Examination of colistin-resistance in Gram-negative bacteria

PhD thesis

Dr. Béla Kádár

Semmelweis University
Doctoral School of Pathological Sciences

Supervisor: Dr. Dóra Szabó, D.Sc.

Official reviewers: Dr. József Kónya, Ph.D.
Dr. Szabolcs Sipeki, Ph.D.

Head of the Final Examination Committee: Dr. Károly Cseh, D.Sc.
Members of the Final Examination Committee: Dr. Gábor Pálos, Ph.D.
Dr. János Sinkó, Ph.D.

Budapest
2017

INTRODUCTION

Antibiotic resistant pathogens are an increasing healthcare problem worldwide. WHO predicts that by 2050 antibiotic-resistant microbes will have caused the death of ten million people every year, of which 390,000 will be Europeans. Infections caused by multidrug-resistant and extensively drug-resistant Gram-negative are typically nosocomial infections – catheter-associated urinary tract infections, bloodstream infections related to use of intravenous devices, lower respiratory tract infections caused by prolonged lying or mechanical ventilation. Primary pathogens are non-fermenting Gram-negative bacteria (*Acinetobacter* spp., *Pseudomonas aeruginosa*, *Stenotrophonomonas maltophilia*), members of *Enterobacteriaceae* family (*Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, as well as *Proteus*, *Enterobacter*, *Citrobacter* spp.), and Gram-positives e.g. methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* strains. For decades these bacteria have been developing resistance to different antibiotic families.

Treatment options for infections caused by multiresistant Gram-negative bacterial strains are limited. Long-existing but previously unheeded antibiotics like polymyxins (polymyxin B and colistin), nitrofurantoin and fosfomycin are living their renaissance, but besides these agents there are newer antimicrobials at disposal, as well, e.g. tigecycline, ceftazidime/avibactam and ceftolozane/tazobactam.

Polymyxins have been subjects of intensive studying for years, because their selective antimicrobial activity against Gram-negatives is making them „last resort" antibiotics against multiresistant bacteria. Acquired resistance to them was relatively rare in the past due to scarce usage. However, the more frequent employment has led to the appearance of colistin-resistant strains (since colistin is more widely used in clinical practice than polymyxin B, the term „colistin-resistance" became general). These strains have shown increased tolerance to antimicrobial peptides, as well. The molecular basis of resistance is the enzymatic modification of lipopolysaccharide which is an integral part of Gram-negative's outer membrane. These modifications enable the outer membrane to electrostatically repel polymyxin molecules. Alterations are attained by addition of either phosphoethanolamine or 4-amino-4-deoxy-L-arabinose to the free phosphate groups. Expression of catalyzing enzymes is influenced by a multiple-level

regulatory mechanism: (i) direct regulation is performed by PmrA-PmrB system – it activates the expression of genes responsible for phosphoethanolamine addition (*pmrC*, *cptA*), and of *arn* operon carrying out 4-amino-4-deoxy-L-arabinose synthesis and transfer, (ii) PhoP-PhoQ system enhances activity of PmrA-PmrB through PmrD connector protein, (iii) MgrB membrane protein stimulates PhoP-PhoQ system. All the aforementioned proteins are products of chromosomal genes, but in 2015 the first representative of a plasmid-encoded, mobile colistin-resistance gene family (*mcr* gene family) coding phosphoethanolamine-transferases was identified.

One possible option for treating infections caused by colistin-resistant strains is antibiotic combinations. *In vitro* synergism of polymyxins and rifampin against colistin-resistant Gram-negative bacteria was proved in numerous studies. Colistin–imipenem and colistin–meropenem were proved to be efficient against carbapenemase-producing *K. pneumoniae* strains. The combinaton of colistin and tetracycline were *in vivo* succesfully used for treatment of bloodstream infection caused by panresistant *K. pneumoniae*.

In our studies we examined the susceptibility of acquired colistin-resistant bacterial strains isolated in Hungary to antibiotic combinations, the genetic factors behind their resistance, their tolerance to cationic antimicrobial peptides, and the changes in the composition of their outer membrane proteins.

OBJECTIVES

- Defining the antibiotic susceptibility of available bacterial strains.

- Finding antibiotic combinations of synergistic interactions that are *in vitro* effective against colistin-resistant bacterial strains.

- Examining the susceptibility of colistin-resistant bacterial strains to lactoferrin, lysozyme, and protamine.

- Identifying the presence of genes responsible for developing colistin-resistance with PCR (*phoP*, *phoQ*, *pmrA*, *pmrD*, *mgrB*, *mcr-1*), then measuring expression of present genes with reverse transcription PCR.

- Analyzing the outer membrane protein composition of colistin-susceptible and colistin-resistant bacterial strains.

MATERIALS AND METHODS

Bacterial strains

Klebsiella pneumoniae and *Enterobacter asburiae* strains isolated from clinical samples were investigated in our studies. The *K. pneumoniae* ATCC 700603 strain was used as control in the antibiotic susceptibility tests.

All eight *K. pneumoniae* strains belonged to international clone ST258. They were multiresistant, KPC-2 enzyme producers isolated and identified in 2008–2009, during the first colistin-resistant *K. pneumoniae* epidemic in Hungary. The two examined *E. asburiae* strains originated from sporadic cases.

Determination of antibiotic susceptibility

Antibiotic susceptibility of the strains was checked by microdilution method and E-test. The following agents were tested by microdilution: ceftazidime, cefotaxime, ceftriaxone, imipenem, ertapenem, amikacin, tobramycin, ciprofloxacin, levofloxacin, moxifloxacin, rifampin, polymyxin B and colistin. E-test was used for separating colistin-heteroresistant subpopulations within colistin-susceptible strains. After overnight incubation the valid recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were followed for minimal inhibitory concentration (MIC) value interpretation.

