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Artículos originales

Comparative study of in vitro activities of polymyxin B commercial products on *Pseudomonas aeruginosa* isolated from hospitalized patients

Estudio comparativo de las actividades in vitro de productos comerciales de polimixina B sobre *Pseudomonas aeruginosa* aislada de pacientes hospitalizados

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Conflict of interest

The authors declare no conflict of interest.

Resumen

Introducción: La polimixina B se ha aplicado como uno de los antibióticos de último recurso para el tratamiento de la multirresistencia entre las infecciones bacterianas Gram negativas. Debido a efectos secundarios como toxicidad renal, el uso de polimixina se asocia con limitaciones. El presente estudio evalúa la actividad antibacteriana in vitro de varios productos comerciales de polimixina B contra *Pseudomonas aeruginosa*.

Métodos: Este estudio incluyó 63 aislados de *P. aeruginosa* no duplicados que se examinaron para la prueba de susceptibilidad in vitro a la polimixina B utilizando los siguientes discos de polvo: sulfato de polimixina B, otosporina, Poly-Mxb y Myxacort. También se han identificado las MIC₅₀ y MIC₉₀ para los antibióticos de polimixina B.

Resultados: Myxacort tuvo una actividad funcional contra la mayoría de los aislados de *P. aeruginosa*, y sólo siete aislados tuvieron una CIM relativamente alta. Las actividades de Poly-MXb y Myxacort fueron las mismas que las de otosporina.

Conclusiones: Nuestros resultados revelaron que el producto genérico nacional de polimixina B (Myxacort), y dos productos externos (Otosporin, Poly-MXb) son similares en términos de actividad microbiológica.

Palabras clave: *Pseudomonas aeruginosa*; productos con polimixina B; método de microdilución en caldo.

Abstract

Introduction: Polymyxin B has been applied as one of the last-resort antibiotics for the treatment of multidrug resistance among Gram-negative bacterial infections. Due to side effects such as renal toxicity, the use of polymyxin is associated with limitations. The present study evaluates in vitro antibacterial activity of a number of polymyxin B commercial products against *Pseudomonas aeruginosa*.

Methods: This study included 63 non-duplicated *P. aeruginosa* isolates examined for in vitro polymyxin B susceptibility testing using the following powder disks: polymyxin B sulfate, otosporin, Poly-Mxb, and Myxacort. MIC₅₀ and MIC₉₀ have also been identified for polymyxin B antibiotics.

Results: Myxacort had functional activity against most *P. aeruginosa* isolates, and only seven isolates had a relatively high MIC. The activities of Poly-MXb and Myxacort were the same as otosporin.

Conclusions: Our findings revealed that the national generic polymyxin B product (Myxacort), and two external products (Otosporin, Poly-MXb) are similar in terms of microbiological activity.

Key words: *Pseudomonas aeruginosa*, Polymyxin B products, Broth microdilution method.

Introduction

Pseudomonas aeruginosa has been recognized as an important opportunistic pathogen in clinical settings and a major source of multidrug resistance⁽¹⁾. This bacterium causes high mortality in immunocompromised patients and has currently been known as a “superbug” owing to the limited effectiveness of antimicrobial drugs⁽²⁾. Infections due to multidrug-resistant (MDR) *P. aeruginosa*, in particular carbapenem-resistant isolates, are escalating in healthcare facilities and are responsible for nosocomial infections, which may give a rise to fatal outcomes because of limited therapeutic options. The World Health Organization and the Center for Disease Control in Atlanta in the United States unanimously identified carbapenem-resistant *P. aeruginosa* as one of the most significant multidrug resistance pathogenic bacteria. Old antibiotics such as polymyxin (colistin) are growingly used as a last resort treatment for MDR *P. aeruginosa*^(3,4,5).

