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Membrane permeabilization of colistin toward pan-drug resistant Gram-negative isolates☆

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ABSTRACT

Pan-drug resistant Gram-negative bacteria, being resistant to most available antibiotics, represent a huge threat to the medical community. Colistin is considered the last therapeutic option for patients in hospital settings. Thus, we were concerned in this study to demonstrate the membrane permeabilizing activity of colistin focusing on investigating its efficiency toward those pan-drug resistant isolates which represent a critical situation. We determined the killing dynamics of colistin against pan-drug resistant isolates. The permeability alteration was confirmed by different techniques as: leakage, electron microscopy and construction of an artificial membrane model; liposomes. Moreover, selectivity of colistin against microbial cells was also elucidated. Colistin was proved to be rapid bactericidal against pan-drug resistant isolates. It interacts with the outer bacterial membrane leading to deformation of its outline, pore formation, leakage of internal contents, cell lysis and finally death. Furthermore, variations in membrane composition of eukaryotic and microbial cells provide a key for colistin selectivity toward bacterial cells. Colistin selectively alters membrane permeability of pan-drug resistant isolates which leads to cell lysis. Colistin was proved to be an efficient last line treatment for pan-drug resistant infections which are hard to treat.

Introduction

Recently, it has been witnessed worldwide that Gram-negative bacteria resistant to many classes of antibiotics represents a fearful situation toward the emergence of a future medical disaster.[1] There are 2 terms commonly describing those superbugs; which are multi-drug resistant (MDR) and pan-drug resistant (PDR). An isolate is considered MDR if it exhibited resistance toward 5 out of the 7 anti-pseudomonal classes of antimicrobial agents, i.e. anti-pseudomonal penicillins, cephalosporins, carbapenems, monobactams, quinolones, aminoglycosides, and colistin, while it is a PDR if it showed resistance toward all 7 anti-pseudomonal antimicrobial

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agents, including colistin.[2] There was another view considering that PDR isolates were those resistant to all antibiotics but only susceptible to polymyxins.[3,4] Although there is no apparent definition for the term PDR throughout literature, it generally denotes resistance against a variety of antibiotics excluding polymyxins.[5] Such view is adopted in the present work. In the past few decades there have been a tremendous increase in resistance to currently available antibiotics and a significant decline in development of new ones.[6] This leads to the revival of older agents as polymyxins, for the treatment of such PDR infections.[2]

Polymyxins are a group of polypeptide cationic antibiotics that were isolated from *Bacillus polymyxa* in the 1940s.[7] Since then, polymyxin E (colistin) and polymyxin B were extensively used in clinical practice for Gram-negative organisms.[8,9] However, they were gradually withdrawn from the market and abandoned during the last two decades due to claimed reports of toxicity. Therefore, during that time, there have been limited studies on the clinical use, pharmacokinetics and pharmacodynamics of colistin.[10] Emergence of the PDR pathogens, necessitated the re-evaluation of polymyxin therapies.[11]

Colistin has been recently considered as last option treatment for patients with nosocomial PDR infections, which have become an important public health issue, owing to its favorable properties of rapid bacterial killing, a narrow spectrum of activity, and slow development of resistance.[12,13] Colistin interacts electrostatically with the outer membrane of Gram-negative bacteria and competitively displaces divalent cations which stabilize the lipopolysaccharide layer thus disrupting the membrane integrity. It is then subsequently taken up via the self-promoted uptake pathway.[13] It is believed that colistin forms cracks in the outer membrane which promotes its uptake inside the cell and permits the passage of different molecules.[14] Thus, polymyxins produce a disruptive detergent effect, leading to increased permeability in the outer membrane, leakage of the absorbing cytoplasmic contents, cell lysis and finally death.[15] The chemical composition of bacterial membranes being rich in phosphatidylethanolamine and negatively charged lipids allows such electrostatic attraction with cationic peptides in contrast to eukaryotic cells in which cholesterol is the predominant component providing a clue for the selectivity of action toward microbial versus host cells.[16] Such a situation prompted the present microbiological study to investigate the membrane permeability alteration of colistin and it bactericidal effect on PDR Gram-negative clinical isolates including *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli*.

Materials and methods

Microorganisms and antibiotics

Four clinical bacterial isolates, identified by classical microscopic and biochemical procedures,[17,18] were used in this study. These are: *A. baumannii* (A182), *P. aeruginosa* (P103), *E. coli* (E9) and *K. pneumoniae* (K103). The identified isolates were maintained by freezing in 15% glycerol broth [19] (Oxoid Ltd.; Basingostok; Hampshire, England). Colistin sulphate was obtained as powder from Pharco pharmaceutical Co., Egypt. It was dissolved in water to prepare stock solutions. The susceptibility of the tested isolates, to the different classes of antibiotics was determined by the standard disc agar diffusion technique according to Bauer et al.[20] with some modifications.[21] MIC of colistin was determined by the standard broth dilution technique.[22]

Bactericidal activity of colistin using the viable count technique

For each tested isolate, two concentrations of colistin (1/2MIC and MIC) were prepared in sterile nutrient broth. Each concentration was inoculated with overnight culture to give a final inoculum of 10^6 cfu/mL. A control without antibiotic was prepared for each of the tested isolates. The systems were mixed well and incubated at 37 °C with shaking. Samples were aseptically withdrawn from each test tube at 0, 1, 3, 6 and 24 h and serially diluted with sterile saline. Then, 10 μL portions were dropped onto the surface of overdried nutrient agar plates. The plates were left to dry and incubated inverted at 37 °C for 24 h, the resulting colonies were counted and the original viable count was determined.[23]

Effect of colistin on the cytoplasmic membrane by leakage technique

Bacterial suspensions of the selected isolates were prepared by streaking an overnight broth culture onto nutrient agar slants (Oxoid Ltd.; Basingostok; Hampshire, England). The slants were incubated at 37 °C for 16–18 h. The resulting growth of 3 slants was resuspended in 5 mL sterile 0.9% saline to produce heavy inoculum ($O.D_{600}$ adjusted to 2) and transferred into sterile test tubes. The obtained bacterial suspensions were centrifuged at 12,000 × *g* for 5 min. The formed pellets were washed twice with sterile saline and then were resuspended in 5 mL sterile saline. Aliquots of the prepared bacterial suspension of each isolate were treated with 50 mg/L of colistin. A control was included in each test containing untreated bacterial suspension. Both the treated and untreated bacterial suspensions were incubated at 37 °C for 24 h. After incubation, the bacterial suspensions were centrifuged at 12,000 × *g* for 5 min. The absorbance of the clear supernatant was estimated at 260 and 280 nm against saline solution using the spectrophotometer (Thermospectronic, Helios alpha, NC 9423UV A 1002E, England).[24]

Effect of colistin on the leakage of red blood cells

One milliliter of fresh human blood was centrifuged at 2000 × *g* for 5 min and the cells were washed 4× with sterile 0.9% saline discarding the supernatant every time. The sedimented red blood cells (RBCs) were resuspended in 1 mL buffer (5 mM sodium phosphate and 150 mM sodium chloride [pH 7.4]). The RBCs suspension in buffer was distributed in sterile eppendorf tubes in 25 μL aliquots and 1 mL of colistin solution dissolved in the same buffer was added to each eppendorf in concentrations ranging from 0.78 mg/L to 100 mg/L. The resulting suspensions were incubated at room temperature for 2 h. The systems were then centrifuged at 2000 × *g* for 10 min. The release of hemoglobin was monitored by measuring the