the role of *cfr*(B) in linezolid resistance in *E. faecium* or *E. faecalis* (Bender et al., 2016; Liu et al., 2014).

An important step in preventing the dissemination of linezolid-resistant staphylococci and enterococci in the nosocomial setting is the reliable identification and early recognition of colonized hosts. In 2018, the German Commission for Hospital Hygiene and Infection Prevention (KRINKO) published a recommendation for the prevention of infections with “enterococci with special antibiotic resistances” (KRINKO, 2018). The guideline recommended screening for linezolid-resistant enterococci (LRE), when more than a single case is notified within 3 months and an epidemiological link cannot be excluded. However, the technical implementation of an appropriate screening has not been specified. In the last 2 years 2 studies introduced screening agar media for the detection of linezolid-resistant staphylococci and enterococci. The first study, published by Nordmann et al., introduced a selective screening agar called “SuperLinezolid” medium (Nordmann et al., 2019). The authors used a non-selective Mueller-Hinton agar supplemented with aztreonam, colistin and amphotericin B to suppress the normal flora and a linezolid concentration of 1.5 mg/L. The study sample mainly included *S. epidermidis* isolates, for which, performance was very good. In the same year, Werner et al. introduced a selective LRE screening agar, which was based on Enterococcoselagar™ supplemented with 2 mg/L linezolid. Reliable screening to detect all LRE required a prolonged incubation of up to 48 h at 37°C (Werner et al., 2019). The agar revealed a sensitivity and specificity of 96.6% and 94.4%, respectively.

With the present study we assessed the performance of a novel, commercial screening agar called CHROMagar™ LIN-R. The agar allows a species- and genus-specific diagnostics based on a cleavage of chromogenic substrates and a detection of linezolid-resistant staphylococci and enterococci by a supplementation of linezolid. We tested this agar with a well-characterized collection of 48 clinical enterococci (40 *E. faecium* (29 linezolid-resistant [LIN-r]), 8 *E. faecalis* (7 LIN-r), 17 *S. aureus* isolates (14 LIN-r) and 70 coagulase-negative staphylococci (50 *S. epidermidis* (39 LIN-r), 20 *S. hominis* (15 LIN-r)).

2. Materials and methods

2.1. Strain collection

For CHROMagar™ LIN-R validation, we included 40 isolates of *E. faecium* (29 LIN-r) and 8 of *E. faecalis* (7 LIN-r). These strains have already been described and used in a previous assay validation (Werner et al., 2019). In addition, we compiled the following staphylococcal isolates' collection: 17 *S. aureus* (14 LIN-r), 50 *S. epidermidis* (all methicillin-resistant [MRSE]; 39 LIN-r), and 20 *S. hominis* (15 linezolid- and methicillin-resistant [MRSH]; 5 linezolid- and methicillin-susceptible [MSSH]). Isolates were partly described in recent publications (Bender et al., 2015; Layer et al., 2018; Wessels et al., 2018). All isolates had been sent previously from diagnostic laboratories to the National Reference Centre for strain characterization, typing and/or resistance confirmation as part of the routine work of the reference center activities (Supplementary Table S1). The following reference isolates were used: *E. faecalis* ATCC 29212, *E. faecium* ATCC 19434^T, *S. aureus* ATCC 25923, and *E. coli* ATCC 25922.

2.2. Susceptibility testing

Susceptibilities to vancomycin and linezolid were determined by broth microdilution using cation-adjusted Mueller-Hinton broth (Becton-Dickinson, Heidelberg, Germany) according to EUCAST guidelines (EUCAST v8 to v10). Oxacillin resistance was deduced from results of a cefoxitin screen, oxacillin broth dilution MICs according to EUCAST and the detection of the *mecA* gene by PCR. Linezolid resistance is defined as R >4 mg/L for *Enterococcus* spp. and *Staphylococcus* spp. In case of contradictory results, linezolid MIC

results were additionally confirmed by Etest® (bioMérieux, Nuertingen, Germany).

