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Molekularna charakterystyka lekooporności oraz czynników wirulencji szczepów *Klebsiella pneumoniae* opornych na kolistynę, izolowanych z dróg oddechowych pacjentów hospitalizowanych w południowej Polsce

Molecular analysis of resistance and virulence of colistin-resistant *Klebsiella pneumoniae* strains isolated from respiratory tract infections from patients hospitalized in Southern Poland

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W pracy przeanalizowano wyniki badań 19 szczepów *Klebsiella pneumoniae,* opornych na kolistynę, wyizolowanych od pacjentów hospitalizowanych w południowej Polsce. Szczepy te poddane zostały wnikliwej charakterystyce – sprawdzono zarówno ich wrażliwość na szeroki panel antybiotyków, wliczając antybiotyki najnowsze, rekomendowane przez EUCAST, jak również zbadano występowanie u nich genów oporności i wirulencji. Weryfikowano obecność najczęstszych determinant oporności na beta-laktamy, a także plazmidowo kodowanych genów oporności na kolistynę. Wśród genów wirulencji, poszukiwano zarówno tych związanych z syntezą otoczki, jak również tych odpowiedzialnych za pozyskiwanie żelaza (m.in. sideroforów). Wykazano, że geny oporności i wirulencji często występują w badanej populacji szczepów.

Słowa kluczowe: *Klebsiella pneumoniae* oporna na kolistynę, czynniki wirulencji, lekooporność

ABSTRACT

Objectives: Colistin resistance is reported in *K.pneumoniae* isolates worldwide. Due to the overuse of antimicrobial agents, colistin has become the only alternative treatment option. The aim of this study was to analyze the resistance and virulence of 19 colistin-resistant *K.pneumoniae* strains isolated from patients with pneumonia in four Polish hospitals.

Methods: All strains were screened for antibiotic susceptibilities by the disc diffusion method. Resistance and virulence genes were detected with PCR.

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Results: Seventeen strains were considered as XDR (Extensively Drug Resistant). Fourteen (75%) strains were ESBL-positive, all possessed bla_{CTX-M}. KPC gene was detected in one strain, *mcr-1, mcr-2, mcr-3, mcr-4* and *mcr-5* genes was not found. One strain (5%) was of capsular serotype K1, four (21%) of serotype K2. *entB* gene was detected in 19 strains (100%), *iutA* in 5 (26%), *ybtS* in 10 (53%), whereas *mrkD* in 19 (100%). *uge* was detected in 15 (79%) of strains, *rmpA* – in 7 (37%).
Conclusions: Colistin resistance in *K.pneumoniae* becomes common pattern that must be monitored even in patients not previously treated with colistin. As we demonstrated, virulence genes are ubiquitous in colistin-resistant strains.

Keywords: colistin-resistant *Klebsiella pneumoniae*, virulence factors, antimicrobial resistance

INTRODUCTION

Colistin resistance is reported in *Klebsiella pneumoniae* isolates worldwide (10, 23, 35), outbreaks have been reported (19, 20). Due to the overuse of antimicrobial agents and emergence of MDR (Multidrug Resistance) or XDR (Extensive Drug Resistance) isolates, colistin has become the only alternative treatment option, regarding the fact that for a long time it had been kept as a last resort drug due to the safety reasons.

The main mechanism responsible for colistin resistance is modification of the lipid A phosphate moieties of the bacterial LPS (Lipopolysaccharide) that reduces the electrostatic interaction between cationic polimyxins and anionic LPS (26). This modification is associated with the mutations in genes that regulate the expression of *pmr*C gene (26). There are also other molecular mechanisms and mutations that may lead to colistin-resistance phenotype. Characterization of the structural alterations of LPSs with regard to polymyxin resistance has shown the involvement of phoP/phoQ and pmrA/pmrB. The phoP/phoQ and pmrA/pmrB systems are upregulated in *Klebsiella pneumoniae* exposed to polymyxins, indicating that these systems are involved in polymyxin resistance in this bacterium (24). Some latest studies describe the pattern that colistin resistance is not associated with decreased virulence (1, 6). Colistin resistance may be encoded by *mcr-1* gene, that is localized on the transferrable plasmid, often together with KPC gene (25). There are also other *mcr* genes connected with colistin resistance, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* and their variants, but those genes are detected more rarely in *K. pneumoniae* (27).