Multidrug resistance has led to a renewed interest in polymyxins, colistin and polymyxin B, as therapeutic options for clinically important Gram-negative bacilli⁽⁶⁾. The toxicity of such agents was the main reason for the low clinical use in early years. Previously, there has been no need for the detailed investigation of the microbiological and pharmacological properties of polymyxins since more active and efficient alternatives were readily available. These membrane-active drugs have accordingly been replaced by newer and more potent antimicrobials^(2,7,8). However, enhancing resistance to various antibiotics has rendered new drugs unsuccessful against nosocomial pathogens. Thus, exhaustive studies on the efficacy, toxicity, and pharmacokinetics of these agents are necessary. In medical and veterinary medicine, the broad use of antibiotics has contributed to the development of drug resistance. As a result, combination therapy and more successful treatments are being sought for serious infections.

Pharmaceutical products, especially antibiotics, must comply with the quality, efficacy, and reliability standards set by competent authorities. In recent decades, the quality and efficacy of generic antibiotics are topics extensively discussed in research communities. Hence, investigation of these antibiotics using biological assays is of paramount importance, and commercial products necessarily have to be similar to the global composite reference standard^(9,10). Although polymyxin B has been available for decades, the pharmacological knowledge of this drug remains considerably limited and constitutes a major barrier to its effective use⁽¹¹⁾. To understand the susceptibility of commonly isolated *P. aeruginosa* to polymyxin in Iran, we investigated the evaluation of in vitro antibacterial activity of polymyxin products, including those frequently cause various infections in Iranian hospitals, against *P. aeruginosa*.

Materials and methods

Materials

Generic polymyxin products were acquired from local pharmacies approved by Iran Food and Drug Administration for the industrial use. The generic drugs applied in this study include Otopsporin (GlaxoSmithKline, UK), Poly-Mxb (Bharat Serums & Vaccines Ltd., India), and Myxacort (Sina, Iran), which their detailed information is available in Table 1. A reference standard, polymyxin B sulfate (CAS number 1405-20-5; Sigma-Aldrich, USA), was also used for biological tests.

Table 1. Polymyxin products, pharmaceutical dosage form, and manufacturer

Polymyxin product (code)	Presentation	Manufacturer
Analytical standard	Polymyxin B sulfate, powder	Sigma-Aldrich
Generic A	Polymyxin B sulphate 10,000 U/mL + Neomycin sulphate 3,400 U/ mL + Hydrocortisone 1.0% w/v	GlaxoSmithKline
Generic B	Polymyxin B 500000 IU	Bharat Serums & Vaccines Ltd, India
Generic C	Polymyxin B sulfate: 10'000 U/mL+ Neomycin sulfate: 5 mg/mL + Hydrocortisone: 10 mg/ML	Sina, Iran

Bacterial strains

A total of 63 isolates of *P. aeruginosa* were collected from inpatients in Besat Hospital in Hamadan, Iran from June 2019 to September 2019. Conventional biochemical tests, such as oxidase, growth in oxidation fermentation (OF) medium, and pigment production, identified the isolates obtained. The API 20NE system (bioMérieux, Marcy l'Etoile, France) was then used for the final identification of isolates.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was conducted with the following antibiotics: amoxicillin/clavulanic acid, cefotaxime, ceftriaxone, aztreonam, tetracycline, ceftazidime, and cefepime (30 µg of each), colistin, gentamicin, imipenem, and Polymyxin B (10 µg of each), and ciprofloxacin (5 µg). The test was accomplished as per the Clinical and Laboratory Standards Institute (CLSI) 2018 guidelines⁽¹²⁾.

Potency determination using the disk diffusion (DD) method

All the isolates were tested for in vitro polymyxin B susceptibility using the powder disks made from polymyxin B sulfate, Otosporin, Poly-Mxb, and Myxacort products. Sterile blank disks were saturated with 20 µl of each stock polymyxin B product. After being dried, the disks were ready for DD test. The standard product was utilized to compare commercially available antibiotic disks (polymyxin B; MAST, UK)⁽¹³⁾.

