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# EFFECT OF ANTIMICROBIAL PEPTIDES ON COLISTIN-SUSCEPTIBLE AND COLISTIN-RESISTANT STRAINS OF *KLEBSIELLA PNEUMONIAE* AND *ENTEROBACTER ASBURIAE*

BÉLA KÁDÁR[1], BÉLA KOCSIS[1], KATALIN KRISTOF[2], ÁKOS TÓTH[3, 4] and DÓRA SZABÓ[1]*

[1]Institute of Medical Microbiology, Semmelweis University, Budapest, Hungary
[2]Institute of Laboratory Medicine, Diagnostic Laboratory of Clinical Microbiology, Budapest, Hungary
[3]National Center for Epidemiology, Budapest, Hungary
[4]European Program for Public Health Microbiology Training (EUPHEM), European Centre for Disease Prevention and Control (ECDC), Stockholm, Sweden

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In this study susceptibility to different antimicrobial peptides was investigated on colistin-susceptible and colistin-resistant identical pulsotype strains of KPC-2 producing *Klebsiella pneumoniae* ST258 as well as colistin-susceptible and colistin-resistant *Enterobacter asburiae* strains isolated from clinical samples. In our test, bacteria were exposed to 50 mg/ml lactoferrin, lysozyme and protamine – cationic antimicrobial peptides belonging to innate immune system and having structural similarity to polymyxins – in separate reactions. After 18 hours incubation of colonies were counted. 40% of colistin-resistant *K. pneumoniae* strains and 97% of colistin-susceptible counterpart strains were lysed by protamine whereas 87% and 100% colony forming unit decrease by lysozyme was seen, respectively. In the case of colistin-resistant *E. asburiae* strains 1 $\log_{10}$ cell count increase were observed after treatment with lysozyme and 1.56 $\log_{10}$ after lactoferrin exposure compared to the initial number whereas the colistin-susceptible showed no relevant cell count increase. Our findings suggest that acquired colistin-resistance in Enterobacteriaceae is associated with tolerance against antimicrobial peptides.

**Keywords:** antibiotic-resistance, antimicrobial peptides, colistin, Enterobacteriaceae

*Corresponding author; E-mail: szabo.dora@med.semmelweis-univ.hu

## Introduction

Antimicrobial peptides and proteins are mainly cationic (i.e. rich in lysine and arginine residues), amphipathic polymers usually comprised of 12–45 amino acids, and they are part of the innate immune system of plants and animals. However, there are certain antimicrobial peptides that possess dominantly neutral or negative charge, but their spectrum is narrower than the cationic ones [1–3]. Different studies found that their mode of action in Gram-positive bacteria is forming channels through the cytoplasmic membrane, causing structural disruption and finally, osmotic lysis. In Gram-negative microbes they initially interact with the lipopolysaccharide (LPS) of the outer membrane, leading to local membrane disruption, then carry on forming pores through the inner cytoplasmic membrane [4–6]. In this capacity the antimicrobial peptides produced by multicellular organisms are very similar to polymyxins (cationic decapeptides) synthetized by *Bacillus* and *Paenibacillus spp.* [7–10].

Multidrug-resistant Gram-negative bacteria are being often identified as causative agents in nosocomial infections, representing an increasing healthcare problem [11, 12]. These emerging multidrug-resistant pathogens set clinicians to constant challenges concerning the adequate therapy, and lately previously unheeded antibiotics such as polymyxins have been utilized against them [13–15].

Nonetheless, polymyxin-resistant Gram-negative pathogens are being reported in several countries in growing frequency [16–18]. The basis of polymyxin-resistance in Gram-negative bacteria is the modification of LPS molecules of the outer membrane in such ways that the otherwise net negative charge of this layer grows, making it more difficult for positively charged agents (polymyxins, cationic antimicrobial peptides) to attach to it [19, 20].

In recent years there were reports about colistin-resistant and colistin-heteroresistant Gram-negative bacteria that developed a certain cross-resistance, cross-tolerance against host (i.e. human) antimicrobial peptides [21, 22]. In our study we describe the aforementioned phenomenon of cross-tolerance in colistin-resistant *K. pneumoniae* and *E. asburiae* strains isolated in Hungary.

## Materials and Methods

### *Bacterial strains*

The study included *K. pneumoniae* and *E. asburiae* strains isolated in Hungary. The two identical pulsetype *Klebsiella* strains belonged to the international clone ST258 were KPC-2 producers: one was colistin-susceptible and the other

one was colistin-resistant. They were isolated during a Hungarian outbreak in 2008 and 2009, from upper respiratory tract and wound infections, respectively [23]. Three *E. asburiae* strains were isolated from sporadic cases of urinary tract infections and were identified by MALDI-TOF/MS.

*Antibiotic susceptibility*

The minimal inhibitory concentration (MIC) values of polymyxin B (Sigma-Aldrich) and colistin (Sigma-Aldrich) were determined by broth microdilution method using Mueller–Hinton broth (Becton Dickinson), and results were interpreted according to EUCAST documents [24].

