LABORATORY DETECTION OF COLISTIN-RESISTANT *ENTEROBACTERALES* IN TANDEM WITH ROUTINE ANTIBIOGRAM

Stefana Sabtcheva

Laboratory for Clinical Microbiology, University specialized hospital for active treatment in oncology (National oncology center), Sofia, Bulgaria

ABSTRACT

Since the emergence and spread of carbapenemase-producing *Enterobacterales*, particularly those carrying metallo-β-lactamases with 16S rRNA methyltransferases for which newly introduced antibiotics are inactive, colistin is a last resort therapy for life-threatening extensively drug-resistant infections. This requires that laboratories use an accurate and reliable method for routine colistin susceptibility testing. The aim of this study was to evaluate the performance and applicability of a colistin screening medium for detection of colistin-resistant isolates simultaneously with routine susceptibility testing. It was evaluated with fifty colistin-resistant and the same number of colistin-susceptible comparator isolates. Our results showed that all colistin-resistant isolates grew on DiaPlate™ EMB Agar + Colistin medium within the standard antibiogram period. In contrast, no growth was observed among the colistin susceptible comparators. DiaPlate™ EMB Agar + Colistin is a screening medium that can detect colistin- resistant *Enterobacterales* isolates when pure bacterial cultures are tested. *As this method for detecting colistin-resistant isolates uses the same inoculum as the Kirby-Bauer method, the screening test for colistin resistance can be conveniently performed on clinical isolates along with routine antimicrobial susceptibility testing.*

Keywords: *Enterobacterales*, colistin resistance, phenotypic detection

ADDRESS FOR CORRESPONDENCE:
Stefana Sabtcheva
Laboratory for Clinical Microbiology, University specialized hospital for active treatment in oncology
6 Plovdivsko pole Str. 1797 Sofia, Bulgaria
phone: +359 2 80 76 293
e-mail: stefanasabtcheva@gmail.com

INTRODUCTION

Polymyxins have a narrow antibacterial spectrum including some Gram-negative bacteria such as *Citrobacter* spp., *Enterobacter* spp., *Escherichia coli*, *Klebsiella* spp., *Salmonella* spp., *Shigella* spp., *Acinetobacter* spp., *Pseudomonas aeruginosa* and most strains of *Stenotrophomonas maltophilia.* They are not active against *Proteus* spp., *Providencia* spp., *Serratia* spp., *Edwardsiella* spp., *Morganella* spp., *Hafnia* spp., Gram-negative cocci, Gram-positive organisms and most anaerobic bacteria (1). In Europe, colistin was first used in 1950, but was gradually abandoned in clinical practice due to its nephrotoxicity and neurotoxicity, as well as to the availability of less toxic and clinically more effective drugs. The renaissance of colistin is linked to the emergence and rapid spread of carbapenemase-producing *Enterobacterales*, particularly those carrying metallo-β-lactamases with 16S rRNA methyltransferases, for which the newly introduced drugs are not active. For many years, colistin-based combinations have been successfully used to treat extensively drug-resistant infections. However, after the overlaying of plasmid-mediated colistin resistance to the mechanisms driven by chromosomal mutations, colistin resistance has steadily increased and it has become imperative to perform accurate colistin susceptibility testing in routine microbiological practice (2).

The recommended method for colistin susceptibility testing, by both EUCAST and CLSI, is broth microdilution (BMD), which should be performed in polystyrene microtitre plates using sulphate salt of colistin without addition of surfactants, according to the ISO standard 20776-1 (3). There are several reasons for this. On the one hand, due to colistin's large molecular weight, diffusion in agar is ineffective, making agar-based methods such as gradient strips or disk diffusion unsuitable (4). On the other hand, colistin binds to polystyrene microtiter plates, reducing the concentration of the active compound in the media. At the same time, the addition of surfactants, such as polysorbate-80, is

not an option due to the synergic effect with colistin (5). Finally, several reports have been published on the unreliability of semi-automatic systems for colistin resistance determination (4, 5). As the use of the reference method is labor-intensive, many routine laboratories still use gradient strips or semi-automated systems, despite EUCAST's warning to use only BMD (6).

Recently, a universal culture medium for screening of colistin-resistant bacteria in stool samples has been developed (7). The SuperPolymyxin™ is based on eosin-methylene blue agar (EMB) and contains colistin at an optimal concentration of 3.5 µg/ml, amphotericin B to suppress fungal growth, and daptomycin to suppress the growth of Gram-positive cocci that may still be growing on EBM medium. This medium was chosen because of its selective-differentiating characteristics, which enable additional distinction between enterobacterial species. In search of an easy and inexpensive method to detect colistin-resistant clinical isolates simultaneously with routine antibiogram, we adopted Proevska's simplified SuperPolymyxin medium. It contains only colistin and its use is convenient when pure bacterial cultures are tested (8).

The aim of this study was to evaluate the performance and applicability of a commercial DiaPlate™ EMB Agar + Colistin medium for detection of colistin-resistant isolates in tandem with routine susceptibility testing.

