

Insertion element mediated *mgrB* disruption and presence of ISKpn28 in colistin-resistant *Klebsiella pneumoniae* isolates from Saudi Arabia

Taher Uz Zaman¹
Maha Albladi¹
Mohammed Ismail Siddique²
Sameera M Aljohani^{3,4}
Hanan H Balkhy^{1,5}

¹Infectious Diseases Research Department, King Abdullah International Medical Research Center, King Saud bin Abdulaziz University for Health Sciences, Riyadh, Saudi Arabia; ²Deccan College of Medical Sciences, Hyderabad, India; ³Microbiology Section, King Abdulaziz Medical City, Riyadh, Saudi Arabia; ⁴Department of Microbiology, King Saud bin Abdulaziz University for Health Sciences, Riyadh, Saudi Arabia; ⁵Department of Infection Prevention and Control, King Abdulaziz Medical City, National Guards Health Affairs, Riyadh, Saudi Arabia

Background: In *Klebsiella pneumoniae*, *mgrB* and components of pmrHFIJKLM operon play a major role in colistin resistance.

Methods: We analyzed 23 nonduplicating colistin-resistant *K. pneumoniae* isolates, collected during the years 2011–2015, for the possible mechanism underlying their nonsusceptibility to colistin. Isolates were tested for their minimum inhibitory concentrations and antibiotic resistance determinants and genotyped by multilocus sequence typing (MLST). The MLST genes, antibiotic-resistant genes, and the genes of two component system (TCS), including *mgrB*, *PhoQ/PhoP*, *pmrA/B*, and *CrrAB*, were investigated by PCR amplification and Sanger sequencing.

Results: All isolates were distributed in eight sequence types (STs) and showed mutations either in *mgrB* or *PhoP* genes. ISKpn14 was found in 10, ISKpn28 in four, and IS903 in three isolates. One isolate showed deletion of a single nucleotide in *mgrB* open reading frame causing premature stop codon. L26Q substitution in *PhoP* was found in five isolates.

Conclusion: The mutations in *mgrB* were mostly mediated by insertion elements (IS). ISKpn14 is the major IS while ISKpn28 is reported for the first time in mediating *mgrB* disruption. IS903, an IS5 family member, involved in *mgrB* disruption in three ST-152 NDM-1-positive isolates, was previously responsible for *omp-36* disruption in our carbapenem-resistant *K. pneumoniae* and appears to contribute to transform the isolates into a pan-drug ones. Also, the abundance of insertion sites in *mgrB* indicates the plasticity of this gene. In our isolates, IS-mediated colistin resistance appears to be a later phenomenon than mutation in *PhoP* gene.

Keywords: colistin resistance, *mgrB* gene mutations, insertion elements, *Klebsiella pneumoniae*

Introduction

Colistin has been the only hope so far for the treatment in worst scenarios of antibiotic-resistant bacterial infections, including carbapenem-resistant Enterobacteriaceae. Recent reports indicate a colistin resistance in multidrug-resistant (MDR) *Klebsiella pneumoniae* to approximately 45%.¹ The main mechanism attributed to colistin resistance is the upregulation of *PhoQ/PhoP*, a two-component system (TCS), which in turn activates the pmrHFIJKLM operon. Mutations in *PhoQ/PhoP* as well as *CrrAB* signaling system are found to exert resistance against colistin.² A significant molecular mechanism that has recently been described in colistin resistance in *K. pneumoniae* is the mutation/inactivation of *mgrB* gene encoding for a 47–amino acid transmembrane protein that exerts a negative-feedback effect on *PhoQ/PhoP*. An inactivation of this gene is reported to upregulate the *PhoQ/PhoP* TCS, thus conferring resistance against colistin.³ A plasmid-mediated mobile colistin resistance gene (*mcr-1*, *-2*, and *-3* variants) has recently been reported from several parts of the globe indicating increased horizontal transfer of this resistance.⁴

Correspondence: Taher Uz Zaman
King Abdullah International Medical Research Center, King Saud bin Abdulaziz University for Health Sciences, MC-1515, PO Box 22490, Riyadh 11426, Saudi Arabia
Tel +966 11 429 4551
Fax +966 11 429 4466
Email taher_zamans@yahoo.com

