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Imipenem-resistance in *Serratia marcescens* is mediated by plasmid expression of KPC-2

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Abstract. – OBJECTIVE: Imipenem is a broad-spectrum carbapenem antibiotic with applications against severe bacterial infections. Here, we describe the identification of imipenem-resistant *Serratia marcescens* in our hospital and the role of plasmid-mediated KPC-2 expression in imipenem resistance.

MATERIALS AND METHODS: We used the modified Hodge test to detect carbapenemase produced in imipenem-resistant strains.

RESULTS: His resistance can be transferred to *E. coli* in co-culture tests, which implicates the plasmid in imipenem resistance. PCR amplification from the plasmid identified two pro consistent with KPC-2 of 583 and 1050 were also present in *E. coli* after co-cultur he restriction pattern for both plasmids was tical, supporting the transfer from the S. m escens isolate to E. coli. Finally, gene sequen ing confirmed KPC-2 in the pla

CONCLUSIONS: Due to th ese of KPC-2 in the imipenem-resista *. marc ens*, we propose that KPC-2 m s anti sis-tance in the S. marces ens

Key Words:
Imipenem, Dru stance, Ser arcescens, Plasmid, KPC-2.

Introduct

solat of bacteria resistant to carbapenem ics bri new challenges to the ic[1]. t st cs indicates that *Escherichia coli*, a pneumoniae, and other ent bacteria the main community- and ho quired pathogenic infections[2]. Drug s[3] in our hospital suggest that drug sistance rates of *K. pneumoniae* to imipenem, penem, and ertapenem are 1.5, 2, and 2.9%, re ively. Multiple drug resistance rate to extended spectrum β-lactamase (ESBLs) and Ampc enzyme are 45%. The drug-resistance mechanism is considered to be mediated mainly by the production of penem enz s that break down t antibiotics e describe the identifi f imipenem tant *Serratia marc ns* a alyze its ug-resistance mechanism, conclud hat plasmid-dependent *Kleb neumoniae* penemase-2 (KPC-2) e main mediator of ipenem resistance.

Mate s and Methods

Sourc g-Resistant Bacteria

We identified 32 multi-drug-resistant bacteria clinical departments of our center after cul- drug sensitivity tests. The bacterial identification by Vitek instrument (bioMerieux, Marcy, l'Etoile, France) identified 20 cases of *E. coli*, eight of *K. pneumoniae,* and four of *S. marcescens*. The average minimum inhibitory concentration (MIC) for *S. marcescens* resistance to imipenem was 46.5 + 7.2 g/ml. We used *E. coli* ATCC25922 as quality control bacteria for the drug sensitivity test.

Detection of Carbapenemase Produced by Modified Hodge test

E. coli ATCC25922 suspension was diluted to 0.5 McF with sterile saline (1:10 dilution) and inoculated into MH agar plates and dried for 5 min. Three strains were inoculated into plates containing 10 µg imipenem paper with a 1 µl inoculating loop. Then, at least 20 mm lines were drawn from the center. The plate was incubated at 35°C for 20 hr. *E. coli* rapid growth near the tested strains indicates the production of carbapenemase. We used as positive control *K. pneumoniae* strains that produce KPC-2 and as negative control *K. pneumoniae* ATCC700603.

Co-culture

E. coli EC600 (rifampicin, MIC> 1000 µg/ml) was inoculated into 5 ml Luria-Bertani broth with

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imipenem-resistant *S. marcescens*. The mixture was shaken overnight. Then, 200 µl of donor strain, 100 µl of recipient strain and 600 µl of fresh LB broth were mixed together in 1.5 ml centrifuge tubes, and incubated at 35˚C for 4 hr. A small volume, mixed and without the mix, was transferred on Mueller-Hinton agar plates (cefotaxime 2.0 µg/ml, rifampin 512 µg/ml), and incubated at 35˚C for 24 hr. Single colonies were picked and incubated at 35˚C for 4 hr. VITEK bacterial identification was used for the biochemical identification of trans-conjugants and drug sensitivity test was carried out to detect the MIC of mixed and non-mixed *E. coli*.

