Infection and Drug Resistance

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REVIEW

Plastic binding feature of polymyxins: the effect on MIC susceptibility measurements

This article was published in the following Dove Press journal:
Infection and Drug Resistance

Infection and Drug Resistance downloaded from https://www.dovepress.com/
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Abstract: The loss of polycationic antimicrobial peptides, polymyxins, due to adhesion to plastics is an important subject matter that influences in vitro susceptibility testing, including minimum inhibitory concentration (MIC) assays. This review reminds us that this issue can serve as a significant source of variation in the MIC values for polymyxins against Gram-negative bacteria.

Keywords: broth microdilution, colistin, Gram-negative bacteria, MIC, polymyxin B

Introduction

Given the rise of multidrug-resistant Gram-negative bacilli, such as *Pseudomonas aeroginosa, Acinetobacter baumannii*, and *Klebsiella penomoniae*, as well as the limited number of effective antibiotics, polymyxin class of antibiotics (polymyxin B and polymyxin E [colistin]) are currently the only remaining treatment options.[1,2] This enforces a significant effort to maintain the antibacterial properties of these antibiotics and delay the emergence of resistance. Development of clear guidance regarding the indications, dosing, duration, and combination of polymyxins appears to be needed to improve clinical outcomes.[1] In addition, standardization of routine susceptibility testing of clinical isolates for polymyxins may be a major contributor to achieve this purpose.[3]

Both the Clinical and Laboratory Standards Institute and the European Committee on Antimicrobial Susceptibility Testing have considered broth microdilution (BMD) as a gold standard reference method for MIC determination of all antibiotics.[4,5] It has been recommended that the assay should be conducted with cation-adjusted Mueller–Hinton broth, a range of two-fold dilutions of an antibiotic, and a final inoculum size of 5×10^5 CFU/mL in round or conical bottom wells of a sterile plastic microdilution plate.[6] Some technical problems have been reported with polymyxin MIC testing, including brand-to-brand heterogeneity in polymyxin formulations, drug binding to plastic materials, the presence of "skipped well" phenomenon, brand-to-brand variation in cation composition of MH media, and the potential antimicrobial activity of additives (eg, polysorbate-80, serum, and proteins) to reduce drug adhesion to microplates.[3] Here, we specifically discuss on the type and nature of the container in which the assay is performed and enlighten their effects on MIC susceptibility to these antimicrobial compounds; the issues which can serve as a source of variability in MIC results and were generally neglected in the most published studies.

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submit your manuscript | www.dovepress.com
DovePress
http://doi.org/10.2147/IDR.S219130

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Nature, type, and surface treatment of microplates

Polypropylene and polystyrene are two types of commonly used polymers for plastic laboratory consumables. Polypropylene is a thermoplastic polymer used to construct plastic laboratory supplies, including beakers, bottles, flasks, specimen containers, test tubes, and microplates. Although they are widely used in laboratories and industries because of their high chemical resistance, as well as high level of temperature tolerance, application of polypropylene plastics in microbiology is unfortunately hampered by their low optical clarity, so it is problematic when growth is evaluated visually or by optical density, eg, in MIC measurements.[7] Polystyrene as a clear, hard, and brittle polymer is used for bottles, flasks, test tubes, microplates, and petri dishes. Due to the low melting point of polystyrene between 64°C and 80°C, polystyrene products are not autoclavable; hence, they are the classic laboratory disposables.[7]

Microplates used in BMD assay to measure MIC values are generally made of polystyrene. As an important issue, because of passive interactions between samples, such as DNA, proteins, or cationic antibiotics, and well surface of microplate, adsorption of samples to the plate surface takes place.[8,9] Due to their material properties, polypropylene products present less biomolecule adsorption than polystyrene. However, surface properties of polystyrene microplates are often modified by manufactures in many ways, whether by physical, chemical, or coating methods, to accommodate requirements for various applications in cell culture, immunosorbent assays, etc. There are different types of surface modification of microplates by applications (Figure 1). Polystyrene microplates without surface treatment (non-treated) are hydrophobic in nature and have low biomolecular binding feature. However, for very sensitive applications, even low amounts of biomolecular binding can interfere with the assay. Tissue culture microplates provide the standard surface for culture of anchorage-dependent cells. These products are subjected to special surface treatment, causing the incorporation of polar groups, such as hydroxyl (–OH), carboxyl (–COOH), and amino ($–NH_2$) residues, to increase the surface hydrophility and introduce a negative or positive charge, consistent with cell attachment. High-binding surface polystyrene microplates feature a relatively high number of ionic groups and/or hydrophobic regions with the ability to bind to large biomolecules. Non-binding surface is another treatment technology for polystyrene microplates that creates a nonionic hydrophilic polyethylene oxide-like surface to minimize passive molecular interactions, leading to reduced non-specific immobilization of biomolecules.

What are the effects of microplate type on polymyxin MIC testing?