Checkerboard analysis

Efficiency of antibiotic combinations was tested with checkerboard method. Using the lowest individual MIC values FIC indices (fractional inhibitory concentration index) were calculated as follows: $\Sigma FICI = FICI_A + FICI_B$, where $FICI_A = MIC_A(c) / MIC_A(a)$, and $FICI_B = MIC_B(c) / MIC_B(a)$. Based on ΣFICI values synergistic ($\Sigma FICI \leq 0,5$), partially synergistic ($0,5 < \Sigma FICI < 1$), additive ($\Sigma FICI = 1$), indifferent ($1 < \Sigma FICI \leq 4$) and antagonistic ($\Sigma FICI > 4$) relations were established.

Susceptibility to lactoferrin, protamine and lysozyme

Bacteria were cultured in Luria-Bertani (LB) broth, then were centrifugalized in the exponential phase of cell division. Bacterial solutions of $2,1 \times 10^5$ CFU/ml (CFU =

colony-forming unit) were prepared, from which 10–10 µls were added to 50–50 mg/ml protamine, lysozyme and lactoferrin, respectively, making 200 µl final volumes. One hundred µl of each solution was inoculated onto LB agar plates after incubation, and after a 18-hour incubation the number of colonies were counted by naked eye (one colony considered as one CFU). Finally, percental changes of germ numbers were calculated.

Examination of colistin-resistance genes with PCR

Two or three colonies of the bacterial strains were taken from Mueller–Hinton (MH) agar, then were solved in distilled water, and the solutions were centrifugalized after a 100°C water bath. Oligonucleotides for amplification of *phoP*, *phoQ*, *pmrA*, *pmrB* and *pmrD* genes were designed with MWG Eurofins Primer Design programme, while the primers of *mcr-1* and *mgrB* genes were produced based on previous publications. PCR amplicons were run in 1,5% agarose gel, then were painted in special gel dye, and finally were detected under UV light. Identification of *pmrB* and *mgrB* amplicons' nucleotide sequence was carried out by BIOMI Ltd. (Gödöllő, Hungary). Results were analyzed using NCBI GenBank database.

Examination of gene expression with RT-qPCR

The complete RNA content of bacterial cells was extracted with RNeasy Mini Kit (QIAGEN, Hilden, Germany), then was processed with RNase-free DNase (QIAGEN). Reverse transcription PCR was performed with LightCycler RNA Master SYBR Green I. kit (Roche Applied Science, Penzberg, Germany). Oligonucleotides for amplification of *phoP*, *pmrD* and *arn* genes were designed with MWG Eurofins Primer Design programme, while the primers of housekeeping genes *rpoB* and *rrsE* were produced based on previous publications.

Isolation of outer membrane proteins

Bacterial strains were cultured in 500 ml MH broth, then were centrifugalized, and at last the sediment was resolved in normal saline solution. After resolving the sediment in Tris-HCl icy cooling was implemented, then ultrasonic disruption was performed for 2x2 minutes with 500 W (MSE Soniprep 150 Ultrasonic Disintegrator,

MSE Ltd., London, UK). After centrifugation the supernatant was ultracentrifugalized, and the resulting sediment was solved in sodium lauryl sarcosine solution (Sigma-Aldrich Ltd., Budapest, Hungary), then was incubated. Following another ultracentrifugation the sarcosine-insoluble outer membrane proteins (OMP) were isolated from the sediment.

One dimensional gel electrophoresis of outer membrane proteins (1-DE)

The outer membrane protein sample was mixed with Lämmli solution [1 M Tris (pH 6,8), 50% glycerol, 10% sodium dodecyl sulfate (SDS), β-mercaptoethanol, bromophenol blue, distilled water (Bio-Rad Magyarország Kft., Budapest, Hungary)], then the mixture was warmed. After cooling electrophoresis was carried out in Bio-Rad Mini Protean 3 system. Gels were incubated in dying solution, then were steeped in differentiating solution.

Analysis of outer membrane proteins with Microchip

Outer membrane proteins were extracted, marked with fluorescent dye, centrifugalized, then separated electrophoretically in Agilent 2100 Bioanalyzer System Microchip (Agilent Technologies, Santa Clara, CA, USA).

Two dimensional gel electrophoresis of outer membrane proteins (2-DE)

Outer membrane proteins were solved in 2-DE sample buffer [8 M urea, 2% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), 50 mM dithiothreitol, 0,2% Bio-Lyte® 3/10 Ampholyte, bromophenol blue (Bio-Rad)], were applied onto immobilized pH gradient (IPG) strips, the were separated according to their charges by isoelectric focusing. After this the strips were washed in equilibration buffer, then the proteins were separated according to their masses by polyacrylamide gel electrophoresis. Protein bands were made visible with dying solution, and the gel areas of interest were cut out for further examination.

In-gel digestion

Gel parts containing protein bands were cut out and sliced into smaller pieces. The dye was removed, gel pieces were dehydrated, disulfide bridges were reduced and

sulfhydryl groups were alkylated, then side-chain-protected trypsin (Promega, Madison, WI, USA) was used for in-gel proteolysis. Digested peptides were extracted from the gel with formic acid solution (Sigma-Aldrich).