Materials and methods

Twenty-three nonrepetitive, confirmed, multidrug, colistin-resistant (ColR) *K. pneumoniae* isolates were studied. These isolates were collected, screened, and stored in the microbiology lab during 2011–2015. Single colonies were picked up and grown in liquid Tryptic Soy Broth (TSB) medium at 37°C overnight. Minimum inhibitory concentrations (MICs) of antibiotics for these isolates were determined using Micro Vitek 2 (bioMérieux, Durham, NC, USA). MIC breakpoints for carbapenemase were defined according to the modified 2010 CLSI guidelines.⁵ ColR *K. pneumoniae* isolates were further subjected to analysis for *mgrB*, *PhoP/PhoQ*, *pmrA/B*, and *CrrAB* genes. Polymerase chain reaction (PCR) amplification of plasmid-borne mobile colistin resistance (*mcr*) genes was carried out using the primers described by Liu et al.⁶ Resistance genes and the genes for multilocus sequence typing, virulence factors, *mgrB*, *PhoP/Q*, *PmrA/B*, and *CrrB* were amplified using their specific primers, reported earlier.^{7–10} The amplicon of these PCR reactions was sequenced on an ABI 3100 DNA analyzer (Life Technologies, USA). The sequences were analyzed using SeqMan software (Lasergene, DNASTar) and blasted against the GenBank (NCBI, USA) sequence database. The type of insertion elements (IS) present in *mgrB* gene was determined by IS Finder.¹¹ The evolutionary histories were inferred using the neighbor-joining method. The evolutionary distances were computed using the maximum composite likelihood method, for ISKpn14 and IS903 nucleotide sequences separately. Evolutionary analyses were conducted using Mega6 software.¹²

Ethics approval

The *K. pneumoniae* isolates studied in this report are part of the collection of pathogens in the Microbiology Laboratory of our hospital collected as routine hospital procedure. Hence, no ethical clearance/patient consent was needed.

Results

Here we present our observations on the 23 ColR *K. pneumoniae* isolates from Saudi Arabia; probably the first report on the mutations in *mgrB* and other regulatory systems involved in colistin resistance.

Demography, MIC, sequence typing, and carbapenem resistance genes

The isolates were collected from different sources including blood, wound, tracheal aspirate, urine, and so on from the patients admitted to different wards of King Abdul-Aziz Medical City, National Guards Hospital Riyadh. All isolates

belonged to MDR group of *K. pneumoniae* (resistant to more than four groups of antibiotics, including carbapenem). OXA-48 and/or NDM-1 genes were the major carbapenemase determinants. None of the isolates was positive for KPC, VIM, or IMP gene. The distribution of resistance genes, virulence factors, and sequence types (STs) among isolates is given in Table 1.

mgrB and other components of colistin resistance mechanism

We were unable to detect the plasmid-borne mobile ColR genes *mcr*-1, -2, or -3 in any of our isolates. Of the 23 resistant isolates, 18 had mutations in their *mgrB* gene. Seventeen isolates carried IS elements while one had a nucleotide deletion in the open reading frame (ORF) of *mgrB* gene. Three types of elements belonging to three different families of IS were encountered in either ORF or the promoter of *mgrB* gene. Ten isolates were carrying ISKpn14, four isolates had ISKpn28, and three had IS903 in their *mgrB* gene. Out of 10 isolates with ISKpn14 insertion, four had it at position +52 of *mgrB* gene, two at +22, and two at -12 (in promoter region, upstream of start codon). All isolates carrying ISKpn28 belonged to ST-14. Three isolates with IS903 element, all belonging to ST-152, had it at +70 of the *mgrB* gene (Figure 1A). Insert sequences were clearly marked for their left and right inverted repeats (IRs). The IR sequences were as follows: ISKpn14 IRL: CCAACTTA, IRR: TAAGTTGG; for ISKpn 903 IRL: GCTTTGTTG, IRR: CAACAAAGC and for ISKpn28 IRL: ATATTGCAATT, IRR: AATTGCAATAT. The *mgrB* sequences with these insertions are deposited in the GenBank with accession numbers MG930931, MG930932, MG930933, MG930934, MG930935, MG930936, and MG930937. One isolate had a deletion of one nucleotide (“T”) at position +22 of the *mgrB* gene sequence, resulting in a premature stop codon at position 9 of the *mgrB* protein. Five isolates had L26Q mutation, whereas one isolate had an additional mutation S72L in the *PhoP* gene of TCS. No mutations were found in other compartments such as *PhoQ*, *PmrA/B*, and *CrrAB* of the signaling system.