Plasmid purification

Plasmid Extraction Kit (Axygen, Union City, CA, USA) was used according to the instructions manual. Isoelectric focusing electrophoresis: we used a polyacrylamide gel pH 3.5-9.5 (Pharmacia co., Kalamazoo, MI, USA) in a PhastSystem electrophoresis instrument and isoelectric focusing electrophoresis, DYY-II electrophoresis instrument (Beijing Six One Instrument Factory). The ultrasonic crushing method was use[illegible] plasmid extraction, and Nitrocefin (Oxoi[illegible] was used as a substrate. In addition, cond[illegible] should follow reference[5]. The enzyme PI[illegible] known β-lactamase TEM-1 (5.4), SHV-1 (7.6) a[illegible] SHV-5 (8.2) PI were compared [illegible]ls.

Plasmid Analysis: PCR a[illegible] DNA Sequencing

BlaKPC and blaOX[illegible]1 g[illegible]e amp[illegible]d with specific prime[illegible]able 1)[illegible] reaction volume was: 50 µl ([illegible]oncentrati[illegible]s 1 x PCR buffer, Mg2+ 2[illegible] dNTPs 2 [illegible]ol/L, 500 mol/L primer, [illegible]q 0.25[illegible]yme and 2 mu l template). Fo[illegible] PCR, we u[illegible]CR System GeneAmp 9[illegible] (ABI co., Oyster [illegible] NY, USA). The reacti[illegible] parame[illegible] were 94˚C 5 min, 94˚C 45 sec. Th[illegible]ealin[illegible]mperature was adjusted based on the primers, and could be extended to 72˚C 1 min, 30 cycles, and at last 72˚C 10 min. Amplified products were resolved by 1.2% gel electrophoresis, EB staining, and photographed under the g[illegible]ging system. PCR products were puri[illegible]ding to Purification Test Kit Manu[illegible]m Shanghai Shenergy gaming Biologi[illegible]echnology. Purified products were sent to Han[illegible]biosune Biotechnology for sequenci[illegible] The [illegible]cing results were compared in G[illegible]bank to ide[illegible] corresponding β-lactam[illegible]genes.

Analysis of plasm[illegible]NA m[illegible] The plasmid DNA was extracte[illegible]b[illegible]lysis, [illegible] was carried out by t[illegible]refere[illegible]t. *S. m[illegible]escens* and Escherich[illegible]i plasmid [illegible]dealt with a restriction[illegible]clease at 3[illegible] 2 hr. Original pla[illegible] and [illegible]mid fragment dealt by restriction enzyme we[illegible]d with a 0.8% agarose ethi[illegible]omide gel, [illegible]ophoresed at 85 V c[illegible]nt voltage for 75 mi[illegible], and observed under [illegible]light.

S[illegible]tical Ana[illegible]is

S[illegible]19.0 [illegible]tical software (SPSS Inc., Chicago, I[illegible]) was used for statistical analysis and data was expressed as mean ± standard deviation. The comparison between groups was made by *t* test, $p<0.05$ was considered to be statistically significant.

Result

Results of the Improved Hodge Test

The Hodge test allows identifying bacterial strains that produce carbapenemases because they permit the growth of carbapenem-sensitive strains towards the antibiotic disc in the center of the plate (Figure 1). Imipenem creates an inhibition zone around the central disc that prevents the growth of *E. coli* ATCC25922. We identified four cases of *S. marcescens* that allow the growth of

[illegible]I. PC[illegible]er [illegible]nces.

Ge[illegible]	Primers	Sequence(5′-3′)	Size(bp)
	KPC-A	TCTAAGTTACCGCGCTGAGG	583
	KPC-B	CCAGACGACCCCATACTCAT	
	KPC-F	GCTACACCTAGCTCCACCTTC	1050
	KPC-R	TCAGTGCTCTACAGAAAACC	
bla_{OXA-1}	OXA-1-S	ACCCCTTAAAATTAAGCCC	
	OXA-1-AS	CTTGATTGAAGGGTTGGGCG	908