MATERIAL AND METHODS

The study involved one hundred clinical Enterobacterales isolates divided into two groups. The first group included a total of 50 colistin-resistant strains. Of these, 40 strains of Klebsiella pneumoniae were already confirmed with acquired colistin resistance (9); 8 isolates (Serratia marcescens, Morganella morganii, Proteus mirabilis, P. vulgaris, P. penneri, Hafnia alvei, Providencia stuartii and P. rettgeri, one isolate each) had intrinsic colistin resistance; and 2 reference strains of Escherichia coli (E. coli NCTC 13846 and EQA 4320) *possessed the mcr-1 gene. The second group comprised the same number of colistin-susceptible isolates, including 20 K.pneumoniae, 10 E.coli, 5 Citrobacter freundii and 5 Enterobacter cloacae complex.*

DiaPlate™ EMB Agar + Colistin medium (Diachim, Bulgaria), evaluated in this study, was developed to detect colistin resistance in pure culture of clinical isolates intended for routine susceptibility testing. It was therefore made from EMB agar to which only colistin sulphate was added at a final concentration of 3.5 µg/ml (7). The colistin agar plate was divided into eight parts with a marker, allowing up to 6 clinical isolates per plate to be tested (Figure 1). Each plate was always inoculated with both quality control strains: *E. coli* NCTC 13846, colistin-resistant and *E. coli* ATCC 25922, colistin-susceptible (10). The test was performed using the standard disk diffusion procedure (11). The bacterial inoculum was prepared in sterile saline to obtain a turbidity of 0.5 McFarland. Using a swab dipped in the suspension, after the excess liquid has been removed, streaks were applied to the appropriate part of the colistin agar plate. The plates were incubated at 35±1°C, 18±2h as for antibiograms. A result was considered valid if the quality control strains demonstrated the expected lack of growth for *E. coli* ATCC 25922 and the presence of confluent growth for *E. coli* NCTC 13846. The presence of growth, confluent or as isolated colonies, in the inoculated area was considered a positive result. The colistin agar plate was inoculated in parallel with the routine antibiogram using the same 0.5 McFarland bacterial suspension. In addition, the minimum inhibitory concentration (MIC) of colistin was determined by broth microdilution using the MIKROLATEST MIC Colistin strip (ErbaLachema, Czech Republic) following the manufacturer's protocol.

RESULTS AND DISCUSSION

All of the 50 colistin-resistant isolates grew on the DiaPlate™ EMB Agar + Colistin medium in 20h. In contrast, no growth was observed among the colistin-susceptible comparator isolates. The sensitivity and specificity of the DiaPlate™ EMB Agar + Colistin for detecting colistin-resistant isolates were 100%. Figure 1 demonstrates a typical growth image on the DiaPlate™ EMB Agar + Colistin medium for colistin-resistant *E. coli*, *K. pneumoniae*, *P. stuartii* and *S. marcescens*.

Our results confirmed the excellent screening capabilities of EMB agar containing colistin at an optimal concentration of 3.5 µg/ml, regardless of

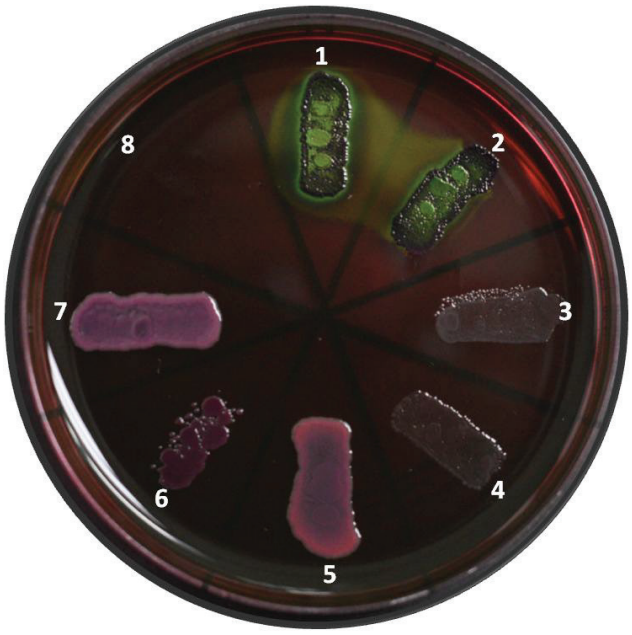

Figure 1. Detection of colistin-resistant *Enterobacterales* using the DiaPlate™ EMB Agar + Colistin medium. Clockwise: *E. coli* NCTC 13846 *mcr-1* positive, colistin MIC = 4 mg/L (1); *E. coli* EQA 4320 *mcr-1* positive, colistin MIC = 4 mg/L (2); *Providencia stuartii,* colistin MIC >16 mg/L *(3); Serratia marcescens* colistin MIC >16 mg/L *(4); Klebsiella pneumoniae* PR3759, colistin MIC >16 mg/L *(5); K. pneumoniae* PR3760, colistin MIC = 8 mg/L *(6); K. pneumoniae* PR3761, colistin MIC >16 mg/L *(7); E. coli* ATCC 25922, colistin MIC = 0.5 mg/L *(8).*

intrinsic, acquired chromosomal or plasmid-mediated resistance (7). Indeed, SuperPolymyxin™ medium is a screening medium for polymyxin-resistant bacteria with a wide range of applications, including detection of colistin-resistant carriers in human medicine and surveillance studies in veterinary medicine. Whereas, the DiaPlate™ EMB Agar + Colistin medium, evaluated here, appears to be a simple and cost-effective method for accurate and reliable detection of colistin-resistant *Enterobacterales* isolates in tandem with routine antibiogram.

CONCLISION

DiaPlate™ EMB Agar + Colistin is a screening medium that can detect all colistin-resistant Enterobacterial isolates, irrespective of resistance mechanism and level of colistin resistance. As this method for detection of colistin-resistant isolates uses the same inoculum as the Kirby-Bauer method, colistin resistance screening test can be conveniently performed on clinical isolates simultaneously with routine antimicrobial susceptibility testing.

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