Discussion

This is probably the first report from Saudi Arabia describing the molecular mechanisms for colistin resistance in *K. pneumoniae*. Although certain mutations appear to be clone-specific; a broad polyclonal involvement of colistin resistance was observed in our isolates. All our 23 ColR *K. pneumoniae* isolates are negative for plasmid-borne *mcr* gene underscoring the involvement of chromosomal-mediated resistance. This is in

Table 1 Showing the details of demography, MICs, virulence factors, and determinants of carbapenem resistance

Isolate #	ST	Index date	Sample source	IS element in mgrB	mgrB/PhoP point mutations	Virulence factors	Colist	Imipen	Merop	Carbapenemase
52	974	22-Oct-11	Wound	No insert	PhoP/L26Q	mrkD, entB	R	R	R	OXA-48
54	37	5-Sep-11	Abdominal drain	No insert	PhoP/L26Q	mrkD, entB	R	32R	32R	OXA-48
62	709	1-Sep-11	Blood	No insert	PhoP/L26Q	iut, kfu, entB	R	4R	4R	OXA-48
67	348	2-Jul-11	Rectal	No insert	PhoP/L26Q	mrkD, entB	R	R	8R	OXA-48
90	37	5-Jun-12	Blood	No insert	PhoP/L26Q	mrkD, entB	R	R	R	OXA-48
114	16	18-Nov-12	Trachea	ISKpn14	No mutation	ybtS, mrkD, entB	R	8R	16R	OXA-48
120	48	3-Nov-12	Rectal	ISKpn14	No mutation	ybtS, mrkD, entB	R	NA	16R	OXA-48
124	152	17-Sep-12	Urine	IS903	No mutation	ybtS, mrkD, entB	R	4R	4R	OXA-48, NDM-I
154	152	31-Jan-13	Trachea	IS903	No mutation	ybtS, mrkD, entB	R	4R	4R	OXA-48, NDM-I
166	15	13-Jul-13	Wound	ISKpn14	PhoP/L26Q	ybtS, mrkD, entB, kfu	R	16R	16R	OXA-48
178	15	22-Nov-13	Other	ISKpn14	No mutation	ybtS, mrkD, entB, kfu	R	8R	16R	OXA-48
197	14	15-May-14	Urine	ISKpn14	No mutation	ybtS, mrkD, entB	R	4R	4R	ND
218	101	20-Oct-14	Urine	ISKpn14	No mutation	ybtS, mrkD, entB, kfu	R	16R	16R	OXA-48, NDM-I
236	22	23-Jan-15	Wound	No insert	mgrB/▲t@22	mrkD, entB	R	16R	16R	OXA-48
261	14	15-May-15	Urine	ISKpn28	No mutation	ybtS, mrkD, entB, K2, kfu	R	16R	16R	OXA-48, NDM-I
278	307	3-Jul-15	Blood	ISKpn14	No mutation	mrkD, entB	R	2I	2I	OXA-48
287	101	21-Jul-15	Trachea	ISKpn14	No mutation	ybtS, mrkD, entB, kfu	R	16R	16R	OXA-48
300	152	25-Oct-15	Blood	IS903	No mutation	ybtS, mrkD, entB	R	1I	4R	ND
303	101	3-Nov-15	Urine	ISKpn14	No mutation	ybtS, mrkD, entB, kfu	R	16R	16R	OXA-48, NDM-I
305	14	27-Oct-15	Trachea	ISKpn28	No mutation	ybtS, mrkD, entB	R	16R	16R	NDM-I
309	14	8-Nov-15	Blood	ISKpn28	No mutation	ybtS, mrkD, entB, K2, kfu	R	16R	16R	NDM-I
313	14	30-Nov-15	Blood	ISKpn28	No mutation	ybtS, mrkD, entB	R	16R	16R	NDM-I
318	14	7-Dec-15	Urine	ISKpn14	No mutation	ybtS, mrkD, entB, K2, kfu	R	2I	16R	OXA-48

Abbreviations: IS, insertion element; MICs, minimum inhibitory concentrations; NA, not available; ND, not detected; ST, sequence type; R, resistant; I, intermediate.

line with the observations made recently by Sonnevend et al.¹³ It has been observed that greater colistin resistance resulted in *K. pneumoniae* by the mutation/inactivation of *mgrB* than the mutations in the genes of the TCSs (*pmrA/pmrB* or *PhoP/PhoQ*).^{3,14} We also encountered a similar situation, and 88% of our ColR isolates showed mutations in their *mgrB* gene alone.