There are known virulence genes that are associated with invasive infections. One of them is *magA*, associated with mucoviscosity, encoding capsular polymerase, located within the gene cluster specifying capsular phenotype K1 (36). The gene *rmpA* is regulating the mucoid phenotype A – strains carrying this gene possess hypermucoviscous phenotype (37). Invasive isolates of *K. pneumoniae* exhibits frequently hypermucoviscous phenotype and belong to serotype K1 or K2 (8). For that reason it is advisable to screen the strains according to their serotype. One of the method that can be use is multiplex PCR for 5 or 6 serotypes (33, 34). Next gene, *uge,* is involved in capsule synthesis, as it encodes uridine diphosphate galacturonate 4-epimerase, responsible for biosynthesis of the capsule and smooth LPS.

Strains that are considered as hypervilurent may possess a number of iron acquisition systems, such as siderophores (aerobactin encoded by *iutA*, enterobactin encoded by *ent*, yersiniabactin encoded by *ybtS*), as well as *kfu*-gene, that mediates the uptake of ferric iron (2). The gene *allS* is correlated with strains isolated from liver abscess (7). This gene is involved in allantoin metabolism. Other gene that is correlated with elevated virulence is *mrkD* – type 3 fimbrial adhesin that mediates binding to the extracellular matrix (16).

The aim of this study was to analyze the resistance and virulence of colistin-resistant *K. pneumoniae* strains isolated from patients with pulmonary infections in four Polish hospitals in 2016 and 2017.

MATERIALS AND METHODS

Bacterial strains. Nineteen colistin-resistant *Klebsiella pneumoniae* isolates were collected in 2016 and 2017 from hospitalized patients. All strains were isolated from clinical samples (respiratory tract infections) and identified with automated systems (VITEK-2Compact, BioMerieux or MALDI-TOFF identification with Maldi Biotyper, Bruker). MICs of colistin (CS-0.064-1024 mg/L) were determined with E-test (Liofilchem Diagnostici, Italy). When the result of MIC testing was above 2 mg/L and the strains should be considered as colistin-resistant, those results were confirmed by microdillution method (SensiTest Colistin; Liofilchem Diagnostici, Italy). String test of hypermucoviscosity was assessed by using inoculation loop on bacterial colonies grown for 24 h at 37°C. This test was considered as positive if the string reached at least 5 mm.

Susceptibility testing. Antibiotic susceptibilities were determined by the disc diffusion method on Mueller-Hinton agar plates according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. Antimicrobial agents tested were: piperacillin (PRL-30µg), piperacillin/tazobactam (TZP-30µg/6µg), ampicillin/sulbactam (SAM-20µg 1:1), amoxicillin/ clavulanic acid (AMC-30µg 2:1), ticarcillin (TIC-75µg), ceftazidime (CAZ-10µg), cefotaxime (CTX-5µg), cefepime (FEP-30µg), cefuroxime sodium (CXM-30µg), ceftaroline (CPT-5µg), ceftriaxone (CRO-30µg), imipenem (IPM-10µg), ertapenem (ETP-10µg), meropenem (MEM-10µg), doripenem (DOR-10µg), aztreonam (ATM-30µg), ciprofloxacin (CIP-5µg), levofloxacin (LEV-5µg), moxifloxacin (MXF-5µg), ofloxacin (OFX-5µg), amikacin (AK-30µg), gentamicin (CN-10µg), netylmicin (NET-10µg), tobramycin (TOB-10µg), sulfamethoxazole/trimethoprim (SXT-25µg 19:1), chloramphenicol (C-30µg) (Oxoid, Ltd., Basingstoke, UK) and ticarcillin/clavulanic acid (TTC-85µg (75+10)), ceftobiprole (BPR-5µg), ceftolozane/tazobactam (T/C-40µg (30+10)) (Liofilchem Diagnostic, Italy). Minimal Inhibitory Concentration (MIC) of tigecyclin (TGC-0,016-256 mg/L) was determined with E-test (Liofilchem Diagnostici, Italy). Extended spectrum beta-lactamases (ESBLs) were detected by E-test (Cefotaxime/Cefotaxime+Clavulanic acid; Liofilchem Diagnostici, Italy). Phenotypic test for the presence of KPC was performed with E-test (MIC Test Strip KPC with meropenem/meropenem+boronic acid; Liofilchem Diagnostici, Italy). Results were interpreted using clinical breakpoints as defined by the current guidelines of the EUCAST – version 8.0 (http://www.eucast.org/clinical_breakpoints/).