Polymyxins, as lipopeptide antibiotics, are well-known biomolecules and their inherent polycationic property causes them to adhere to a wide range of materials, including plastics used in microbiology laboratories.[3] This causes the antibiotic to be lost considerably during the in vitro experiments, such as MIC assays, resulting in unreliable data from standard laboratory procedures. While its implications are potentially serious, neither scientific communities nor literature have satisfactorily characterized the matter of loss of polymyxins during experiments, although a number of recent papers have discussed the problem.[8–12]

In this issue, several studies demonstrated that plate type can lead to significant variations in MIC assay results.[10–12] Bock et al reported that MIC values for cationic antibiotics (polymyxins) and some cationic disinfectants (chlorhexidine digluconate and octenidine hydrochloride) are influenced significantly by plate type.[12] By polypropylene plates, the median MIC values to both polymyxin B and colistin for *E. coli* NCTC 10,418 and *P. aeruginosa* NCTC 13,359 reference stains were >4-fold and ≥4-fold lower, respectively, when compared to polystyrene. Singhal et al found that the validity of BMD with polystyrene plates (BMD-Ps) was lower than that of a reference standard, where this assay was unable to identify three colistin- and polymyxin B-susceptible isolates of carbapenem-resistant *A. baumannii*.[11] In addition, Albur et al evaluated MICs of colistin for the isolates, including 56 *P. aeruginosa*, 29 *Acinetobacter* spp., and 61 *Enterobacteriaceae* using two different types of polystyrene microtitre trays (MTTs), namely non-coated V-bottom MTTs (NMTTs) and tissue-culture-coated round-bottom MTTs (TCMTTs).[10] The authors found an overall 5.3-fold increase in MIC value using TCMTTs, most likely due to decreased free antibiotic concentration within the wells resulted by the enhanced negative electric charge of microplate well surface.[10] The different containers, including 10-mL soda-lime glass tubes, 15-mL polypropylene tubes, 10-mL polystyrene tubes, and 1.5 mL low-protein-binding polypropylene microtubes, have been compared in