Although IS5-like insertion element has been reported to be the main element for the *mgrB* disruption in *K. pneumoniae*, insertion of ISKpn14 (a member of IS1 family) is also sporadically reported.^{8,15} However, we found ISKpn14 to be the major IS and 59% of our isolates have ISKpn14-mediated *mgrB* gene disruption. The high incidence of ISKpn14 positivity in our isolates is not a mere expansion of a single clone because these isolates belonged to at least five different STs. The orientation and site of insertion are also varying (Figure 1A). Though ISKpn14 is inserted at as many

as six locations in the *mgrB* gene, site +52 appears to have a recombination event. Four isolates from two different STs had this IS element in both orientations. As our isolates carrying ISKpn14 ranged from year 2012 to 2015, there are variations in the IS sequences. A phylogenetic tree (Figure 1C) comparing the ISKpn14 sequences from these isolates segregated the older clones from the recent ones indicating their divergence through single-nucleotide polymorphisms.

The novel finding of this study is the incidence of 1100 bp ISKpn28 (a member of IS481 family) in four isolates. To the best of our knowledge, this is the first report on ISKpn28 involvement in *mgrB* disruption in *K. pneumoniae* from any part of the globe. Interestingly, all isolates shared 100% similarities in their ISKpn28 sequence and all had the same integration site (+91) in the *mgrB* gene and could be the result of clonal expansion of a single isolate with

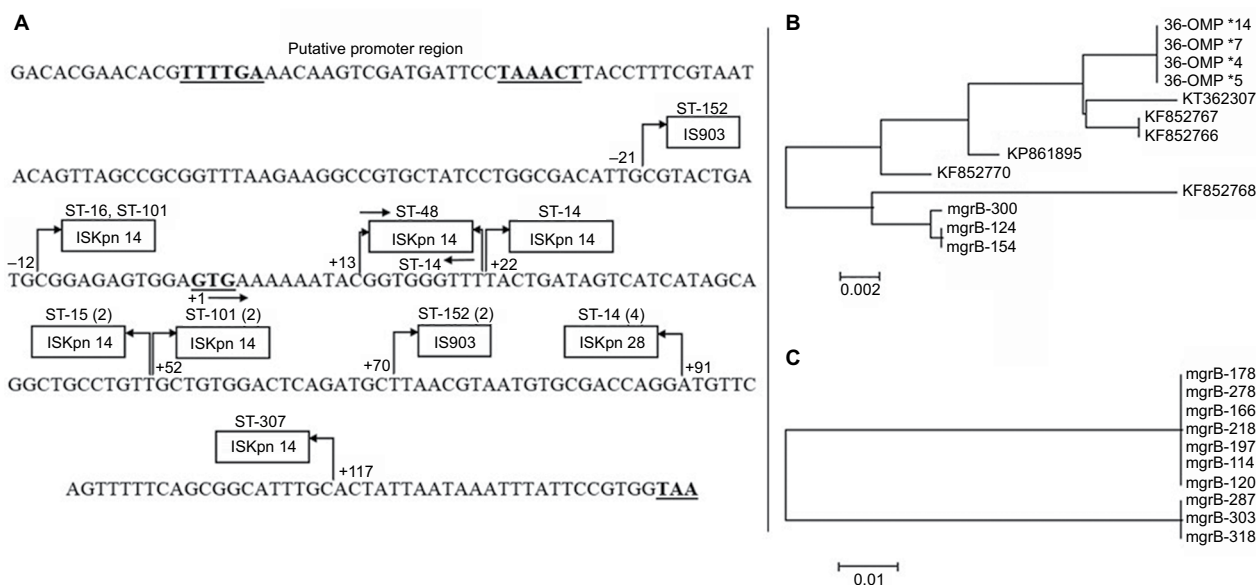


Figure 1 (A) *mgrB* gene sequence with the three ISs inserted at different positions in the open reading frame and the promoter region of the gene. The bold-face TTTTGA and TAAACT are -35 and -10 boxes of the promoter, whereas GTG and TAA are start and stop codons, respectively. The arrows and the numbers at their base indicate the orientation and the upstream (-) and downstream (+) positions of IS from the start codon. The numbers in parentheses indicate the multiples of isolates with the particular sequence type; (B) Phylogenetic relationship based on comparisons of IS903 insert sequence in *omp-36* gene of our 2010 outbreak isolates in and the *mgrB* gene of the present study population and six other GenBank depositions; (C) Phylogenetic relationship based on ISKpn14 sequence among isolates of this study carrying the insert. The bar in (B) and (C) is the divergence scale denoting number of substitutions per site.