2.3. Determination of resistance genotypes

Genetic determinants coding for vancomycin resistance in *E. faecium* (*vanA*, *vanB*) and oxacillin resistance in *S. aureus* (*mecA*) were identified by the use of multiplex PCR (Bender et al., 2015; Bender et al., 2018). For enterococcal isolates, 23S rDNA mutations were determined by an amplification-restriction-based procedure as described recently (Werner et al. 2004). Additionally, the presence of the transferable LIN-r resistance genes *cfr*(B), *optrA* and *poxtA* was assessed using a multiplex PCR (Bender et al., 2019). For isolates where whole genome sequence data were available, we used LRE Finder to confirm the genetic basis of linezolid resistance in enterococci (Hasman et al., 2019). Staphylococcal isolates were screened for presence of the *cfr* gene by PCR according to (Kehrenberg and Schwarz, 2006). For *S. aureus* strains genes, encoding the central loop of domain V of 23S rRNA, were analyzed for mutations conferring linezolid resistance (Tsioupras et al., 2001). Detailed information can be found in Supplementary Table S1.

2.4. Testing CHROMagar™ LIN-R plates

All isolates were tested on CHROMagar™ LIN-R. CHROMagar LIN-r is a brand of CHROMagar (Paris, France), which is distributed in Germany by MAST Diagnostica (Heidelberg, Germany). In brief, 10 µL of a freshly prepared bacterial suspension of McFarland 0.5 (approximately 1.5 × 10⁶ CFU) were inoculated onto the agar plates. Cell density was in a range that allowed single colony growth after streaking the sample out. Growth, colony morphology and color were determined after 24 hours (overnight) and 48 hours (2d) at 35 to 37°C.

2.5. Statistical analyses

Statistical values were calculated according to https://www.medcalc.org/calc/diagnostic_test.php.

3. Results and discussion

3.1. Validating CHROMagar™ LIN-R for enterococci

All linezolid-susceptible reference isolates performed accordingly on CHROMagar LIN-R (no growth after 48 hours). Out of 48 *E. faecium* and *E. faecalis* isolates, 47 performed as expected on CHROMagar LIN-R: While 35 of 36 linezolid-resistant enterococcal isolates grew on CHROMagar, 12 linezolid-susceptible isolates showed no growth. Importantly, to predict a correct screening result, an incubation of up to 48 hours was required. Growth of linezolid-resistant enterococci was delayed on CHROMagar LIN-R revealing only a slight growth after overnight incubation, meaning that growth was not visible as single colonies and colony color was not fully expressed (Fig. 1). A prolonged incubation was also required with the LRE screening agar introduced recently (Werner et al., 2019). For trained and experienced personal a differentiation based on colony color was possible for *E. faecalis* (turquoise blue) and *E. faecium* (dark blue) (Fig. 1).

A single *E. faecium* isolate UW19492 with an initial linezolid MIC of 8 mg/L (broth microdilution; LIN-r) did not grow on CHROMagar LIN-R. Repeated susceptibility testing by broth microdilution and Etest revealed contradictory MIC results for the respective isolate (broth microdilution: 4 mg/L (LIN-s) and 8 mg/L (LIN-r); Etest: 3 mg/L (LIN-s)). A molecular analysis of this isolate revealed no transferable linezolid resistance determinant and a 23S rDNA alleles' ratio of 4 (wildtype) to 2 (mutant). A wildtype to mutant 23S rDNA alleles' ratio of 4:2 is mostly associated with phenotypic linezolid resistance (Marshall et al., 2002; Werner et al., 2019). Due to these conflicting