DNA isolation. Genomic Mini AX Bacteria Kit (A&A Biotechnology) was used for extraction of genomic DNA from *K. pneumoniae* isolates in accordance with the manufacturer's protocol. DNA extracted from pure cultures were stored at -20°C for further examination.

Detection of resistance genes. Isolates showing ESBL activity were screened with multiplex-PCR for the presence of bla_{CTX-M}, bla_{SHV} *and* bla_{TEM} genes and a simplex PCR for bla_{OXA-1} gene using previously published primers (21). KPC gene was detected with PCR using method published by *Schechner* et al. (2009) (30). Colistin resistant isolates were screened by PCR for the presence of *mcr-1, mcr-2, mcr-3, mcr-4, mcr-5* genes according to previously published method by *Rebelo et al.* (2018) (27).

Virulence genes detection. Common virulence genes were detected with PCR according to previously published protocol: magA (mucoviscosity-associated gene A) (11) and uge (the gene involved in capsule synthesis) (28), rmpA (4), ybtS (yersiniabactin), entB (enterobactin), iutA (aerobactin), allS, mrkD (8). Capsule types (K2, K5, K20, K54, K57) were detected with previously published primers (33, 34).

RESULTS

Antimicrobial susceptibility testing. Altogether, 19 unique *Klebsiella pneumoniae* strains resistant to colistin were studied – all of them were isolated from hospitalized patients. The range for colistin resistance was 2-16 mg/mL, MIC50 = 3 mg/mL. Sixty-three percent of the strains came from male patients (n=12) and the median patient age was 77 years (Quartile 1: 39; Quartile 3: 86). All strains came from respiratory tract infections (sputum, bronchoalveolar lavage, bronchial aspirate, bronchial secretion) – patients were diagnosed with pneumonia. Five strains were considered as hypemucoviscous, according to string test. Seventeen strains (90%) were considered to be extensively drug-resistant (XDR), while 2 were classified as multidrug resistant (MDR). Fourteen (74%) strains were able to produce extended spectrum beta-lactamases (ESBLs). All strains were resistant to ticarcillin. One strain was non-susceptible to all studied carbapenems (Table 1). Almost all strains were resistant to the most common cephalosporins, as well as the newest cephalosporins with inhibitors (ceftolozan/tazobactam).

Resistance genes. Fourteen strains (74%) were able to produce EBSL, according to phenotypic results. All of those strains possessed bla_{CTX-M} gene. Bla_{SHV-1} gene were present in all 19 strains. TEM-1 gene was present in 13 strains (68%), whereas bla_{OXA-1} in 9 (47%). KPC gene was detected in 1 strain – this strains was resistant to all antimicrobials tested except tygecycline. The presence of KPC was proven by phenotypic test, described in Method section. *mcr-1, mcr-2, mcr-3, mcr-4, mcr-5* genes were not detected among the strains. The gene encoding an aminoglycoside 6'-N-acetyltransferase (*aac-6'-Ib*) was present in 13 (68%) strains – all of them were non-susceptible to amikacin and tobramycin.

Virulence genes. One strain (5%) was of capsular serotype K1, whereas four (21%) – of serotype K2. None of the strains were of capsular serotype K5, K20, K54 or K57. *entB* gene was detected in 19 strains (100%), aerobactin gene (*iutA*) was present in 5 strains (26%), *ybtS* in 10 (53%). *mrkD* was prevalent in 19 (100%) of strains. *uge* was detected in 15 (79%) of strains, whereas *rmpA* – in 7 (37%). No strains with *allS* were found.

Table 1. Number and percentage of strains non-susceptible to particular antimicrobials.

