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Cross-resistance to human cationic antimicrobial peptides and to polymyxins mediated by the plasmid-encoded MCR-1?

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Running Head: antimicrobial peptides resistance

Key words; colistin, defensins. *Enterobacteriaceae, Escherichia coli*, *Klebsiella pneumoniae*, MCR-1

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Abstract

Objectives. To evaluate whether acquired resistance to cationic antimicrobial peptides (CAMP) group molecules, being normal components of the human immune system, may select co-resistance to antibiotic peptides such as polymyxins, considering they share the same mechanism of action. We aimed to evaluate strains producing the recently identified plasmid-encoded polymyxin resistance determinant MCR-1, which is a phosphoethanolamine transferase that modifies the lipopolysaccharide structure of Gram-negative bacteria.

Methods. In-vitro susceptibility studies using human CAMPs, namely cathelicidin LL-37, α-defensin 5 (HD5) and β-defensin 3 (HDB3), towards MCR-1-producing and colistin-resistant *Escherichia coli* or *Klebsiella pneumoniae* were performed.

Results. Cross-resistance to CAMPs and colistin mediated by MCR-1 or chromosomal mechanisms was neither observed in *E. coli* nor in *K. pneumoniae*.

Conclusion. Therefore, the future therapeutic development of human CAMPs may likely not be impeded by the spread of MCR-1 plasmid-mediated resistance to polymyxins, at least in *E. coli*.

Introduction

Polymyxin antibiotics, including colistin, are currently considered as last resort antibiotics drugs for treating infections due to multidrug resistant Gram-negative pathogens [1]. Colistin is a cationic antimicrobial peptide (CAMP) derived from the Gram-positive bacterium *Bacillus polymyxa*, and acts by disrupting the outer membrane of Gram-negative bacteria [2], a trait shared with other CAMPs [3]. Emergence of polymyxin-resistance is increasingly observed [2] and the recent identification of a plasmid-mediated colistin resistance determinant MCR-1 is an additional source of concern [4]. MCR-1 is a phosphoethanolamine transferase that modifies the ionic charge of the lipopolysaccharide by adding a phosphoethanolamine group to the lipid A, leading to resistance to polymyxins [4]. This resistance trait has been reported first from animal, human isolates and food in *Enterobacteriaceae* in China and then worldwide, mostly in *Escherichia coli* [4, 5].

Structurally related CAMPs are part of the intrinsic human immune system and may target Gram-negative bacteria [6, 7]. They are mostly cathelicidins and defensins [6, 7] that are small peptides of 18-45 and 15-80 amino acids, respectively. They act by disrupting the outer and inner membranes of Gram-negative bacteria through electrostatic interaction and do not possess a catalytic or enzymatic activity. Cross-resistance between several humans CAMPs and polymyxins has rarely been evidenced in *Enterobacteriaceae* [7, 8]. In addition, resistance to polymyxins and to the human CAMPs has been associated with the production of a phosphoethanolamine transferase in the Gram-negative species *Haemophilus ducreyi* and *Campylobacter jejuni* [9, 10]. Therefore, our working hypothesis was that a crossed resistance to CAMPs and polymyxins might be observed among MCR-1 producing *E. coli*.

The selected CAMPs, LL-37, α-defensin 5 (HD5) and β-defensin 3 (HDB3) are among the most studied and widely distributed CAMPs in humans, and they are known to be active against *E. coli* [6, 7]. LL-37 is a human CAMP found at sites of inflammation where it is a primary defense gate against Gram-negatives [11, 12]. HD5 is abundant in neutrophils of epithelia of mucosal surfaces such as those of the intestine, respiratory tract, urinary tract, and vagina. HBD3, as most β-defensins, is inducibly produced, usually in response to pro-inflammatory stimuli [13], and is found in epithelial cells of gut and lungs [6, 7, 13]. HBD3 exhibits antibacterial activity against both Gram-negative and Gram-positive bacteria, and also possesses the ability to act as a chemo-attractant [6, 7].