Abbreviations: IS, insertion element; KP, *Klebsiella pneumoniae*; ST, sequence type.

this mutation. According to our records, ST-14 started circulating in our hospital only from mid-2014 and the isolates carrying ISKpn28 in this study are from year 2015, indicating the insertion to be a recent event. ST-14 with *mgrB* mutations has recently been reported from several parts of the world and appears to have become an endemic ColR clone.¹⁴

Another significant finding of this study is the presence of IS903 at +70 of *mgrB*, in three *K. pneumoniae* isolates belonging to ST-152, a clone found to be typically an NDM-1 gene carrier in our carbapenem-resistant (CR) *K. pneumoniae* isolates (data not shown). IS903, a member of IS5 family, is implicated in antibiotic resistance, either as a carrier of resistance gene or an element for modifying the targets of antibiotic action.¹⁶ Position +70 (from start codon) of the *mgrB* gene sequence appears to be a hotspot for insertion of IS903. GenBank DNA sequence depositions from different countries including China, Greece, and France reported the same +70 position, as the site of integration, for IS903 into the *mgrB* gene of their isolates.^{3,16} It suggests that there had been a recombination event at this location in the chromosome of the bacteria. We have previously reported a disruption of *omp-36* gene in four CR *K. pneumoniae* isolates from ST-29 because of IS903 insertion of similar size and the same IR sequences as found in our present ColR isolates, in carbapenem-resistant but colistin-susceptible

K. pneumoniae isolates from year 2010.⁹ However, one of the three isolates with IS903-mediated *mgrB* disruption also showed an insertion of IS903 in its *omp-36* gene indicating its potential role not only in colistin resistance but also for carbapenem. A similar mechanism for tigecycline resistance in *K. pneumoniae* has been reported, where IS5 is integrated into the promoter region of a putative efflux pump operon called *kpgABC*.¹⁷ More studies are needed to evaluate the spacers that possibly induce the mobilization of these elements from plasmids to the chromosome to transform the MDR pathogen into a pan-drug-resistant one.¹⁸ The phylogenetic analysis comparing the IS903 sequences from *omp-36* and *mgrB* in our isolates as well as the GenBank-deposited IS5 insert sequences for *mgrB* disruption from other countries showed a largely congruent distribution of the isolates (Figure 1B).

Deletions of single nucleotide causing a frame shift resulting in premature stop codon are widely reported in colistin resistance in *K. pneumoniae* and other bacteria.^{3,8,15} We found that one isolate belonging to ST-22 carried a deletion of a nucleotide ("T" at +22) in *mgrB* ORF causing a frame shift and premature stop codon and resulting in a shortened nonfunctional protein of only nine amino acids.

Several nonsynonymous mutations including one at L26Q in *PhoP* gene have been reported to cause colistin resistance;³ five of our isolates had L26Q mutation in *PhoP*

gene. Due to the abundance of studies reporting the involvement of IS elements in disruption of *mgrB* as well as PhoP mutations in colistin resistance, we assume that both the insertional inactivation of *mgrB* and the L26Q mutation in PhoP are the cause for colistin resistance in these isolates. It is interesting to note that in all our isolates from year 2011 till mid-2012, L26Q mutation was the only probable mechanism of colistin resistance and the isolates showed no insertional element in their *mgrB* gene. *MgrB* disruption through ISs is a later phenomenon than the *PhoP* mutations. These data, though on a small number of isolates, support the notion that under stressful situations such as antibiotic exposure, the bacteria require a more robust structural change in the genome to bring crucial beneficial variations, and this change is impossible through simple point mutations; ISs do this job more efficiently.^{19,20} In conclusion, our report depicts the increased role of IS elements in colistin resistance in *K. pneumoniae* through *mgrB* disruption. Also, the abundance of insertion sites (as many as nine places) in *mgrB* indicates the plasticity of this gene.

Disclosure

The authors report no conflicts of interest in this work.

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