Determination of minimum inhibitory concentration (MIC)

The susceptibility testing was conducted using the broth microdilution (BMD) method as recommended by the CLSI guideline⁽¹²⁾. Mueller Hinton Broth (MHB; 100 µl) was added to the rows 2-12 of a 96-well microtitre plate. Thereafter, 200 µl of the various polymyxin B products was added to row 1 and then serially diluted through row 10. No polymyxin B products were added to row 11, which served as a growth control. After culturing on the Mueller Hinton Agar plates, *P. aeruginosa* was incubated at 37 °C for 18 h. Subsequently, colonies were separately transferred to a test tube. The separated colonies were inoculated into the MHB (5 mL), which was then incubated at 35 °C for 4 h. By applying the 0.5 McFarland standard, the adjustment of the turbidity of an active growing broth culture with a sterile broth was accomplished. Then the bacterial suspension was diluted 1:100 using the appropriate broth to achieve a final concentration about 5×10^5 CFU/ml. The bacterial cells (100 µl) was added to each well on the panel, resulting in a final volume of 200 µl. The purity of each isolate was checked by adding a sample of each isolate to the microtitre panel and inoculating a fresh agar plate. Both the purity plates and the microtitre panels were incubated at 35 °C for 16-20 h. In the end, broth microdilution method was conducted, and assays were performed by the aid of MHB. The concentrations of polymyxin B (i.e. polymyxin B sulfate, Otosporin, Poly-Mxb, and Myxacort products) were in the range of 0.25-1024 µg/µl. The microtitre panels were removed from the incubator after 16-20 h and read. Control strains included *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853.

Statistical analysis

Chi-square was used to compare the in vitro activity of various polymyxin B products in *P. aeruginosa* isolates and was performed triplicate. SPSS version 16 was applied for statistical analysis. Probabilities (p values) less than 0.05 were considered as statistically significant.

Results

The isolates were collected from different infection sites of patients hospitalized in several wards. *P. aeruginosa* isolates were most often recovered from burn wounds (41.26%), blood (20.64%), catheter (15.88%), urine (14.29%), and discharges (7.94%). The frequency of antibiotic resistance in the isolates of *P. aeruginosa* is shown in Figure 1. The highest and lowest resistance was observed for ceftriaxone (65.07%) and amikacin (33.33%), respectively. Polymyxin B demonstrated significant in vitro antimicrobial activity against all the *P. aeruginosa* isolates, which were susceptible to colistin. In addition, 99.1% of the isolates were susceptible to polymyxin B. Among the 63 *P. aeruginosa* isolates, 42 isolates

indicated simultaneous resistance to six antibiotics (aztreonam, cefotaxime, amoxicillin-clavulanic acid, gentamicin, ceftriaxone, and ceftazidime). Disks made of three types of polymyxin B products (Otosporin, Poly-Mxb, and Myxacort) showed similar antibacterial activity compared to the standard polymyxin B disk (MAST, UK; Table 2). Wound infection was the main source of polymyxin-resistant *P. aeruginosa* (Figure 2). MICs of polymyxin B products for all *P. aeruginosa* isolates are shown in Figure 3. Otosporin had functional activity against most *P. aeruginosa* isolates, and seven isolates had a relatively high MIC ($\geq 2 \mu\text{g/mL}$). Poly-Mxb and Myxacort had similar activity with otosporin. Polymyxin B sulfate compared to other generic drugs, e.g. Poly-Mxb and Myxacort (and otosporin, demonstrated a higher MIC₅₀ level (Figure 3).

Table 2. Comparison of the concentration of polymyxin B products in the disks prepared manually with standard polymyxin B disk.