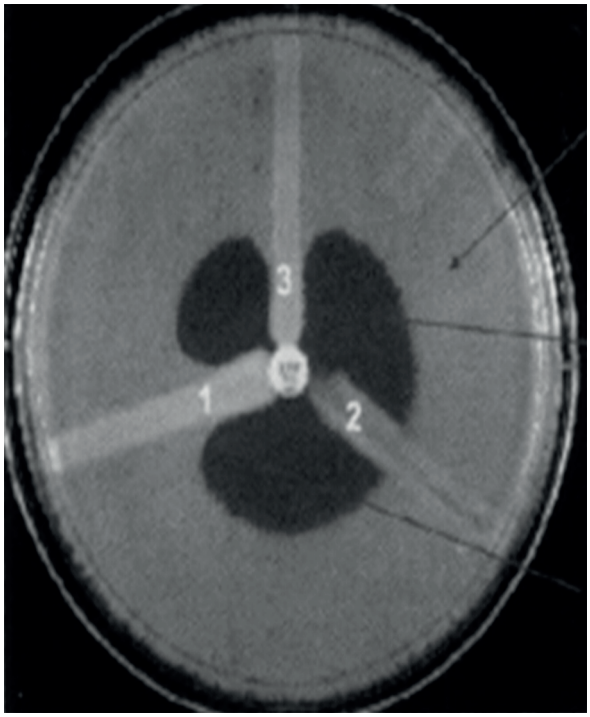

Figure 1. Improved Hodge test to detect carbapenem. 1, *Zuckerberg pneumonia* strains producing KPC-2 typ... Z... *pneumonia* strain ATCC700603. 3, Experimental ... be identified).

E. coli ATCC25922, creating a ...leaf inde... tation in the inhibition area. ... po... e Hodge... test indicates that the *S. m...scens* ...ates can produce carbapenemase.

Co-culture Exper...nt

The co-cultur... the *S. marc...s* isolates and imipenem... *E. coli* EC6... produced similar resista...ce spe... for *S. marcescens* (Table II)... the imipen... esistance rate and MIC fo... *coli* EC600 increa... markedly (Table II). T... e resul... rther support the production of ...pene...e by the *S. marcescens* isolates that re... gnifica... efits for *E. coli* EC600.

Figure 2. J... periment and ...ic focusing electrophore... ...escens*. 2, Jo... carbapenem. 3, Known βphe...ic ami... -1.

... and DNA Sequencing

...llowing co-...ure and isoelectric focusing e... phoresis, ... found a common pattern bet... S. ...escens* and *E. coli*, with two bands ... at 6.5 and 6.7 (Figure 2). PCR am...lification from the purified plasmid produced two ...ts of 583 and 1050 bp. The products iso...ted ...m *E. coli* after co-culture were the same as those purified from *S. marcescens*, but without blaOXA-1 and other fragments (Figure 3). Plasmid analysis showed that the plasmids isolated from *S. marcescens* and *E. coli* after co-culture had the same size, about 60 Kb and showed the same EcoRI restriction map. Gene sequencing of the PCR products confirmed the presence of the *KPC-2* gene (SEQ ID NO AY034847) in both *S. marcescens* isolates and *E. coli* after co-culture.

Discussion

So far, 11 isoforms of KPC enzymes have been identified, KPC-1 to 11[6]. Despite this diversity, the

Tab... Results ... co-culture.

	Drug rate to imipenem (%)		MIC (μg/ml)	
	before	after	before	after
...cescens	92.4±5.5	90.5±6.2	46.5±7.2	45.2±6.9
E... EC600	3.7±0.5	86.6±7.3#	1.7±0.6	42.3±5.8#
t	42.615	0.439	38.652	0.538
p	<0.001	0.626	<0.001	0.714

Notes: #comparison of E. coli EC600 engagement, $p<0.05$.

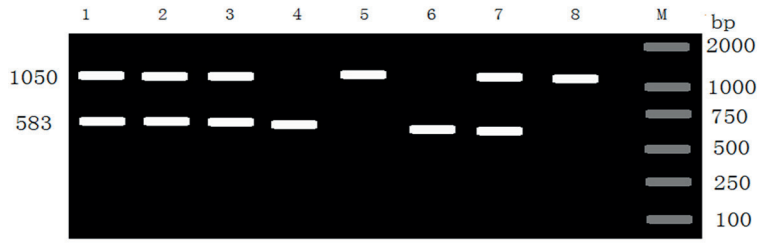

Figure 3. DNA blaKPC gene amplification. 1-4, *S. Marcescens*. 5-8, Joint carbapenem.