Methods

Bacterial strains, media and growth conditions

Strains used in this study are summarized in Table 1 and include: wild-type *E. coli* and *K. pneumoniae* strains, MCR-1-producing *E. coli* strains, *E. coli* transconjugants producing MCR-1, and *E. coli* and *K. pneumoniae* isolates harbouring chromosomal-encoded colistin resistance mechanisms (Table 1 [14, 15]). To assess the role of plasmids carrying the genetic determinant of MCR-1, we tested two *E. coli* J53 transconjugants (TC24 and TC31) respectively obtained from clinical isolates Af24 and Af31 producing MCR-1 (Table 1). All strains were grown at 37°C either in Luria-Bertani (LB) broth or on LB agar plates.

Minimum inhibitory concentration

All strains were tested for colistin and antimicrobial peptides resistance by broth microdilution method (BMD) according to the EUCAST guidelines [20] (Table 3).

Bactericidal assay

Each strain was exposed to variable concentrations of CAMPs (Peptanova Gmbh, Sandhausen, Germany, and Innovagen AB, Lund, Sweden) and plated onto LB agar to evaluate the surviving bacteria by colony counting. Bacterial survival to CAMPs was expressed as the percentage of colony forming unit (CFU) surviving the exposure to various concentrations of CAMPs as previously described [16-19]. Approximately 1,000 CFU of mid-logarithmic phase of each bacterial culture were incubated with 0.2, 0.3, 0.6, 1.25, 2.5, 5, 10 and 20 mg/L of each CAMP for 2 h in a 96-well plate at 37°C in phosphate buffer saline supplemented with 1% LB broth. The upper limit of 20 mg/L of each CAMP was deduced from previous works that showed less than 20% of bacterial survival at that concentration [6, 7]. Samples were plated in triplicate onto LB agar plates to quantify the remaining bacteria. The assays were run at least in duplicate for each peptide concentration. The means were calculated for each experimental condition. The variability of observation was shown by plotting the the maximum and minimum values for each proportion on the chart as vertical bars.

Results

The MCR1-producing *E. coli* clinical strains (Af24 and Af31) showed similar susceptibility patterns for the tested CAMPs, with a lower susceptibility to HDB3 for Af31 as compared to Af24 at 5, 10 and 20 mg/L of peptide concentrations (Figure A, Table 2). The observed susceptibility pattern consisted of a high dose dependent activity for LL-37, an intermediate (dose-dependent) activity for HDB3, and a very low activity for HD5. This is in agreement with previously published work [16-19] except for HD5 which activity could have been higher. Compared to their wild-type counterpart R1436, results for Af24 and Af31 are not significantly different for LL-37 and HD5. Therefore, the *mcr-1* gene did not provide any significant resistance to LL-

37, HDB3 or HD5 for the selected strains under our experimental conditions. This was confirmed with the *E. coli* transconjugant strains (Figure A) TC24 and TC31 which results are similar to those obtained with their parental clinical strains Af24 and Af31. One could observe that TC24 had a slightly increased resistance for HD5 compared to J53 and TC31 strains.

As colistin resistance can be also mediated by chromosomal mechanisms, we tested strains (Af51 and CDF11) with chromosomally by-encoded colistin resistance (Figure B). No difference was observed for these strains and the wild-type 1436 for LL-37 and HD5, in accordance with results obtained with MCR-1-producing strains. Isolates Af51 and CDf11 showed a lowered susceptibility for HDB3 compared to that of the control. Similar results are observed for isolate Af31 (MCR-1 positive), but not for *E. coli* transconjugant TC31. This suggests that a cooperative effect might exist between colistin resistance mechanisms and some specific genetic features modulating the resistance to CAMPs. A more thorough work would be required to clarify that issue.

Finally, we compared the colistin-resistant *E. coli* strains to *K. pneumoniae* harbouring chromosomally-encoded colistin resistance. Figure C shows that Fr-49 and L31 are not sensitive to CAMPs, whereas the control (R192) had a low susceptibility to the three peptides at concentrations >10 mg/L. Compared to the *E. coli* strains, *K. pneumoniae* seems naturally more resistant to human CAMPs. We could not tested MCR-1-producing *K. pneumoniae* strains, as these were not available in our strain collection.