StPolymyxin B	Polymyxin B sulfate	Otosporin	Poly-Mxb	Myxacort
300 U	30 $\mu\text{g}/20\mu\text{l}$	300 U/20 μl	50 U/20 μl	310 U/20 μl

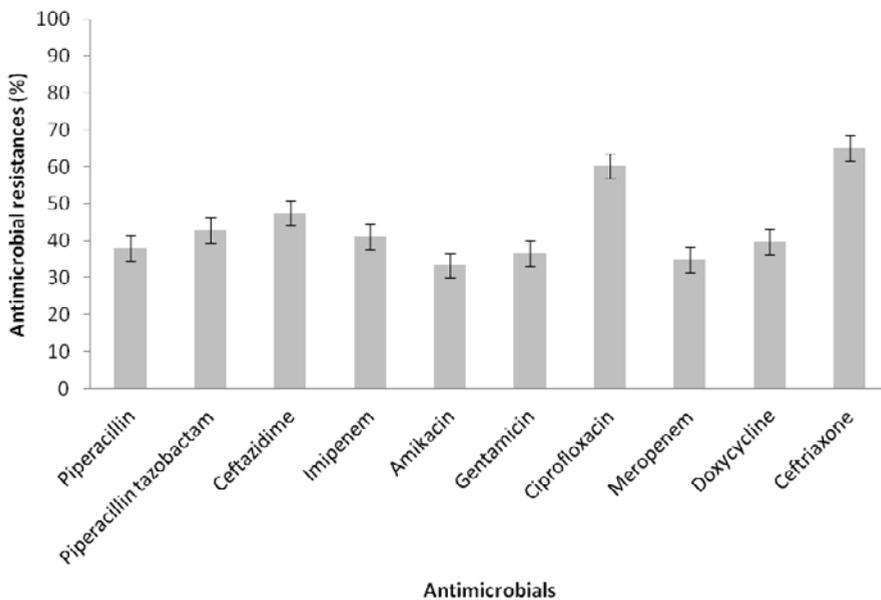


Figure 1. Antibiotic resistances pattern of *Pseudomonas aeruginosa*. No significant difference was seen in antibiotic resistances pattern in *P. aeruginosa* isolates from different sources.

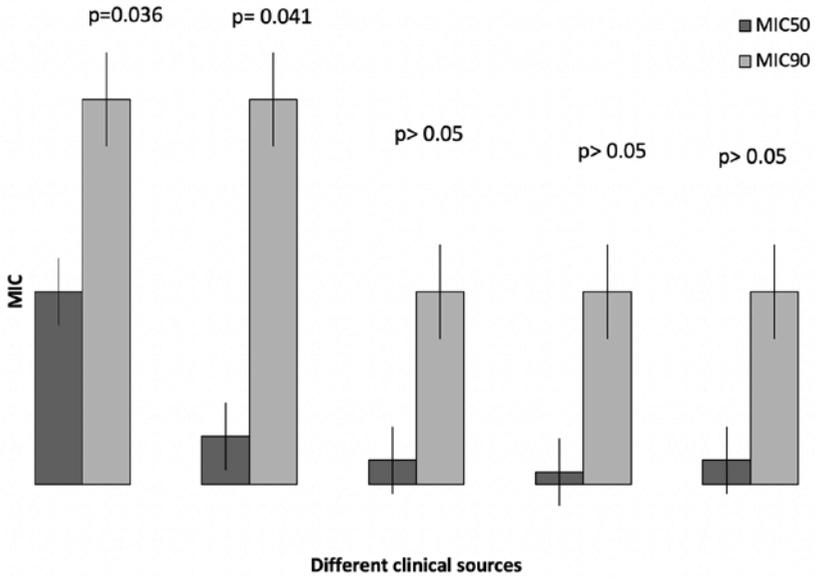


Figure 2. MIC₅₀ and MIC₉₀ of polymyxin B sulfate for *Pseudomonas aeruginosa* isolated from different clinical sources.