Antimicrobial class	Antimicrobial	Number of non-susceptible strains	% of non-susceptible strains
Penicillins	ampicillin/sulbactam (SAM)	15	79%
	amoxycillin/clavulanate (AMC)	19	100%
	Piperacillin (PIP)	18	95%
	piperacillin/tazobactam (TZP)	15	79%
	Ticarcillin (TIC)	19	100%
	ticarcillin/clavulanate (TIM)	18	95%
Cephalosporins	Cefepime (FEP)	13	68%
	Cefotaxime (CTX)	14	74%
	Ceftaroline (CPT)	17	89%
	Ceftazidime (CAZ)	15	79%
	Ceftobiprol (BPR)	19	100%
	Ceftolozan/tazobactam (T/C)	18	95%
	Ceftriaxone (CRO)	17	89%
	Cefuroxime (CXM)	13	68%
Carbapenems	Doripenem (DOR)	8	42%
	Ertapenem (ETP)	14	74%
	Imipenem (IMP)	1	5%
	Meropenem (MEM)	1	5%
Monobactams	Aztreonam (AZT)	17	89%
Fluoroquinolones	Ciprofloxacin (CIP)	15	79%
	Lewofloxacin (LVX)	15	79%
	Moxifloxacin (MOX)	17	89%
	Ofloxacin (OFX)	18	95%
Aminoglycosides	Amikacin (AK)	13	68%
	Gentamycin (CN)	9	47%
	Netilmycin (NET)	13	68%
	Tobramycin (TOB)	14	74%
Tetracyclines	Tigecycline (TGC)	6	32%
Miscellaneous	Chloramphenicol (C)	10	53%
Polymyxins	Colistin (COL)	19	100%
Miscellaneous	trimpetoprim/sulfametoxazol (SXT)	14	74%

Table 2. Virulence genes among colistin-resistant *Klebsiella pneumoniae* strains.

Strain number	Antimicrobial resistance profile*	Identified virulence genes **
7	SAM-AMC-PRL-TZP-TIC-TIM-CTX-CPT-CAZ-BPR-C/T-CRO-CXM-ETP-ATM-CIP-LEV-MXF-OFX-AK-CN-NET-TOB-C-CS-SXT	rmpA, uge, mrkD, entB, iutA
42	AMC-PRL-TIC-TIM-CPT-BPR-C/T-CRO-DOR-ATM-MXF-OFX-CS	K2, rmpA, uge, ybtS, mrkD, entB, iutA
46	AMC-PRL-TIC-TIM-CPT-BPR-C/T-CRO-DOR-ETP-ATM-OFX-CS	K2, rmpA, uge, mrkD, entB, iutA
48	SAM-AMC-PRL-TZP-TIC-TIM-FEP-CTX-CPT-CAZ-BPR-C/T-CRO-CXM-DOR-ETP-IMP-MEM-ATM-CIP-LEV-MXF-OFX-AK-CN-NET-TOB-C-CS-SXT	uge, mrkD, entB
53	AMC-PRL-TIC-TIM-BPR-C/T-CRO-MXF-OFX-TGC-CS	K1, rmpA, uge, ybtS, mrkD, entB
54	SAM-AMC-PRL-TZP-TIC-TIM-CPT-BPR-CIP-LEV-MXF-OFX-C-CS-SXT	uge, mrkD, entB
59	SAM-AMC-PRL-TZP-TIC-TIM-FEP-CTX-CPT-CAZ-BPR-C/T-CRO-CXM-DOR-ETP-ATM-CIP-LEV-MXF-OFX-AK-NET-TOB-TGC-CS-SXT	mrkD, entB
60	AMC-TIC-BPR-C/T-ATM-CS	rmpA, uge, ybtS, mrkD, entB
62	SAM-AMC-PRL-TZP-TIC-TIM-FEP-CTX-CPT-CAZ-BPR-C/T-CRO-CXM-ETP-ATM-CIP-LEV-MXF-OFX-AK-CN-NET-TOB-TGC-C-CS-SXT	uge, ybtS, mrkD, entB
87	SAM-AMC-PRL-TZP-TIC-TIM-FEP-CTX-CPT-CAZ-BPR-C/T-CRO-CXM-ATM-CIP-LEV-MXF-OFX-AK-CN-NET-TOB-CS	K2, rmpA, uge, mkrD, entB, iutA
91	SAM-AMC-PRL-TZP-TIC-TIM-FEP-CTX-CPT-CAZ-BPR-C/T-CRO-CXM-DOR-ETP-ATM-CIP-LEV-MXF-OFX-AK-CN-NET-TOB-C-CS-SXT	uge, ybtS, mrkD, entB
93	SAM-AKC-PRL-TZP-TIC-TIM-FEP-CTX-CPT-CAZ-BPR-C/T-CRO-CXM-DOR-ETP-ATM-CIP-LEV-MXF-OFX-AK-CN-NET-TOB-C-CS-SXT	uge, ybtS, mrkD, entB
97	SAM-AMC-PRL-TZP-TIC-TIM-FEP-CTX-CPT-CAZ-BPR-C/T-CRO-CXM-DOR-ETP-ATM-CIP-LEV-MXF-OFX-AK-CN-NET-TOB-TGC-C-CS-SXT	uge, ybtS, mrkD, entB
98	SAM-AMC-PRL-TZP-TIC-TIM-FEP-CTX-CPT-CAZ-BPR-C/T-CRO-CXM-DOR-ETP-ATM-CIP-LEV-MXF-OFX-TOB-C-CS-SXT	uge, ybtS, mrkD, entB
99	SAM-AMC-PRL-TZP-TIC-TIM-FEP-CTX-CPT-CAZ-BPR-C/T-CRO-CXM-ETP-ATM-CIP-LEV-MXF-OFX-AK-CN-NET-TOB-C-CS-SXT	uge, ybtS, mrkD, entB
105	SAM-AMC-PRL-TZP-TIC-TIM-FEP-CTX-CPT-CAZ-BPR-C/T-CRO-CXM-ETP-ATM-CIP-LEV-MXF-OFX-AK-NET-TOB-CS-SXT	ybtS, mrkD, entB
115	SAM-AMC-PRL-TZP-TIC-TIM-FEP-CTX-CPT-CAZ-BPR-C/T-CRO-CXM-ETP-ATM-CIP-LEV-MXF-OFX-AK-NET-TOB-TGC-C-CS-SXT	mrkD, entB
117	SAM-AMC-PRL-TZP-TIC-TIM-FEP-CTX-CPT-CAZ-BPR-C/T-CRO-CXM-ETP-ATM-CIP-LEV-MXF-OFX-AK-CN-NET-TOB-CS-SXT	K2, rmpA, uge, mrkD, entB , iutA
119	SAM-AMC-PRL-TZP-TIC-TIM-FEP-CTX-CPT-CAZ-BPR-C/T-CRO-CXM-ETP-ATM-CIP-LEV-MXF-OFX-AK-NET-TOB-TGC-CS-SXT	mrkD, entB