With respect to individual peptide toxicity, LL-37 was the most active compound for all strains with no survival at a concentration as low as 2.5 mg/L for all strains with a dose-dependent effect, except for the *K. pneumoniae* strains for which

no lethal effect was observed (Figure, Table 2). A recent study of Kao *et al.* [21] suggests a low bactericidal effect of LL-37, however the methodology used in that study (MICs performed by broth microdilution) differed from that used in our and in previously published studies [16-19]. In order to compare the activity levels of LL-37 between our study and Kao *et al*., we determined the MICs by BMD for LL-37, HDB3 and HD5 for all our strains (Table 3). Table 3 shows that the activity measured in this study is similar to what has been reported by Kao *et al.* [21]. HDB3 also showed a dose dependent activity when activity was observed, but to a lower extent than LL-37 ranging between 10-20 mg/L for a 90% reduction of CFU after 2 hours of incubation. Finally, HD5 had almost no effect on all tested strains except for J53, TC31 and R192. Although, some punctual differences are sometimes observed between the tested strains and the controls exposed to the tested peptides, no general correlation can be deduced between CAMPs activities and strain antibiotic resistance mechanism. The CAMP activity seems therefore to be variable and specific for each CAMPs member and each strain.

Discussion

Our results indicate that the susceptibility of *E. coli* strains to each CAMP may vary depending on the CAMP molecule itself rather than the susceptibility to the entire CAMP family. This is in agreement with a previous study that showed that the bactericidal levels of cathelicidin peptides was specific for each peptide [21]. Under our experimental conditions, although MCR-1 is a phosphoethanolamine transferase that modifies the lipid A, it does not confer cross resistance with human CAMPs for *E. coli* strains. Similar observations were made with strains being resistant through chromosomally-encoded mechanisms. These results do not correlate with previous observations obtained with the non-enterobacterial species *Acinetobacter baumannii*

[8]. This could be explained by three major differences; (1) the bacterial species were different, (2) the level of resistance to colistin was much higher in the other study (128-256 mg/L versus 4-8 mg/L), and (3) the mechanism of colistin resistance corresponded to chromosomal mutations in the *pmrB* gene involved in the LPS biosynthesis pathway [8]. A very recent report showed some cross-resistance between MCR-1 and the cationic host antimicrobial lysozyme in *E. coli* [22]. Nevertheless, lysozyme can not be considered as an antimicrobial peptide such as cathelicidin or defensin, as it is a much bigger molecule (120-200 amino acids) and possess an enzymatic activity targeting specific molecules.

This study has two limitations, the first one being the low number of available MCR-1-producing *E. coli* strains and the lack of MCR-1-producing *Klebsiella* spp. strains. The second limitation is the lack of characterization of the surface properties of the strains which might play a role in the CAMPs-cells interaction and therefore affect the susceptibility of the strains to CAMPs.

The colistin-resistant but MCR-1-negative *Klebsiella pneumoniae* strains that we tested did not show any increased resistance to the selected CAMPs. However, as we had no access to MCR-1-producing *K. pneumoniae* strains, further work will be needed to evaluate the MCR-1 effect with regard to CAMPs resistance in that bacterial species.

For *E. coli*, the variable susceptibility results indicated sharp differential activities for each CAMP, regardless of the resistance mechanism. This underscore that further therapeutic development of CAMPs such as LL-37, magainin or protegrin may not be impeded by the spread of MCR-1-producing *E. coli* as these strains are not systematically more resistant to human CAMPs than their wild-type counterparts.

Transparency declaration

All authors declare that they have no conflicts of interest

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TABLE 1. Bacterial strains used in this study