MALDI-TOF/MS mass spectrometry

Mass spectrometry examination was carried out with Autoflex II MALDI-TOF/MS method (Bruker Daltonics, Bremen, Germany). Digested peptides were solved in a mixture of α-cyano-4-hydroxycinnamic acid (Bruker Daltonics), acetonitrile and trifluoroacetic acid (Scharlau Chemie, Barcelona, Spain). Peptides were identified by 1000 shots, data processing was executed with flexAnalysis software package version 3.1 (Bruker Daltonics), and Sequence Editor software (Bruker Daltonics) was used for analysis. The MASCOT algorithm (http://www.matrixscience.com) and the Swiss-Prot database (Swiss Institute of Bioinformatics, Genf, Svájc) were utilized for protein identification.

RESULTS

Antibiotic susceptibility

All *K. pneumoniae* strains belonging to ST258 clone were resistant to 3rd generation cephalosporins, ertapenem, tobramycin, fluoroquinolones and rifampin. All strains were resistant to polymyxins except the No. 11 (Table 1).

Enterobacter asburiae strains isolated from sporadic cases were susceptible to 3rd generation cephalosporins, carbapenems, fluoroquinolones and amikacin. Within the colistin-susceptible strain No. 0821 a colistin-heteroresistant subpopulation was identified with E-test (Table 2).

Table 1: MIC values of examined *K. pneumoniae* strains

Antibiotics	MIC (µg/ml)										
	EUCAST breakpoints	***K. pneumoniae* ATCC 700603**	***K. pneumoniae* 11**	***K. pneumoniae* 12**	***K. pneumoniae* 97**	***K. pneumoniae* 105**	***K. pneumoniae* 132**	***K. pneumoniae* 153**	***K. pneumoniae* 160**	***K. pneumoniae* 167**	***K. pneumoniae* 168**
ampicillin	**8**	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
ceftazidime	**4**	64	>256	>256	>256	>256	>256	>256	>256	>256	>256
cefotaxime	**2**	16	128	128	32	32-64	32	64	32	32	128
ceftriaxone	**2**	8	256	256	128	128	128	128-256	64-128	128	256
ertapenem	**1**	<0,125	32	64	8-16	16	8-16	16	16-32	16	32
imipenem	**8**	<0,125	256	256	4	4-8	2-4	4	4	4	16
amikacin	**16**	2	32	32	2	2	2	2	16	16	2
tobramycin	**4**	4	32	16	16	16	8-16	16	16	16	16
ciprofloxacin	**1**	0.5	128	128	128	128	128	128	128	128	128
levofloxacin	**2**	1	64	64	64	64	64	64	64	64	64
moxifloxacin	**1**	2	64	64	64	64	64	64	64	64	64
polymyxin B	**-**	2	<0,125	128	32	16	16-32	8-16	16	32	64-128
colistin	**2**	1	<0,125	256	32	32	32-64	32	32-64	32-64	256
rifampin	**-**	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256

Table 2: MIC values of examined *E. asburiae* strains

Antibiotics	MIC (µg/ml)				
	EUCAST breakpoints	***K. pneumoniae* ATCC 700603**	***E. asburiae* 0821**	***E. asburiae* 0821/H**	***E. asburiae* 148**
ampicillin	**8**	>256	256	256	32
ceftazidime	**4**	64	0,25	1	<0,125
cefotaxime	**2**	16	0,5	1	<0,125
ceftriaxone	**2**	8	0,5	1	<0,125
ertapenem	**1**	<0,125	<0,125	<0,125	<0,125
imipenem	**8**	<0,125	0,5	0,5-1	0,25-0,5
amikacin	**16**	2	0,5	0,5	0,5-1
tobramycin	**4**	4	0,25	<0,125	0,5
ciprofloxacin	**1**	0,5	<0,125	<0,125	<0,125
levofloxacin	**2**	1	<0,125	<0,125	<0,125
moxifloxacin	**1**	2	<0,125	<0,125	<0,125
polymyxin B	**-**	2	0,125	>256	64-128
colistin	**2**	1	0,125	>256	256
rifampin	**-**	>256	>256	>256	>256

Results of checkerboard analysis

Antibiotic interactions observed in *K. pneumoniae* strains No. 11 and 12 were different from several aspects (Table 3–4). In case of No. 11 pure synergism was detected only between imipenem and tobramycin, but partial synergism was noted in combinations comprised of rifampin, ciprofloxacin, imipenem and ceftazidime. Imipenem combined with rifampin, tobramycin or ciprofloxacin was synergistic against the colistin-resistant strain No. 12. Rifampin combined with colistin or polymyxin B was also synergistic against it.

Table 3:

MIC values and FIC indices of antibiotics tested on *K. pneumoniae* strain 11

***K. pneumoniae* 11**					
Antibiotic combinations	MIC (µg/ml)				FICI
	1. AB_{alone}	2. AB_{alone}	1. $AB_{combination}$	2. $AB_{combination}$	
colistin - ceftazidime	0,125	256	0,25	1	2,004
colistin - ciprofloxacin	0,125	128	0,25	1	2,008
colistin - imipenem	0,125	8	0,25	1	2,125
colistin - rifampin	0,125	256	0,25	1	2,004
polymyxin B - ceftazidime	0,125	256	0,25	1	2,004
polymyxin B - ciprofloxacin	0,125	128	0,25	1	2,008
polymyxin B - imipenem	0,125	8	0,25	1	2,125
polymyxin B - rifampin	0,125	256	0,25	1	2,004
rifampin - ciprofloxacin	256	128	0,25	64	0,501
rifampin - imipenem	256	8	4	4	0,516
imipenem - ciprofloxacin	8	128	4	1	0,508
imipenem - tobramycin	8	32	1	4	0,250
ceftazidime - ciprofloxacin	256	128	0,25	64	0,501
ceftazidime - tobramycin	256	32	1	16	0,504
tobramycin - ciprofloxacin	32	128	32	1	1,008