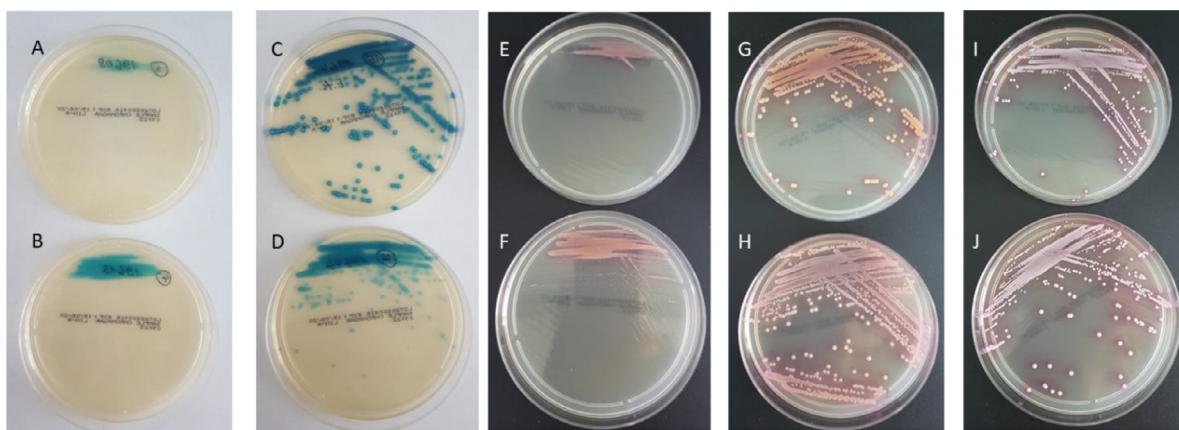


Fig. 1. Growth behavior of linezolid-resistant isolates of *E. faecium*, *E. faecalis*, *S. aureus* and *S. epidermidis*. *E. faecium* with linezolid (LIN) MICs of 8 (A) and 32 mg/L (B) after overnight incubation. *E. faecalis* (C; LIN MIC = 32 mg/L) and *E. faecium* (D; LIN MIC = 8 mg/L) grown for 48 hours. Isolates of *S. aureus* grown for 24 hours (E; LIN MIC = 16 mg/L) and (F, LIN MIC = 8 mg/L) and 48 hours (G, H; LIN MICs = 8 mg/L). Isolates of *S. epidermidis* with LIN MICs of >16 mg/L after 48 hours (I, J).

Table 1
Performance of CHROMagar™ LIN-R to identify linezolid-resistant staphylococci and enterococci.

| Bacterium | LIN-r | LIN-s | Grown | Not grown | Specificity (%) | 95% CI (%) | Sensitivity(%) | 95% CI (%) |
|-----------------------------------|-------|-------|-------|-----------|-----------------|------------|----------------|------------|
| <i>Enterococcus</i> spp., n = 47* | 35 | 12 | 35 | 12 | 100% | 73.5–100 | 100% | 90–100 |
| <i>S. aureus</i> , n = 17 | 13 | 4 | 13 | 4 | 100% | 47.8–100 | 100% | 73.5–100 |
| <i>S. epidermidis</i> , n = 50 | 39 | 11 | 38 | 12 | 100% | 71.5–100 | 97.4% | 86.5–99.94 |
| <i>S. hominis</i> , n = 20 | 15 | 5 | 15 | 5 | 100% | 47.8–100 | 100% | 78.2–100 |
| Sum | 101 | 33 | 100 | 34 | 100% | 89.7–100 | 99.01 | 94.6–99.97 |

E. faecium and *E. faecalis* were summarized as *Enterococcus* spp. LIN-r/-s, linezolid-resistant/-susceptible.

* An *E. faecium* isolate which appeared linezolid-resistant (8 mg/L) and linezolid-susceptible (4 mg/L) in repeated MIC tests and with different methods could not be categorized and thus was excluded from this analysis.

phenotypic and genotypic results we were unable to classify this isolate unambiguously as LIN-r or LIN-s and have excluded it from subsequent analyses and calculations (Table 1).

3.2. Validating CHROMagar™ LIN-R for staphylococci

All 4 linezolid-susceptible *S. aureus* isolates did not grow on CHROMagar LIN-R after up to 48 hours incubation at 35 to 37°C. As expected 16 of 17 linezolid-resistant *S. aureus* isolates grew on CHROMagar LIN-R (Table 1). The morphology of *S. aureus* colonies was mainly characterized by yellowish-pink colony color (Fig. 1). A single isolate with an initial linezolid MIC of 8 mg/L (LIN-r) showed no colony formation on the screening agar. In repeated MIC determinations the isolate revealed linezolid MIC values of 2 and 4 mg/L (LIN-s). Subsequent molecular analyses revealed a loss of the *cfr* encoding resistance plasmid. This single case demonstrates the excellent predictive power of the tested screening agar by identifying an isolate with an incorrect categorization which has been re-tested and then re-classified as “linezolid-susceptible”.