enterobacteriaceae of clinical relevance in China mainly produce KPC-2[7]. Rasheed and other American scholars isolated *Citrobacter freundii* and *Klebsiella oxytoca* from two patients that also produce the same KPC-2 isoform[8]. Sequencing the flanking sequence around *KPC-2* from *K. pneumoniae* isolated by Naas and cols[9], confirmed that it is the exact same gene as in *KPC-2* from American *Salmonella enterica*. The presence of the exact same *KPC-2* gene in the plasmids from two different bacteria is due to plasmid transfer, a phenomenon known as bacterial conjugation. *KPC-2* is almost identical to KPC-1 except for a Ser to Gly substitution at position 174 (S174G)[10]. The imipenem resistance of *S. marcesce* is mainly due to the production of β-lact (KPCs), which can hydrolyze the carbapene tibiotics IMP-1, Imp-6, VIM-2, sme-1, and S 2. This resistance is also associated with redu levels of penicillin binding prot P) comb ned with the loss or decreased hich are critical for the transport of iotics[1].

The modified Hodge reco CLSI to detect the car en nteroc riaceae. The test s vity fo penem is higher than for m nem and nem. The false positive be due to lack of the ESBL ou membr otein or the too large inocula[11] conclusion t all four cases of clay *Se a* bacteria isolates duce carbapenemase njugati experiments conferred *E. coli* EC he s drug resistance spectrum of *S. marce* ter th -culture, we found the sam ds fr *. marcescens* and *E. coli*, is di om previous results[13]. PCR am cation a solated the same products from *S ens* isolates and *E. coli* co-cultures, uced blaOXA-1 and other fragments similar to previous reports[14]. Gene sequencing firmed the presence of *KPC-2* in *S. marcesc* solates and *E. coli* co-cultures, supporting the role of *KPC-2* in the imipenem resistance.

At present, the infections caused by KPC-positive strains are rare and the number of effective antibacterial drugs is very limited ough t drug s vity results show resist to carb em antibiotics and other kinds of an n as q ones and aminoglycos the f the r cement antibiotic is le n ideal[15]. and development mase inhibit ng hope for treating st s tha ce KPC. NXL104, LK-157 and BLI-489 are still development, but preliminary results show that XL104 can recover the a cterial activity of various β-lactam antibiotics to strains producing KPC[16]. Also, the tricyclic penem LK has inhibitory activity for β mase in cl A and C[17]. BLI-489 is a bicyclic carb m n le with inhibitory activity over various β-lactamase[18].

Conclusions

We suggest that the mechanism mediating *S. marcescens* resistance to imipenem requires plasmid-mediated KPC-2 expression.

Acknowledgements

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Conflict of interest

The authors declare no conflicts of interest.

References

1) Dhar S, Martin ET, Lephart PR, McRoberts JP, Chopra T, Burger TT, Tal-Jasper R, Hayakawa K, Ofer-Friedman H, Lazarovitch T, Zaidenstein R, Perez F, Bonomo RA, Kaye KS, Marchaim D. Risk factors and outcomes for carbapenem-resistant klebsiella pneumoniae isolation, stratified by its multilocus sequence typing: ST258 versus non-ST258. Open Forum Infect Dis 2016; 3: ofv213.

2) Akturk H, Sutcu M, Somer A, Aydin D, Cihan R, Ozdemir A, Coban A, Ince Z, Citak A, Salman N. Carbapenem-resistant Klebsiella pneumoniae coloniza-

tion in pediatric and neonatal intensive care units: risk factors for progression to infection. Braz J Infect Dis 2016; 8: 10-11.

3) Meletis G, Oustas E, Botziori C, Kakasi E, Koteli A. Containment of carbapenem resistance rates of Klebsiella pneumoniae and Acinetobacter baumannii in a Greek hospital with a concomitant increase in colistin, gentamicin and tigecycline resistance. New Microbiol 2015; 38: 417-421.

4) Lübbert C, Becker-Rux D, Rodloff AC, Laudi S, Busch T, Bartels M, Kaisers UX. Colonization of liver transplant recipients with KPC-producing Klebsiella pneumoniae is associated with high infection rates and excess mortality: a case-control analysis. Infection 2014; 42: 309-316.