* - all abbreviations of antimicrobial names are presented in Table 1.

** - *mag*A - mucoviscosity-associated gene A; *uge* - the gene involved in capsule synthesis; *rmp*A - positive regulator of extracapsular polysaccharide synthesis; *ybt*S – yersiniabactin; *ent*B – enterobactin; *iut*A – aerobactin; allS - gene involved in allantoin metabolism; mrkD - type 3 fimbrial adhesion; K2, K5, K20, K54, K57 - capsule types genes.

DISCUSSION

Klebsiella pneumoniae is widely known as an etiological factor of community acquired and hospital acquired infections, responsible for bacteremia, liver abscesses, pneumonia or meningitis. As the incidence of such infections has increased in recent years, it led to overuse of antimicrobials and – as following – to the occurrence of multidrug or extensively drug resistant pathogens. *Klebsiella pneumoniae* strains resistant to all antimicrobial drugs, including carbapenems and colistin were noticed worldwide (17, 19, 20, 26). In this study we analyzed the resistance and virulence of selected 19 strains, isolated from patients with pneumonia.

mcr-1, mcr-2, mcr-3, mcr-4, mcr-5 genes, connected with plasmid mediated colistin resistance, were not detected among the strains, similarly to previous studies (3, 10). We suppose that colistin resistance in our strains was due to molecular changes in *pmrA, pmrB,* and *mgrB* genes. Unfortunately we have no possibility to conduct deeper molecular studies.