All eleven linezolid-susceptible *S. epidermidis* and 5 linezolid-susceptible *S. hominis* isolates did not grow on CHROMagar LIN-R after 48 hours incubation at 35 to 37°C. Altogether 42 of 43 linezolid-resistant *S. epidermidis* and *S. hominis* isolates grew on CHROMagar LIN-R. A single *S. epidermidis* isolate showed no colony formation but revealed a broth microdilution MIC of 16 mg/L (LIN-r) and an Etest MIC of 12 mg/L in repeated tests. Thus, this result must be considered “false negative”. Growth of coagulase-negative staphylococci was mainly characterized by whitish-pink colonies (mainly white colonies and a pink surrounding; Fig. 1).

3.3. Comparison to previously published linezolid screening media

In 2019, Nordmann *et al.* introduced a Super Linezolid agar which is based on Mueller-Hinton agar supplemented with 1.5 mg/L linezolid. The medium was additionally supplemented with aztreonam

(2 mg/L), colistin (15 mg/L) and amphotericin B (5 mg/L) to suppress microorganisms of the normal intestinal flora. The agar was supposed to be used to detect linezolid-resistant Gram-positive bacteria. However, the collection contained primarily linezolid-resistant *S. epidermidis* (n = 13) and only a limited number of linezolid-resistant isolates of other genera and species such as *S. aureus* (n = 2), *S. capitis* (n = 1), and *E. faecium* (n = 1). Due to the low number of isolates other than *S. epidermidis*, a general assumption about performance of this agar with a concentration of linezolid of 1.5 mg/L cannot be made. Recently, our group introduced a LRE screening agar which was based on an enterococcal screening agar (Enterococcoselagar™, Becton-Dickinson, Heidelberg, Germany) supplemented with 2 mg/L linezolid (Werner *et al.*, 2019). The agar was validated with several reference strains and 48 well-characterized enterococcal isolates (the same that were used for this study), which revealed a high sensitivity (98.8%) and specificity (100%). A feasibility study using 400 swab samples of enterococci sent to the Reference Centre for further analyses but of unknown linezolid status also revealed good results. An application study with a clinical partner is currently underway and preliminary results confirm the good specificity and sensitivity of predicting LRE directly from swabs (Weber *et al.*, unpublished results).

There are reports about staphylococcal and enterococcal isolates containing linezolid resistance genes and which exhibited linezolid MICs of 4 mg/L or less (interpreted as linezolid-susceptible) (Li *et al.*, 2015; Wardenburg *et al.*, 2019). Whereas the clinical significance of this observation remains to be determined, it is obvious that such isolates represent a diagnostic challenge in general and in particular for the present CHROMagar™ LIN-R and all other corresponding screening agar media.

4. Conclusion

The present study includes the largest number of linezolid-resistant isolates of *Enterococcus* spp. and *Staphylococcus* spp. which has

been tested so far with a novel, chromogenic linezolid screening agar. CHROMagar LIN-R demonstrated an excellent performance to selectively grow linezolid-resistant isolates of *E. faecium*, *E. faecalis*, *S. aureus*, *S. epidermidis* and *S. hominis* while retaining sufficient specificity to suppress growth of linezolid-susceptible variants of the same species also after incubation of up to 48 hours and at 35 to 37°C. Summarizing all available data from this study, the agar revealed a specificity of 100% and a sensitivity of 99% (Table 1). Even species prediction from plates after 2 times overnight incubation was possible for the experienced and trained expert (Fig. 1). We did not recognize growth differences in relation to the linezolid MIC or a corresponding linezolid resistance mechanism. However, about half of the linezolid-resistant staphylococci already demonstrated single colony growth after overnight incubation, whereas the other half and almost all linezolid-resistant enterococci required longer incubation of up to 2 times overnight. The CHROMagar LIN-r will be commercially available soon. The instruction leaflet (package insert) will consider the results of the internal and external (this study) evaluations. Further analysis must now prove performance under routine clinical conditions with swab samples taken from hospital patients.

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Author contributions

F.L., G.W., conceptualization and project supervision; C.F., R.W., laboratory analyses and raw data collection; B.S., C.C., F.L., R.W., resources and sample pre-assessment; F.L., G.W., R.W., data curation and validation; G.W., writing of the original draft; all authors, review and editing.

Declaration of competing interest

All authors have nothing to declare.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.diagmicrobio.2020.115301.

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