Table 4:

MIC values and FIC indices of antibiotics tested on *K. pneumoniae* strain 12

K. pneumoniae 12					
Antibiotic combinations	MIC (μg/ml)				FICI
	1. AB_{alone}	2. AB_{alone}	1. $AB_{combination}$	2. $AB_{combination}$	
colistin - ceftazidime	256	256	64	2	0,258
colistin - ciprofloxacin	256	128	2	16	0,133
colistin - imipenem	256	8	32	8	1,125
colistin - rifampin	256	256	0,25	1	0,005
polymyxin B - ceftazidime	128	256	64	1	0,504
polymyxin B - ciprofloxacin	128	128	1	16	0,133
polymyxin B - imipenem	128	8	2	4	0,516
polymyxin B - rifampin	128	256	0,25	1	0,006
rifampin - ciprofloxacin	256	128	64	64	0,750
rifampin - imipenem	256	8	1	4	0,504
imipenem - ciprofloxacin	8	128	1	2	0,141
imipenem - tobramycin	8	32	1	2	0,188
ceftazidime - ciprofloxacin	256	128	256	64	1,500
ceftazidime - tobramycin	256	32	0,25	16	0,501
tobramycin - ciprofloxacin	32	128	16	1	0,508

Susceptibility to lactoferrin, protamine and lysozyme

The *K. pneumoniae* strains were proved to be resistant to lactoferrin – we did not observe any decrease of colony forming units after exposure. Protamine caused 97% colony forming unit fall in the colistin-susceptible *K. pneumoniae* strain, however, CFU number only decresed with 40% in the colistin-resistant strain. Lysozyme exhibited 100% bactericidal effect on the colistin-susceptible strain, and the effect was also notable albeit of lesser extent in case of the colistin-resistant one (Table 5).

Protamine did not cause any significant change in CFU numbers of the *E. asburiae* strains. On the other hand, the colistin-resistant strains showed high tolerance to lactoferrin and lysozyme (Table 6).

Table 5: CFU changes in *K. pneumoniae* strains after exposition to serum, liquor, lactoferrin, lysozyme and protamine (Col S = colistin-susceptible; Col R = colistin-resistan)

		K. pneumoniae **Col S**	***K. pneumoniae*** **Col R**
Initial germ number (CFU/ml)		$2{,}1 \times 10^5$ (100%)	$2{,}1 \times 10^5$ (100%)
Germ number change	**Serum (rat)**	$2{,}52 \times 10^4$ (-88%)	$2{,}1 \times 10^4$ (-90%)
	Liquor (rat)	$6{,}3 \times 10^3$ (-97%)	$3{,}15 \times 10^4$ (-85%)
	Protamine (50 mg/ml)	$6{,}3 \times 10^3$ (-97%)	$1{,}26 \times 10^5$ (-40%)
	Lactoferrin (50 mg/ml)	$2{,}1 \times 10^5$ (0%)	$2{,}1 \times 10^5$ (0%)
	Lysozyme (50 mg/ml)	0 (-100%)	$2{,}73 \times 10^4$ (-87%)

Table 6: CFU changes in *E. asburiae* strains after exposition to lactoferrin, lysozyme and protamine (Col S = colistin-susceptible; Col R = colistin-resistan)

		E. asb **0821** **Col S**	***E. asb*** **0821/H** **Col R**	***E. asb*** **148** **Col R**
Initial germ number (CFU/ml)		$2{,}1 \times 10^5$ (100%)	$2{,}1 \times 10^5$ (100%)	$2{,}1 \times 10^5$ (100%)
Germ number change	**Protamine (50 mg/ml)**	$1{,}995 \times 10^5$ (-5%)	$2{,}1 \times 10^5$ (0%)	$2{,}1 \times 10^5$ (0%)
	Lactoferrin (50 mg/ml)	$2{,}1 \times 10^5$ (0%)	$2{,}919 \times 10^5$ (+39%)	$2{,}373 \times 10^5$ (+13%)
	Lysozyme (50 mg/ml)	$2{,}184 \times 10^5$ (+4%)	$2{,}646 \times 10^5$ (+26%)	$2{,}163 \times 10^5$ (+3%)

Examination of colistin-resistance genes with PCR

Presence of *phoP*, *phoQ*, *pmrA*, *pmrB* and *pmrD* genes were verified in the colistin-susceptible and colistin-resistant *K. pneumoniae* strains, as well. Neither the *E. asburiae* nor the *K. pneumoniae* strains carried the *mcr-1* gene.

The *mgrB* gene was detected in all *K. pneumoniae* strains. A 954 basepair-long amplicon was detected in the colistin-susceptible strain. Sequence analysis showed that the amplicon contained a new, previously undescribed *mgrB* variant, and a type 5 insertion sequence (*IS5*). Uniformly 540 basepair-long amplicons lacking *IS5* were detected in the colistin-resistant strains, and these identical *mgrB* genes also coded a new variant of the protein (Figure 1).

Sequence of PmrB demonstrated wild-type protein in the colistin-susceptible and colistin-resistant strains, as well.