Fortunately, all isolates except one (18/19) were susceptible to imipenem and meropenem, but the vast majority of isolated strains was non-susceptible to most commonly used drugs, such as penicillins and cephalosporins. What may be of serious concern, the vast majority of strains were resistant to ceftolozan/tazobactam that is considered as quite new treatment option for ESKAPE pathogens. In contrast to previous studies this antimicrobial was less efficient than cefepime, cefuroxime or cefotaxim (32). Similar results were obtained for ceftaroline, drug approved by FDA in 2010 – only 2 strains were susceptible for this antimicrobial. Three-fourth of the strains produced ESBLs, all of them possessed CTX-M gene, similarly to previous report (23). Nine out of 19 strains had all four beta-lactamase genes studied: CTX-M together with TEM, SHV-1 and OXA-1. The only one carbapenem-resistant, colistin-resistant strain possessed KPC gene – such strains have been reported previously (12). Previous studies have shown that hypermucoviscous strains are not resistant to many antimicrobials (15, 31). Here, 5 of 7 strains possessed both genes. KPC strains are detected in Poland – the number of such strains has been growing since 2017.

Klebsiella pneumoniae serotypes K1 and K2 are associated with highly virulent strains. Both are the most prevalent serotypes found in pyogenic liver abscess and are also frequent in strains isolated from community acquired pneumonia (9). In our study, K1 and K2 represented 26% of all colistin resistant strains, similarly to the data provided by *Wasfi R* et al. (2016) (35), where only MDR strains were studied. K1 and K2 were less prevalent among studies conducted in Japan on general *K. pneumoniae* strains (11.8%) (13). Capsular serotype K2 plays an important role in determining virulence and may worsen the course of infection caused by *K. pneumoniae* (11).

Remarkably, *rmp*A gene, that is a positive regulator of extracapsular polysaccharide synthesis (it confers a mucoid phenotype), was present in 7 strains studied (37%), what was consistent with previous data (37). Aforementioned studies conducted by Yu W et al. have shown a strong correlation between the *rmp*A gene and virulence in terms of abscess formation for hypermucoviscous *K. pneumoniae* strains (37). Here we noted that *magA* gene together with *rmpA* was present in two isolates. Such strains may be admitted as hypervirulent and may lead to worsening the progress of infection. It is worth mentioning that *mag*A gene may be useful as a marker for the diagnosis of invasive *K. pneumoniae* infections. Here, the only one strain with *magA* gene was also hypermucoviscous according to string test results.

Type 3 fimbrial adhesin (encoded by *mrkD* gene) is involved in binding of bacterial cells to endothelial cells of the respiratory tract. Interestingly, *mrkD* gene was detected in all strains, what is consistent to previous studies (29, 35).

Iron acquisition systems are essential for the growth of bacterial pathogens especially *in vivo* during the infection (22). What is more, siderophore secretion by *K. pneumoniae* may contribute to inflammation and bacterial dissemination during pneumonia (14). The most prevalent gene connected with iron uptake was *entB*, found in 100% of strains. It is consistent with the data shown by Wasfi R et al. (2016) and Candan et al (2015) (5, 35). Previous data published by Lawlor M et al suggested that yersiniabactin may be a key virulence factor for *Klebsiella pneumoniae* during pulmonary infection (18). Here, we detected *ybtS* gene in 53% and *iutA* gene in 26% of strains. Notably, the vast majority (74%) of studied strains contained at least 2 siderophore-related-genes.

The presence of *uge* gene is connected with colonization ability and virulence of *K. pneumoniae* strains, what was previously confirmed - *K. pneumoniae uge* mutants were unable to produce experimental urinary tract infections in rats and were completely avirulent in two different animal models (septicemia and pneumonia) (28). In our study, in 4 strains resistant to colistin *uge* was not detected what is not consistent to previous data provided by Yu et al. (37).

As none of those strains was isolated from patients with liver abscess, the absence of *allS* gene was expected and this trait was confirmed (38).

This study has several limitations. We have no data about mortality in patients with pneumonia. The number of samples was relatively small what may give selection bias. We were also not able to give detailed information about mechanisms of colistin-resistance in those particular *K. pneumoniae* strains.

CONCLUSIONS

Colistin resistance in *K. pneumoniae* strains becomes more and more common pattern. Some authors conclude that colistin resistance must be monitored even in patients not previously treated with colistin (3). As we demonstrated, virulence genes are ubiquitous in colistin-resistant strains.

CONFLICT OF INTEREST

None to declare

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