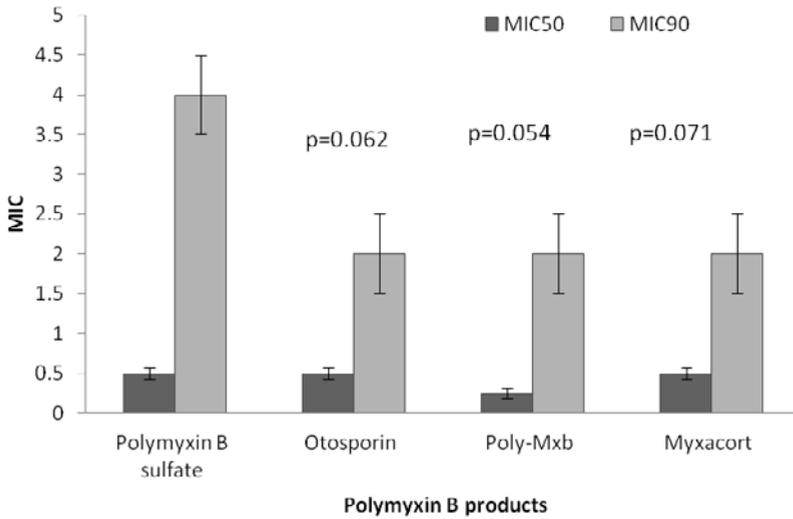


Figure 3. Comparative in vitro activity of polymyxin B products against *Pseudomonas aeruginosa* isolates.

Discussion

Polymyxins are known to target the outer membrane of Gram-negative bacteria by displacing divalent calcium and magnesium ions from the negatively charged phosphate groups of membrane lipids. This process causes the destabilization of the lipopolysaccharide (LPS) and the membrane, leading to the leakage of cytoplasmic contents and bacterial cell death. Although a clear mechanism of action is not known, polymyxins have been suggested to be capable of binding to endotoxin, which is the lipid A portion of the LPS and neutralizes LPS during cell lysis. Polymyxins have bactericidal effect against several Gram-negative pathogens such as *E. coli*, *A. baumannii*, *P. aeruginosa*, *K. pneumoniae*, *Enterobacter spp.*, *Salmonella spp.*, and *Shigella spp.* Until recently, the mechanisms of reported polymyxin resistance have been chromosomally mediated that result in LPS modifications, formation of capsules, and overexpression of outer membrane protein OprH, thereby conferring resistance in Gram-negative bacteria^(13,14). In 2015, a plasmid-mediated polymyxin resistance mechanism (MCR-1) was reported in *Enterobacteriaceae*, which is further compromising and threatening the treatment using polymyxin antibiotics⁽¹⁵⁾.

Antibiotic resistance is a growing public health problem worldwide, and the resistance pattern of microorganisms mainly differs in varied communities. Therefore, it is crucial to specifically plan for diminishing the resistance of antibiotics, particularly those most widely used for treatment^(17,18). Studies have suggested that polymyxin B is an effective agent for treating *P. aeruginosa* infections. Polymyxin B has long been utilized as a topical agent for the treatment of conjunctivitis, otitis, and infectious surgical complications, especially osteomyelitis^(19,20). The emergence of polymyxin resistance among *P. aeruginosa* clinical isolates has recently raised the concern that effective antimicrobial treatment options for these isolates can severely be limited in the future.

A comparison of our study results those from similar studies in other countries displays that in vitro polymyxin B is highly active against all clinical isolates of *P. aeruginosa*^(21,28). Only for *P. aeruginosa* isolates, an excellent level of concordance has been found between the two generic polymyxin B products (Myxacort and Poly-Mxb) and otosporin. The level of essential agreement achieved for all *P. aeruginosa* isolates from otosporin was more than 90%. For all *P. aeruginosa* isolates, similar results were obtained from the antimicrobial susceptibility test, which was performed by Mast Company.

Our studies uncovered that Myxacort and Poly-Mxb have the same antibacterial potential. Disks made from different polymyxin B products seem to have problems interpreting the results due to the combination of additional compounds with polymyxin. MIC testing was performed using commercially available polymyxin B sulfate powders. The discrepancy in the relative proportions of the mixture constituents between the powder disks and the producers seems to be due to the additional components in the generic polymyxin B compared to polymyxin B sulfate.