*Susceptibility to antimicrobial peptides*

Each bacterial strain was incubated in 5 ml Luria–Bertani (LB) broth on 37 °C was centrifugated with 5000 G for 15 minutes on 5 °C in their exponential growth phase. Bacterium solutions of $2.1\times10^5$ CFU/ml were prepared in 1 wt/vol% tryptone phosphate-buffered saline (T-PBS) buffer. Ten µl inocula of each bacterium solution was distributed and complemented to 200 µl with protamine (Sigma-Aldrich), lysozyme (Sigma-Aldrich) and lactoferrin (Sigma-Aldrich), each with an end-concentration of 50 mg/ml. The mixtures containing lysozyme and protamine were incubated on 37 °C for 60 minutes, while the compounds with lactoferrin were incubated on 37 °C for 180 minutes. One hundred µl of each solution was inoculated on sterile LB agar plates, and was incubated on 37 °C for 18 hours, then colonies were calculated $\log_{10}$ CFU/ml. Colony numbers of bacteria treated with antimicrobial peptides were compared to that of "untreated" ones [25].

## Results

One of the two KPC-2 producing *K. pneumoniae* strains was resistant to both polymyxin B and colistin, while the other one was susceptible to the two agents, as interpreted according to the latest EUCAST documents. One *E. asburiae* was susceptible to polymyxins, and two were resistant however, we have managed to detect a sub-strain of the *E. asburiae* 0821 which demonstrated high-degree colistin-heteroresistance with E-test (Table I).

**Table I.** MIC values of polymyxin B and colistin in the investigated strains

| | MIC (μg/ml) | |
|---|---|---|
| | polymyxin B | colistin |
| EUCAST breakpoints | not determined | 2 |
| *K. pneumoniae* 11 | <0.125 | <0.125 |
| *K. pneumoniae* 12 | 128 | 256 |
| *E. asburiae* 0821 | 0.125 | 0.125 |
| *E. asburiae* 0821/H | >256 | >256 |
| *E. asburiae* 148 | 64–128 | 256 |

**Table II.** Percentile changes of *K. pneumoniae* CFU decrease after treatment with different antimicrobial peptides

| | After treatment with protamine (50 mg/ml) | After treatment with lactoferrin (50 mg/ml) | After treatment with lysozyme (50 mg/ml) |
|---|---|---|---|
| *K. pneumoniae* col S | 97% | 0% | 100% |
| *K. pneumoniae* col R | 40% | 0% | 87% |
| Difference | +57% | 0% | +13% |

**Table III.** *E. asburiae* CFU/ml differences after treatment with different antimicrobial peptides

| | Initial | After treatment with protamine (50 mg/ml) | After treatment with lactoferrin (50 mg/ml) | After treatment with lysozyme (50 mg/ml) |
|---|---|---|---|---|
| *E. asburiae* 0821 col S | 4.9 $\log_{10}$ | 4.69 $\log_{10}$ | 4.9 $\log_{10}$ | 5.11 $\log_{10}$ |
| *E. asburiae* 0821/H col R | 4 $\log_{10}$ | 4 $\log_{10}$ | 5.56 $\log_{10}$ | 5.04 $\log_{10}$ |
| *E. asburiae* 148 col R | 4.85 $\log_{10}$ | 4.85 $\log_{10}$ | 5.48 $\log_{10}$ | 5 $\log_{10}$ |

Lactoferrin did not demonstrate any effect on either *Klebsiella* strains as there was practically no change in the colony forming unit (CFU) after treatment with it. Protamine exposition caused a 97% decrease in CFU of the colistin-susceptible *K. pneumoniae* strain, while only 40% reduction was observed with the colistin-resistant strain. Lysozyme showed complete bactericidal effect on the colistin-susceptible isolate, and its effect on the colistin-resistant one was also relevant, but it resulted in an only 87% diminution of CFU. These results are shown on Table II.

The CFU/ml changes of the *E. asburiae* strains are listed in Table III. Exposure to protamine caused no relevant difference between the initial and post-expositional CFU. High-level tolerance to lactoferrin was observed in isolates 0821/H and 148. Lysozyme-tolerance was detected in isolates 0821/H and 148.

## Discussion

Our findings on the appearance of colistin-heteroresistance within Gram-negative bacterial strains correlate with various studies of recent years [26–28]. Colistin-heteroresistance concomitant with resistance to lysozyme was also previously observed in *Enterobacter cloacae* species [22]. The treatment of these polymyxin-resistant enterobacteria is a great challenge, although some recent studies showed that antibiotic combinations involving e.g. polymyxins, rifampicin, carbapenems and aminoglycosides could be efficient [29].

Cationic antimicrobial peptides are known to bind to certain outer membrane proteins (OMP), which play integral part in the effects of polymyxin antibiotics, as well. In *Salmonella enterica* serovar Typhimurium and other Gram-negative bacteria the outer membrane bound sensor kinase PhoQ is directly activated by antimicrobial peptides, succeeding in phosphorylation of transcription regulator PhoP, which activates a number of genes being important in virulence, taking part in modification of LPS molecules, and thus being responsible for developing resistance to antimicrobial peptides and polymyxins [30–34].

According to our knowledge, this is the first description of simultaneous resistance to polymyxin-type antibiotics and cationic antimicrobial peptides in Hungary.

Our study suggests that prior exposure to antimicrobial peptides can influence the development of resistance to polymyxins in bacteria. Furthermore, bacteria with resistance to polymyxins could also develop tolerance to antimicrobial peptides, which subsequently could influence the host defensive abilities in case of infections (especially in bloodstream infections), the therapeutical possibilities and choices, as well as the clinical outcomes.

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## Conflict of Interest

No conflict of interest.

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