5) Matthew M, Harris AM. Identification of beta-lactamases by analytical isoelectric focusing: correlation with bacterical taxonomy. J Gen Microbiol 1976; 94: 55-67.

6) Gong L, Xu L, Diao M, Guo F, Bian FF, Min J, Liu R, Zhang CL. Clinical effect of treating secondary asthma attacks of children Mycoplasma pneumoniae with combined therapy of montelukast and azithromycin. Eur Rev Med Pharmacol Sci 2016; 20: 5256.

7) Cheng L, Cao XL, Zhang ZF, Ning MZ, Xu XJ, Zhou W, Chen JH, Zhang JH, Shen H, Zhang K. Clonal dissemination of KPC-2 producing Klebsiella pneumoniae ST11 clone with high prevale oqxAB and rmtB in a tertiary hospital i results from a 3-year period. Ann Clin M Antimicrob 2016; 15: 1.

8) Rasheed JK, Biddle JW, Anderson KF, Washer L, noweth C, Perrin J, Newton DW, B. Detecti of the Klebsiella pneumoni enemas type-2 carbapenem-hydro g enz in clini- cal isolates of Citrobact undii ar . Oxyto- ca carrying a commo mid. J 2008; 46: 2066-206

9) Naas T, Nordmann del G, Plasmid-mediated carbape hydrolyzing amase KPC in a Klebsiel moniae isola France. Antimicrob ent mother 200 49: 4423-4424.

10) Piazz Caltagirone M, I, Nucleo E, Spalla M. to E, D'Angelo R, L, Migliavacca R. gence Escherichia coli sequence Type (ST13 d ST3948 with KPC-2, KPC-3 and KPC-8 carbapenemases from a long-term care and rehabilitation facility (LTCRF) in northern Italy. Adv Exp Med Biol 2016; 26: 2-4.

11) Motayo BO, Akinduti PA, Adeyakinu FA, Okeren- tugba PO, Nwanze JC, Onoh CC, Inn HC, Okonko IO. Antibiogram and p a p ling of carbapenemase and exte spectrum Beta-lactamase (ESBL) produ Escherichia coli and Klebsiella pneumoni Abeoku- ta, Southwestern Nigeria. fr Hea 2013; 13:1091-7.

12) McGettigan SE, Andrea K, Edelstein P cificity of ertapenem ceptibi creenin detection of Kleb pueu ae carbapene- mases. J Clin M ro 0 785-7

13) Silva KE, Cayô arvalh Patus reia Sac- chi F, Rodr Costa F, Ra AC, Croda J, Gales natto S. Co on of KPC- 2 and arbapenem-r stant Serratia marce ens iso om an outbreak in a Brazi- lian Teaching Hosp Clin Microbiol 2015; 53: 28.

14) sakris A, Voulgari E, Poulou A, Kimouli M, Pour- naras S, Ranel u K, Kosmopoulou O, Petropou- lou D. In viv quisition of a plasmid-mediated la(KPC-2) g among clonal isolates of Serra- marcesc J Clin Microbiol 2010; 48: 2546-

15) Hirsc am VH. Detection and treatment options for Klebsiella pneumoniae carbapenemases (KPCs): an emerging cause of multidrug-resistant ion. J Antimicrob Chemother 2010; 65: 1119-1125.

16) Stachyra T, Levasseur P, Pechereau MC, Girard AM, Claudon M, Miossec C, Black MT. In vitro activity of the beta-lactamase inhibitor NXL104 against KPC-2 carbapenemase and Enterobacteriaceae expressing KPC carbapenemases. J Antimicrob Chemother 2009; 64: 326-329.

17) Paukner S, Hesse L, Prezelj A, Solmajer T, Urleb U. In vitro activity of LK-157, a novel tricyclic carbapenem as broad-spetrum beta-lactamase inhibitor. Antimicrob Agents Chemother 2009; 53: 505-511.

18) Petersen PJ, Jones CH, Venkatesan AM, Bradford PA. Efficacy of piperacillin combined with the penem beta-lactamase inhibitor BLI-489 in murine models of systemic infection. Antimicrob Agents Chemother 2009; 53: 1698-1700.