Name	Species	Origin [reference]	Source[a]	MICs of colistin[b]	Mechanism of colistin resistance	Plasmid type[c]	Resistance*	Sequence Type
R1436	*E. coli*	Wild type clinical strain	NA	0.25	-	-	TET	ND
J53	*E. coli*	Wild type reference strain	NA	0.25	-	-	-	ST735
Af24	*E. coli*	South Africa [14]	Human	4	Plasmid, *mcr-1*	IncI2	AMX, CHL, CIP, CST, NAL, SUL, SXT, TET, TIC	ST1007
Af31	*E. coli*	South Africa [14]	Human	8	Plasmid, *mcr-1*	IncHI2	CHL, CIP, CST, FLO, KAN, NAL, SXT, SUL, TET	ST624
Af51	*E. coli*	South African	Human	16	Chromosomal		AMX, ATM, CEF, CIP, CST, CTX, GEN, KAN, FEP, NAL, PIP, SXT, SUL, TET, TIC, TOB	ND
CDF11	*E. coli*	Switzerland	Human	8	Chromosomal		AMX, ATM, CEF, CIP, CST, CTX, FEP, KAN, NAL, PIP, SXT, TEM, TET, TIC, TOB	ND
TC24	*E. coli*	Transconjugant of Af24 in J53	NA	4	Plasmid, *mcr-1*	IncI2	CST	ND
TC31	*E. coli*	Transconjugant of Af31 in J53	NA	8	Plasmid, *mcr-1*	IncHI2	CHL, CST, SUL, SXT	ND
R192	*K. pneumoniae*		NA	0.25	-	-		ND
FR-49	*K. pneumoniae*	France [15]	Human	64	*mgrB* truncated by IS5-like	-	AMC, AMK, AMX, ATM, CAZ, CEF, CIP, CST, CTX, ETP, FEP, FOF, FOX, KAN, IPM, MEM, NAL, PIP, PPT, SXT, SUL, TCC, TEM, TIC, TOB	ND
L31	*K. pneumoniae*	France	Rabbit	64	Chromosomal	-	AMC, AMX, ATM, CAZ, CEF, CIP, CST, CTX, ETP, FEP, KAN, MEM, NAL, PIP, PPT, TEM, TCC, TIC, TOB	ND

- none; ND not determined; NA not available

[a]Human strains are from clinical source and the rabbit from a veterinary one

[b]MIC values are in mg/L

[c]Plasmid types only correspond to those carrying the *mcr-1* gene

* AMC, Amoxicillin-clavulanic acid ; AMK, Amikacin ; AMX, Amoxicillin ; ATM, Aztreonam ; CAZ, Ceftazidime ; CEF, Cephalothin ; CIP, Ciprofloxacin; CHL, Chloramphenicol; CST, Colistin; CTX, Cefotaxime ; ETP, Ertapenem, FEP, Cefepime ; FLO, Florfenicol; FOF, Fosfomycin ; FOX, Cefoxitin ; GEN, Gentamycin ; KAN,

Kanamycin ; IPM, Imipenem, MEM, Meropenem ; NAL, Nalidixic acid ; PIP, Piperacillin ; TZP, Piperacillin-Tazobactam ; SPH, Sulphonamides ; SXT, Trimethoprim-sulfametoxazole ; TEM, Temocilin ; TET, Tetracycline ; TIC, Ticarcilin ;TIM, Ticarcilin-clavulanic acid ; TOB, Tobramycin

TABLE 2. Survival percentage of tested strains exposed to 20 mg/L of human CAMPs

Name	Strain	LL37[a]			HD5[a]			HDB3[a]		
		min	$\bar{x}$	max	min	$\bar{x}$	max	min	$\bar{x}$	max
w	*E. coli*	0	0	0	50	74	134	3	9	15
J53	*E. coli*	0	0	0	32	40	46	0	1	4
Af24	*E. coli*	0	0	0	84	95	105	9	11	13
Af31	*E. coli*	0	0	0	61	83	106	32	49	66
TC24	*E. coli*	0	0	0	58	75	91	0	0	0
TC31	*E. coli*	0	0	0	23	49	69	0	0	1
Af51	*E. coli*	0	0	0	87	95	104	28	38	49
CDF11	*E. coli*	0	0	0	67	74	81	63	77	91
R192	*K. pneumoniae*	56	68	80	40	56	72	34	54	74
FR-49	*K. pneumoniae*	71	83	95	104	110	116	94	107	120
L31	*K. pneumoniae*	84	100	116	63	97	130	94	105	116

[a]Calculated means ($\bar{x}$) of survival percentage are given for each peptide with respective minimum (min) and maximum (max) values of the calculated proportion.

TABLE 3: Antimicrobial peptide MICs (mg/L) for tested strains.