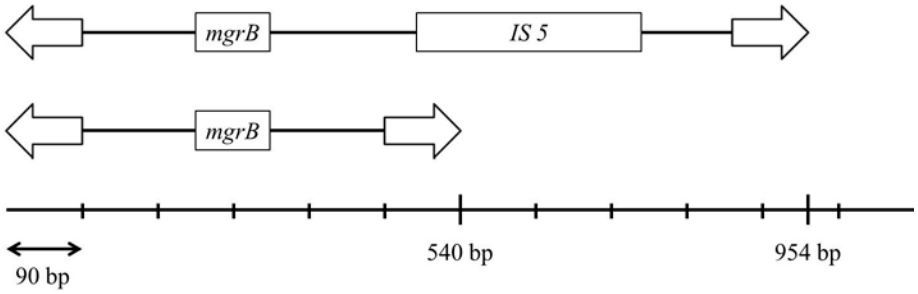

Figure 1:

Length of *mgrB* genes isolated from *K. pneumoniae* strains (upper amplicon: colistin-susceptible *K. pneumoniae* strain; lower amplicon: colistin-resistant *K. pneumoniae* strain)

Examination of gene expression with RT-qPCR

The PhoP-PmrD-*arn* regulatory system's role in colistin-resistance was confirmed in the colistin-resistant *K. pneumoniae* strains: increased expression of *phoP* and *arn* genes was detected (Figure 2).

One dimensional gel electrophoresis of outer membrane proteins (1-DE)

Absence of a ca. 15–16 kDa protein fraction was detected in the colistin-resistant *K. pneumoniae* and colistin-resistant *E. asburiae* strains (Figures 3–4).

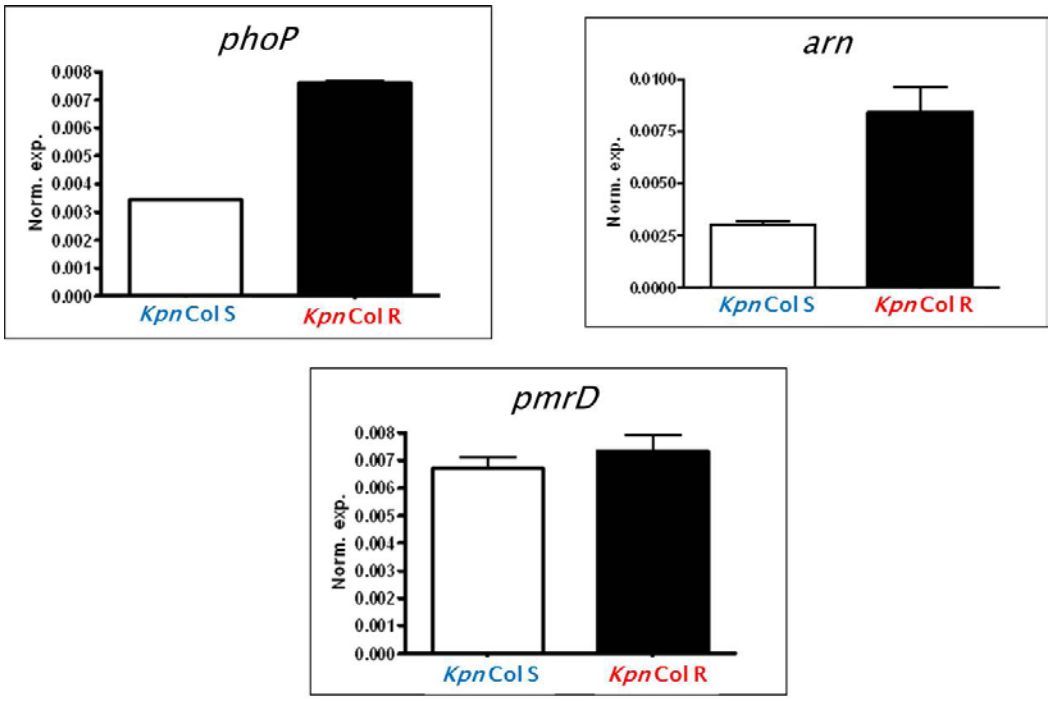

Figure 2: Relative gene expression of *K. pneumoniae* colistin-resistance genes

(*Kpn* Col S = colistin-susceptible *K. pneumoniae*; *Kpn* Col R = colistin-resistant *K. pneumoniae*)