The DD test, a simple and an inexpensive method for screening a large number of isolates, is used to determine the antimicrobial susceptibility in many clinical laboratories⁽²⁵⁾. However, the weak and slow diffusion of polymyxins through agar is associated with small zones of growth inhibition and significant assay variation, negating the use of this method for susceptibility testing. In fact, the predictive accuracy of the DD test is unacceptable, and consequently, no reliable correlation of zone diameters with MICs has been found in many previous studies. The difficulty in differentiating the inhibition zone diameters has been illustrated by Jerke et al.⁽²⁶⁾ who compared a *P. aeruginosa*-resistant isolate of 10 mm diameter with a susceptible isolate of 11 mm diameter; both isolates were categorized as susceptible with BMD MICs of ≤ 0.25 $\mu\text{g/ml}$.

At the same time, MICs achieved by the broth microdilution with refined forms of the main constituents of polymyxin B were found to be within a \log_2 dilution of the MICs achieved from the US Pharmacopoeia polymyxin B sulfate powder mixture⁽²¹⁾. These data implies that the composition of the powder possibly does not affect polymyxin susceptibility testing. Broth dilution is a method in which a predetermined concentration of the bacterial suspension is tested against different levels of the antimicrobial agent in a predetermined liquid medium. For testing polymyxin antimicrobial susceptibility, it is currently the only procedure approved by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST)⁽⁵⁾. The broth microdilution method is per-

formed by a cation-adjusted MHB, twofold dilutions of polymyxin B (ranging from 0.12 to 512 µg/mL), and a final bacterial inoculum of $5 \sim 10^5$ CFU/mL in each well, according to the CLSI guidelines. Broth microdilution is recognized as an optimal method and is presently recommended for susceptibility testing in the recent document proposed by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group^(23,27). However, this approach is time-consuming, and antibiotic solutions can cause significant errors in manual preparation (if the technique used is unautomated). It is, therefore, unadaptable to most clinical microbiology laboratories. Non-reproductive and non-interpretable MIC findings for *Enterobacter* species, *P. aeruginosa* and *A. baumannii*, have also been reported owing to the presence of skip wells, i.e. those that do not show growth, but growth is observed in wells with higher antibiotic concentrations^(18,23,24). Moreover, some technical issues for testing have been reported using Broth microdilution method. Polymyxins readily adhere to the plastic trays, leading to decreased antibiotic concentrations actually being present in the MHB dispensed in the wells. The addition of a surfactant, such as polysorbate 80 (P-80), limits polymyxin adhesion to the BMD panels. It has also been demonstrated that it possesses a synergistic effect on polymyxins and has antibacterial activity of its own. Nonetheless, CLSI or EUCAST guidelines do not currently recommend using P-80 for polymyxin B susceptibility testing by BMD⁽²⁵⁾.

Antibiotic activities should be tested *in vitro* and *in vivo* to confirm their suitability for clinical application. Pharmaceutical equivalence or MIC value systems of any generic product is/are invalid requirements for equal treatment. In view the fact that MIC breakpoints for Gram-negative and Gram-positive bacteria in polymyxin B products (Myxacort, Poly-Mxb, and Otosporin) have not yet been acquired, it remains unclear whether the *in vitro* efficacy of antibacterial drugs predicts the clinical outcome. To achieve this goal, all the generic polymyxin B products are required to be checked *in vivo*. It would be better to perform further tests for *in vivo* use of Myxacort and Poly-Mxb, an activity against different species, stability, etc.

Considering the similarity of the national generic polymyxin B product (Myxacort) and the external products (Poly-MXb and otosporin), it is rationale to conclude that these products have the same microbiological actions, as evaluated by DD and MIC tests. We suggest the preparation of antibiotic powder disks in developing countries that can be used in the absence of standard disks in microbiological activity. These findings need to be further investigated and confirmed *in vivo*.

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