Name	Strain	HD5	HBD3	LL-37
R1436	*E. coli*	>64	64	32
J53	*E. coli*	>64	64	32
Af24	*E. coli*	>64	64	32
Af31	*E. coli*	>64	64	32
TC24	*E. coli*	>64	64	>64
TC31	*E. coli*	>64	64	16
Af51	*E. coli*	>64	>64	32
CDF11	*E. coli*	>64	64	16
R192	*K. pneumoniae*	>64	>64	>64
FR-49	*K. pneumoniae*	>64	>64	>64
L31	*K. pneumoniae*	>64	>64	>64

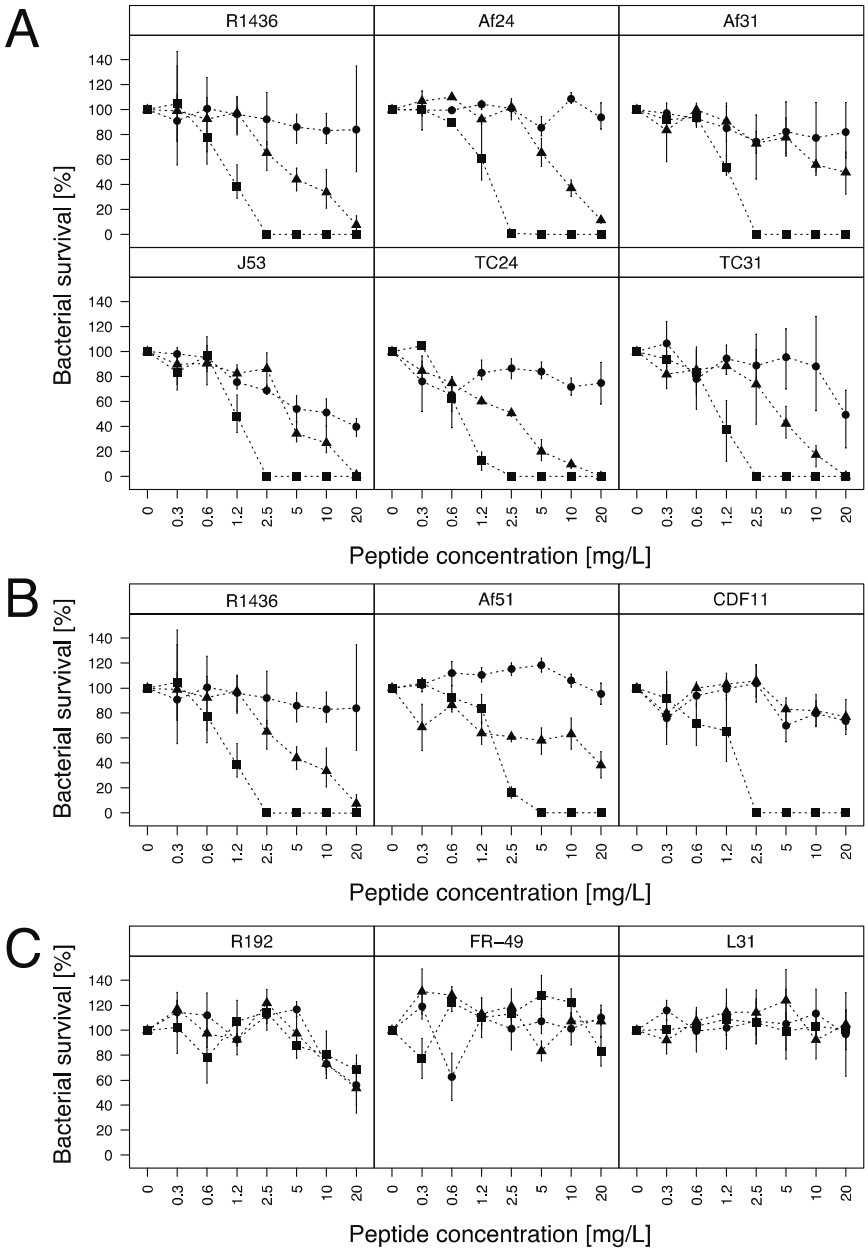

Figure. Percentage of bacterial survival as a function of peptide concentration. Panel A shows data for wild-type and MCR-1 *E.coli* strains, panel B and C respectively for *E. coli* and *K. pneumoniae* strains with chromosomal colistin resistance mechanism and wild-type strains. TC24 and TC31 are *E. coli* J53 transconjugants obtained from donor strains Af24 and Af31, respectively. Data represent the experimental means (plain symbols) with respective range of the calculated means (vertical bars) for the cationic antimicrobial peptides LL-37(■), HD5 (●) and HDB3 (▲).