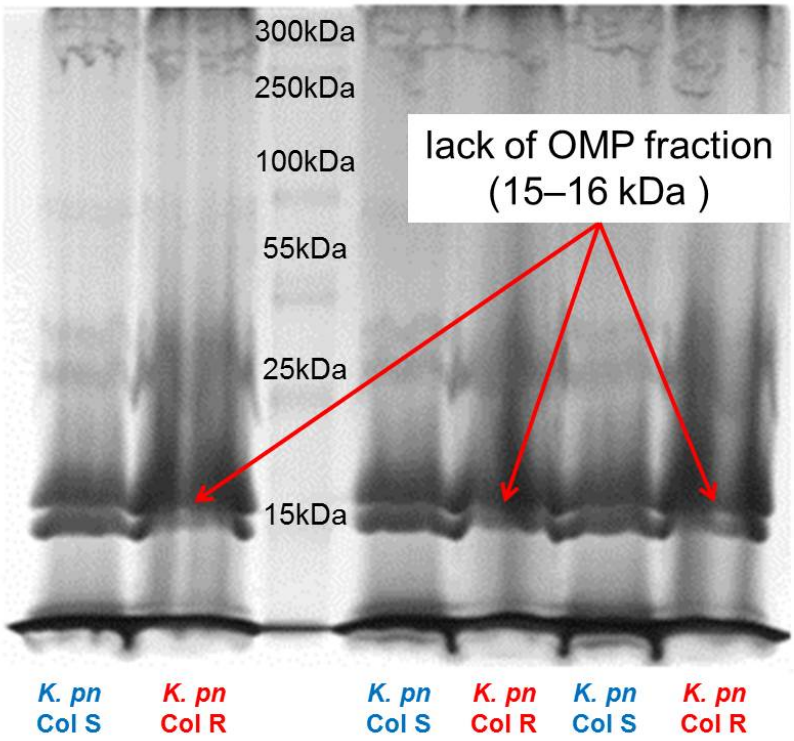

Figure 3: 1D gel electrophoresis of *K. pneumoniae* outer membrane proteins

(*K. pn* Col S = colistin-susceptible *K. pneumoniae*; *K. pn* Col R = colistin-resistant *K. pneumoniae*)

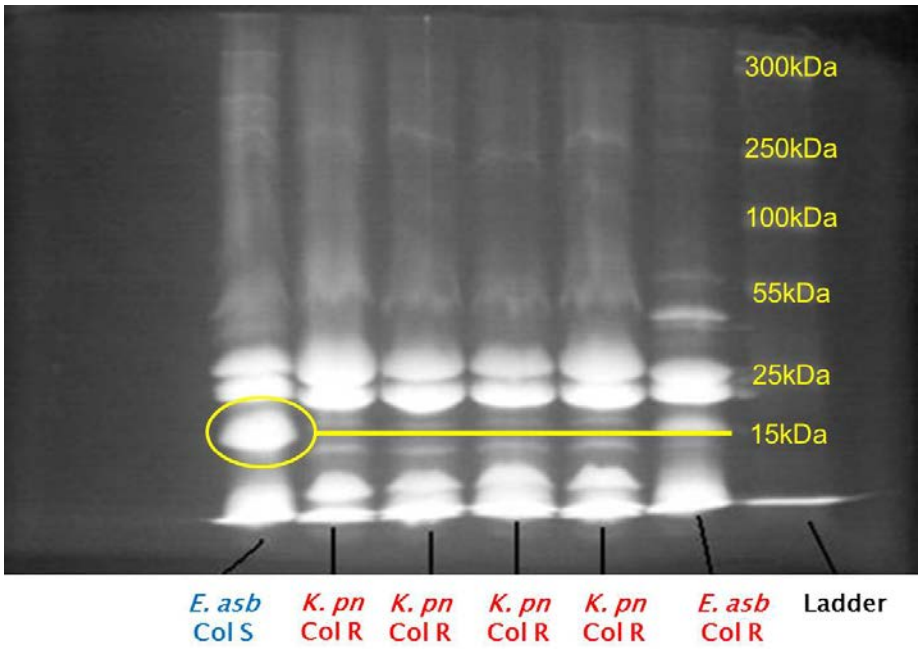

Figure 4: 1D gel electrophoresis of *E. asburiae* outer membrane proteins

(*K. pn* Col R = colistin-resistant *K. pneumoniae*;

E. asb Col S = colistin-susceptible *E. asburiae*; *E. asb* Col R = colistin-resistant *E. asburiae*)

Analysis of outer membrane proteins with Microchip

Running of outer membrane proteins marked with fluorescent dye in Agilent 2100 Bioanalyzer System microchip demonstrated absence of a protein fraction in the colistin-resistant *K. pneumoniae* and colistin-resistant *E. asburiae* strains, respectively, 20–25 seconds into the run (Figures 5–6).

Analysis of outer membrane proteins with MALDI-TOF/MS mass spectrometry

Substantial amount of DNA starvation/stationary phase protection proteins (Dps) and LysM domain/BON superfamily proteins was found in the colistin-susceptible *K. pneumoniae* strain, while the colistin-resistant strain lacked these.

Outer membrane proteins OmpC and OmpW were present in the colistin-susceptible *E. asburiae* strain, but were absent in the colistin-resistant ones. OmpA and OmpX were only detected in the colistin-resistant strains.

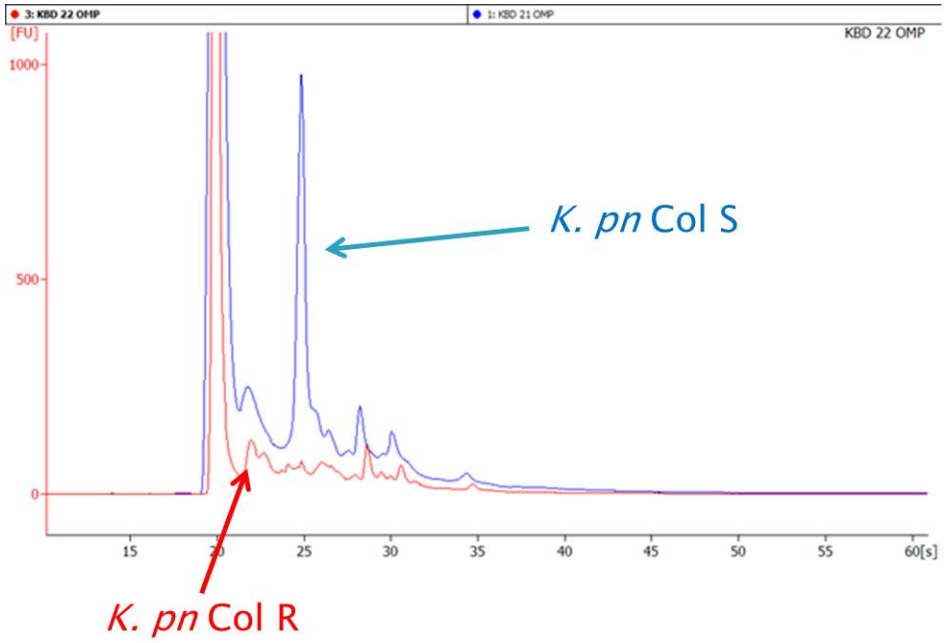

Figure 5: Analysis of *K. pneumoniae* outer membrane proteins by Microchip

(*K. pn* Col S = colistin-susceptible *K. pneumoniae*; *K. pn* Col R = colistin-resistant *K. pneumoniae*)