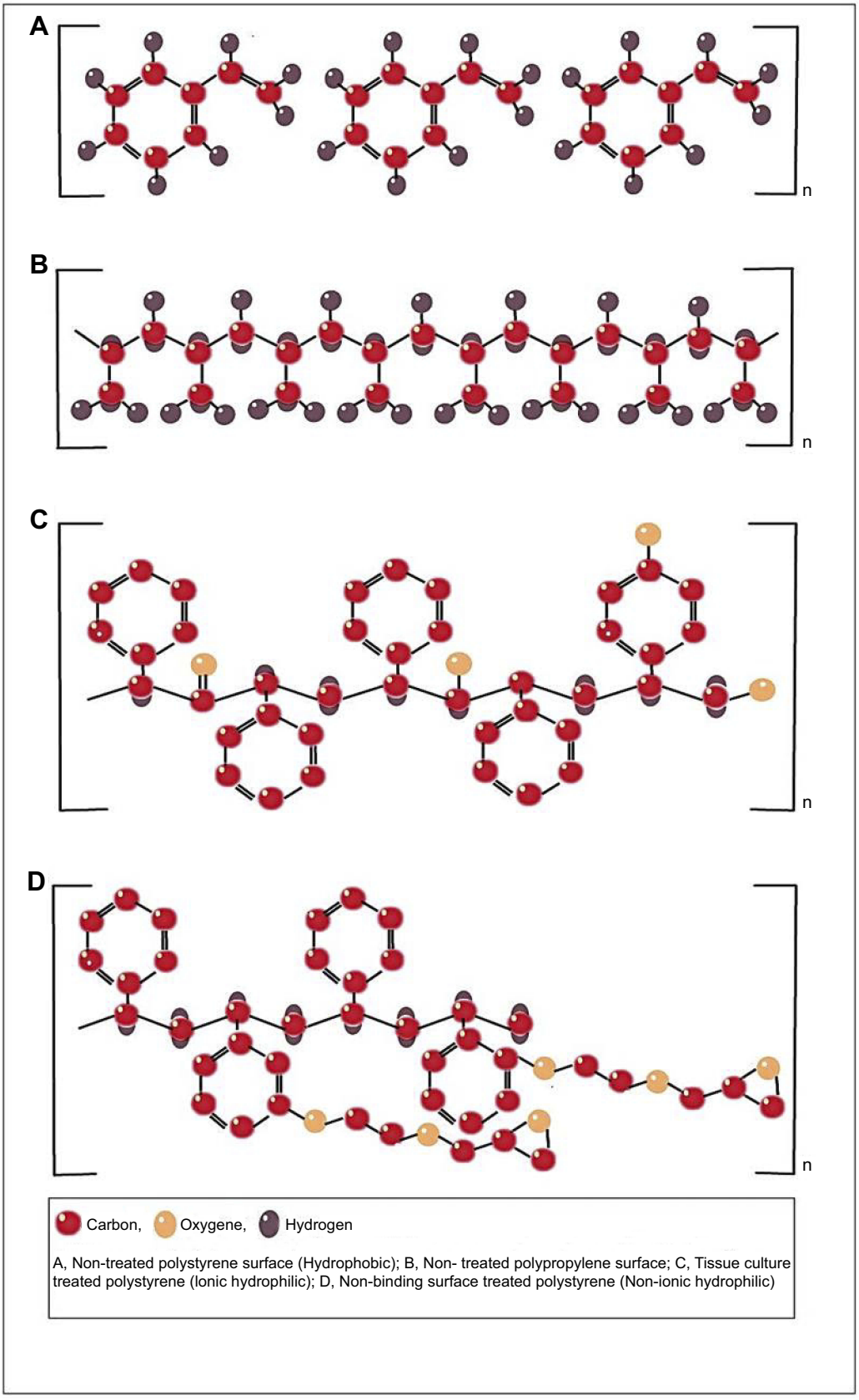


Figure 1 Different types of surface modification of plates used in broth microdilution assay.

a comprehensive study with regard to the total colistin binding.[8] At different initial concentrations of colistin ranging from 0.125 μg/mL to 8 μg/mL, low-protein-binding polypropylene microtube showed the least loss (with 59–90% of expected values left at 24 hrs), followed by polypropylene tubes (with 23–90% of expected values left at 24 hrs), glass tubes (with 25–80% of expected values left at 24 hrs), and polystyrene tubes (with 8.4–84% of expected values left at 24 hrs). Taken together, these findings indicate that the polymyxin concentration will appear to be lower than the actual concentration, so care should be taken during the all the steps of experiments to reduce the effect of adsorption. Low-protein binding or non-binding surface plastic materials appear to be suitable for cationic compound susceptibility testing. A study by Kavanagh et al demonstrated that non-binding surface plates provided significantly more potent MIC values for some cationic antimicrobial peptides, including lipoglycopeptides and polymyxins in comparison with other types of plates used.[9] Regarding the polymyxins, non-binding plates resulted in MIC values of ≤0.03 μg/mL for both colistin and polymyxin B versus *E. coli* ATCC 25,922, while untreated and tissue culture-treated polystyrene plates gave less potent MICs.[9] Likewise, MIC assays for cationic antimicrobial peptides can be performed with some additives, such as broth containing 50–95% human or mouse serum, 3–4% human or bovine serum albumin as a protein supplement, 2% lysed horse blood, and 0.002% polysorbate-80 to improve drug availability.[13–16] In the case of serum or protein supplement, it seems that because of the adsorption of proteins to plate surfaces, binding sites of antibiotics are occupied, resulting in avoiding or minimizing the possibility of the cationic molecule adsorption to plates. In addition, polysorbate-80 is a dispersing agent that has been shown to inhibit the binding of drug to plastics at a final concentration of 0.002%.[17,18] Nevertheless, it is important to note that high concentrations of serum as well as polysorbate-80 may show a synergistic effect in combination with antibiotics on bacterial growth.[14,19] In addition, MIC assessments of cationic antibiotics that bind to both protein and plastic may be confused by contrary effects. Protein binding of the polymyxin lipopeptides is estimated to be 50–60%[20] that reduces the concentration of free antibiotics available for antimicrobial activity, lessening their MIC potency; while the added protein also decreases non-specific binding to plastic, leading to more potent MIC values.

As for the type of test container, loss of polymyxins during the experiment has been shown to vary depending on the manufacturer. Two recent studies found that the brand of microplates effects on polymyxin activity.[8,9] The Thermo Scientific Nunc, Corning Life Sciences, and Trek Diagnostics Systems plates were compared in BMD assay against the *E. coli* ATCC 25,922.[9] For colistin, Treck non-treated polystyrene plate led to at least MIC value (≤0.03 μg/mL), followed by Corning (0.06 μg/mL) and Nunc (0.125–1 μg/mL) plates. For polymyxin B, Corning nontreated polystyrene plate gave more potent MIC value (0.06 μg/mL) than Treck (0.06–0.125 μg/mL) and Nunc (0.125–0.25 μg/mL) plates.[9] The study by Karvanen et al found remarkable differences in colistin concentrations between different brands of noncoated round-bottom polystyrene microplates.[8] The author observed that after 24-hrs incubation, only 2% and 70% of an expected 8 μg/mL concentration of colistin remained in the Greiner Bio-One and Nunc polystyrene microplates, respectively.

Conclusion

In summary, the loss of polymyxins during the time course of BMD susceptibility assays serves as a great condition-specific systematic error in microbiology laboratories, emphasizing the need to characterize relevant issues, such as plate types and suppliers and any additives used. Out of materials employed in different studies, low-protein binding or non-binding surface microplates appear to be beneficial to improve the problem of antibiotic adsorption and can exclude the need for adding serum or albumin protein. Future investigations should be conducted to determine which type of plate provides true polymyxin MIC that is most relevant to the clinical activity of drug.

Disclosure

The authors report no conflicts of interest in this work.

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