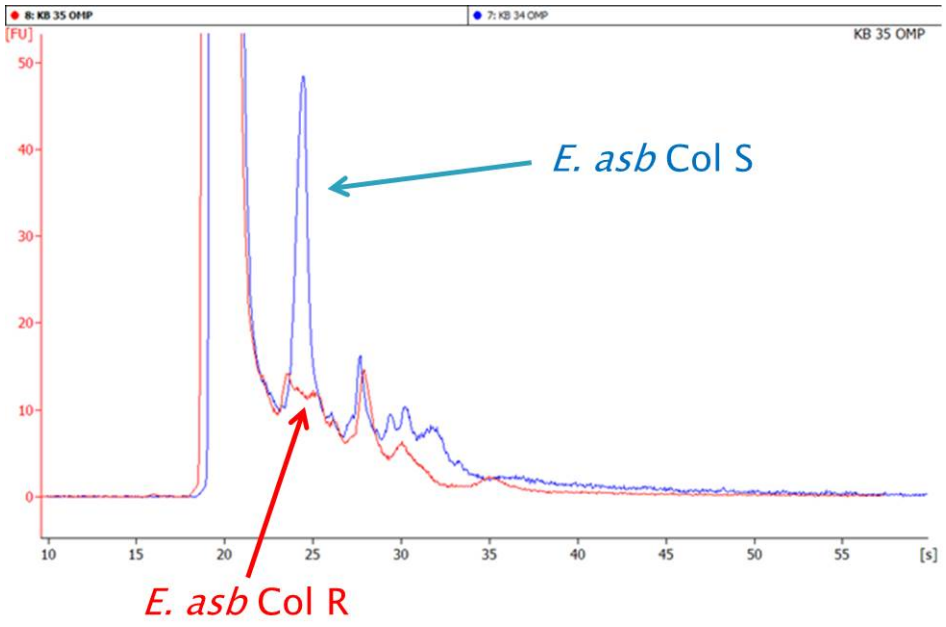

Figure 6: Analysis of *E. asburiae* outer membrane proteins by Microchip

(*E. asb* Col S = colistin-susceptible *E. asburiae*; *E. asb* Col R = colistin-resistant *E. asburiae*)

DISCUSSION

Our experiments on colistin-resistant, KPC-2 producing *K. pneumoniae* strains confirm previous reports about importance of antibiotic combination therapy versus monotherapy. Synergistic interaction was detected between the following antibiotic combinations: rifampin–colistin, rifampin–polymyxin B, imipenem–rifampin, imipenem–tobramycin and imipenem–ciprofloxacin.

Our findings on the presence of a colistin-heteroresistant subpopulation within a colistin-susceptible *E. asburiae* strain correlate with international reports of recent years. Colistin-heteroresistance concomitant with lysozyme-tolerance was previously observed in *Enterobacter cloacae*. Cationic antimicrobial peptides bind to the PhoP-PhoQ proteins in the outer membrane of Gram-negative bacteria similarly to polymyxins. This regulatory protein pair has an important role in the development of colistin-resistance. Our *K. pneumoniae* and *E. asburiae* strains developed colistin-resistance without previous polymyxin exposure, which suggests the possibility of cross-tolerance/-resistance. Tolerance to human antimicrobial peptides (e.g. lactoferrin, lysozyme) may contribute to development of colistin-resistance. This phenomenon might applies backwards, too – bacteria may become tolerant to host antimicrobial peptides due to colistin-resistance.

Presence of several chromosomal genes responsible for colistin-resistance (*phoP*, *phoQ*, *pmrA*, *pmrB*, *pmrD* and *mgrB*) was successfully verified with PCR in *K. pneumoniae* strains. However, the *mcr-1* plasmid-encoded resistance gene first described in November 2015 was not detectable in any examined strains.

Changes in amino-acid sequence of PmrB are knowingly contribute to development of colistin-resistance, but PmrB proteins of our colistin-resistant *K. pneumoniae* strains were all wild-type. On the other hand, MgrB proteins of the colistin-susceptible and colistin-resistant *K. pneumoniae* strains were equally different from wild-type, and there was an *IS5* nucleotide portion adjoining *mgrB* gene in the susceptible strain which was absent in colistin-resistant ones. This is remarkable because insertion sequences usually lead to colistin-resistance by inserting themselves into *mgrB*, deactivating it.

Role of PhoPQ and *arn* systems in developing colistin-resistance in our strains was verified with RT-qPCR. Although expression of *pmrD* gene did not demonstrate

significant difference between colistin-susceptible and colistin-resistant *K. pneumoniae* strains, substantial overexpression of *arn* and *phoP* was detected in the colistin-resistant strain.

During gel electrophoresis of colistin-resistant *K. pneumoniae* and *E. asburiae* strains' outer membrane proteins absence of a ca. 15-16 kDa protein fraction was detected. Absent proteins in case of *K. pneumoniae* were DNA starvation/stationary phase protection proteins (Dps) and LysM domain/BON superfamily proteins. Colistin-resistant *E. asburiae* strains lacked OmpC and OmpW, but OmpA and OmpX were present.

Main function of Dps and their homologues is the protection of bacterial cells during stationary phase of cell division. The bind to bacterial chromosome in a non-specific way, creating a stable, protected Dps-DNA complex. They bind and oxidize intracellular Fe^{2+} ions, decreasing the amount of reactive oxygen species.

LysM and BON domains are conserved protein sections. These are mainly structural proteins and enzymes responsible for maintaining cell membrane integrity. As colistin-resistance is based on molecular changes in the cell wall, the alterations in the expression of these proteins is logical, although its background is not clear, yet.

OmpC and its homologues are porin-type transport proteins found in the outer membrane of *Enterobacteriaceae*. They are responsible for transporting several types of molecules into the cells, including antibiotics. Their loss or decreased expression leads to antibiotic resistance and diminished susceptibility to serum antimicrobial activity in *E. coli* and *Enterobacter* spp.

OmpA is a multifunctional membrane protein: in addition to maintaining integrity of the outer membrane, it is responsible for serum resistance in *E. coli* and antimicrobial peptide resistance in *K. pneumoniae*.

OmpX is a protein structurally similar to OmpA. Its overproduction was observed in multiresistant *Enterobacter aerogenes* strains with the simultaneous underproduction of OmpF and Omp36 porins, as well as structural changes of LPS. Upregulation of *ompX* and downregulation of *omp36* together cause the decrease of outer membrane permeability.

CONCLUSIONS

We have reached the following conclusions and results in our studies:

- *Klebsiella pneumoniae* strains
  - ST258 clone is prone to developing colistin-resistance
  - combinations of polymyxins and rifampin, as well as imipenem–rifampin, imipenem–tobramycin and imipenem–ciprofloxacin have *in vitro* antibacterial activity against colistin-resistant strains
  - colistin-resistant strains develop tolerance to antimicrobial peptides
  - colistin-resistant strains are overexpressing *phoP, pmrD* and *arn* genes
  - new MgrB variants were identified in ST258 clone
  - outer membrane protein assortment changes in colistin-resistant strains (loss of LysM/BON superfamily proteins and DNA starvation proteins)
- *Enterobacter asburiae* strains
  - certain *E. asburiae* strains are able to develop colistin-resistance while retaining their susceptibility to β-lactams, aminoglycosides and fluoroquinolones
  - colistin-resistant strains develop tolerance to antimicrobial peptides
  - outer membrane protein assortment changes in colistin-resistant strains (absence of OmpC and OmpW, and presence of OmpA and OmpX)

LIST OF OWN PUBLICATIONS

Articles published in the subject of dissertation

Kádár B, Kocsis B, Tóth Á, Damjanova I, Szász M, Kristóf K, Nagy K, Szabó D. (2013) Synergistic antibiotic combinations for colistin-resistant *Klebsiella pneumoniae*. Acta Microbiol Immunol Hung, 60(2): 201-209., **IF: 0,780**

Kádár B, Kocsis B, Kristóf K, Tóth Á, Szabó D. (2015) Effect of antimicrobial peptides on colistin-susceptible and colistin-resistant strains of *Klebsiella pneumoniae* and *Enterobacter asburiae*. Acta Microbiol Immunol Hung, 62(4): 501-508., **IF: 0,568**

Kocsis B, Kádár B, Tóth Á, Fullár A, Szabó D. (2017) MgrB variants in colistin-susceptible and colistin-resistant *Klebsiella pneumoniae* ST258. J Microbiol Immunol Infect, 50(5): 735-736., **IF: 2,973**

Kádár B, Kocsis B, Tóth Á, Kristóf K, Felső P, Kocsis B, Böddi K, Szabó D. (2017) Colistin resistance associated with outer membrane protein change in *Klebsiella pneumoniae* and *Enterobacter asburiae*. Acta Microbiol Immunol Hung, 64(2): 217-227., **IF: 0,921***

Articles published in other subjects than the dissertation

Kádár B, Szász M, Kristóf K, Pesti N, Krizsán G, Szentandrássy J, Rókusz L, Nagy K, Szabó D: *In vitro* activity of clarithromycin in combination with other antimicrobial agents against biofilm-forming *Pseudomonas aeruginosa* strains. *ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA* 57:(3) pp. 235-245. (2010), **IF: 0,625** (Kádár B és Szász M megosztott első szerzők)

Kádár B, Kocsis B, Nagy K, Szabó D: The renaissance of polymyxins. *CURRENT MEDICINAL CHEMISTRY* 20:(30) pp. 3759-3773. (2013), **IF: 3,715**

Szabó Bálint Gergely, Lénárt Katalin Szidónia, Kádár Béla, Gombos Andrea, Dezsényi Balázs, Szanka Judit, Bobek Ilona, Prinz Gyula: A *Streptococcus pneumoniae* (pneumococcus) -infekciók ezer arca. *ORVOSI HETILAP* 156:(44) pp. 1769-1777. (2015), **IF: 0,291**

Szabó BG, Kádár B, Lénárt KS, Dezsényi B, Kunovszki P, Fried K, Kamotsay K, Nikolova R, Prinz G: Use of intravenous tigecycline in patients with severe *Clostridium difficile* infection: a retrospective observational cohort study. *CLINICAL MICROBIOLOGY AND INFECTION* 22:(12) pp. 990-995. (2016), **IF: 5,292**