Official journal of the Spanish Society of Chemotherapy

Original

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Colistin resistance due to insertional inactivation of the *mgrB* in *Klebsiella pneumoniae* of clinical origin: First report from India

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Article history
Received: 29 January 2018; Revision Requested: 5 February 2018; Revision Received: 9 May 2018; Accepted: 9 July 2018

ABSTRACT

Objectives. Mutations in *mgrB, phoP/phoQ, pmrA, pmrB, pmrC,* and *crrABC* regulatory systems have been found responsible for colistin resistance. The aim of our study was to investigate the role of alteration in *mgrB* gene and plasmid mediate *mcr*-1 and *mcr*-2 genes as a source of colistin resistance in 17 non duplicate *Klebsiella pneumoniae* clinical isolates.

Methods. All isolates classified as resistant to colistin by VITEK 2 system (BioMerieux, Marcy l' Etoile, France) were included. Susceptibility to colistin was also determined by broth microdilution using breakpoints recommended by EUCAST (>2mg/L resistant; and ≤2mg/L susceptible). PCR amplification of *mgrB* gene was performed and sequenced using specific primers. Presence of *mcr-1* and *mcr-2* was also investigated using PCR.

Results. PCR amplification of the *mgrB* gene of the 17 *K.pneumoniae* isolates revealed a larger (~1000bp) amplicon in three isolates when compared with the wild type *mgrB* ampiclon (250 bp). Sequencing of these amplicons showed that *mgrB* was disrupted by the insertion of IS*Kpn14*, a IS element belonging to the IS*1* family. Sequencing, of the 250 bp *mgrB* gene in the remaining 14 isolates revealed frame shift mutation after the second codon leading to a premature stop codon in only one isolate.

Conclusions. The study showed that colistin resistance in 20% of the *K. pneumoniae* isolates was due to loss of function of *mgrB*. We describe for the first-time from India, insertional inactivation of *mgrB* by IS*Kpn14* inserted at different sites, responsible for colistin resistance.

Key words: *mgrB*; colistin; insertion sequence; polymyxins; *Klebsiella pneumoniae*.

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Resistencia a colistina debido a inactivación insercional del gen *mgrB* en aislados clínicos de *Klebsiella pneumoniae*: Primera notificación en India

RESUMEN

Objetivos. Las mutaciones en los sistemas de regulación *mgrB, phoP/phoQ, pmrA, pmrB, pmrC* y *crrABC* se han asociado a la resistencia a colistina. El objetivo del estudio fue investigar el papel de la alteración en el gen *mgrB* y los genes mediados por plásmidos *mcr*-1 y *mcr*-2 como fuente de la resistencia a colistina en 17 aislados clínicos de *Klebsiella pneumoniae*.

Material y métodos. Todos los aislados que fueron clasificados como resistentes a colistina por el sistema VITEK 2 system (BioMerieux, Marcy l' Etoile, France) fueron incluidos. La sensibilidad a colistina fue también determinada por microdilución en caldo empleando los puntos de corte recomendados por el EUCAST (> 2mg/L resistente y ≤ 2mg/L sensible). Se realizó la amplificación del gen *mgrB* por PCR empleando cebadores específicos. La presencia de los genes *mcr-1* y m*cr-2* fue también realizada empleando la PCR.

Resultados. La amplificación por PCR del gen *mgrB* de 17 aislados clínicos de *K. pneumoniae* mostró un amplicón más grande (~1000pb) en 3 cepas cuando se comparó con el amplicón salvaje (250 pb). La secuenciación de estos 3 amplicones mostró que el gen *mgrB* estaba alterado por la inserción de IS*Kpn14*, un elemento IS que pertenece a la familia IS*1*. La secuenciación de los 250 pb del gen *mgrB* en el resto de los 14 aislados reveló mutaciones con desplazamiento del marco de lectura después del segundo codón que conducía a una interrupción de la lectura en sólo un aislado.

Conclusiones. Este estudio mostró que la resistencia a colistina en el 20% de los aislados de *K. pneumoniae* fue debida a la pérdida de la función del gen *mgrB*. Describimos

por primera vez en India que la inactivación insercional en el gen *mgrB* por IS*Kpn14* es responsable de la resistencia a colistina.

Palabras clave: *mgrB*; colistina; secuencia inserción; polimixinas; *Klebsiella pneumoniae*.

INTRODUCTION

Due to high rates of infections due to ESBL producing *Enterobacteriaceae* in India, carbapenems are extensively used for their treatment [1]. This lead to the emergence of carbapenem-resistant Enterobacteriaceae (CRE) due to plasmid mediated NDM-1 metallo-betalactamase in 2010 [2]. Soon after, NDM-1 producing CRE were reported from all parts of India, including the remote islands of Andaman & Nicobar. Subsequently polymyxins (colistin & polymyxin B) were launched as an effective option to treat CRE. Polymyxins are cationic compounds that bind to the negatively charged phosphate group of the lipopolysaccharide (LPS) causing cell death by disruption and loss of integrity of cell membrane [3]. They are active against a wide variety of Gram-negative pathogens but has no activity against Gram-positive and anaerobic pathogens. For the past few years, colistin and polymyxin B have been used mostly in combination with a broad spectrum betalactam to treat infections due to CRE [4]. Though colistin was used extensively, the dosing, most often would have been sub-therapeutic due to reasons like lack of clarity on the optimum dose, high cost, absence of loading dose and poor renal function leading to emergence of colistin resistance. Colistin resistance is primarily due decrease in the negative charge of the outer membrane due to addition of positively charged L-Ara-N and PEtN molecules thereby decreasing the affinity between colistin and its target [5]. This modification is mediated by the *pmrHFIJKLM* operon which in turn is regulated by the,*phoP/phoQ* two component system [5]. A small transmembrane protein *mgrB* negatively regulates the *phoP/phoQ*system preventing activation of *pmrHFIJKLM* operon in *K. pneumoniae*. Previous studies have reported that insertional inactivation of *mgrB* gene in *K.pneumoniae* lead to upregulation of the *phoP/phoQ* system, causing overexpression of the *pmrHFIJKLM* operon, resulting in colistin resistance [6]. It was also found that insertion of different types of insertion sequence (IS) at different locations into the *mgrB* gene lead to its inactivation [5,6]. Mutations in *mgrB, phoP/phoQ, pmrA, pmrB, pmrC,* and *crrABC* regulatory systems have also been found responsible for colistin resistance [7]. Plasmid mediated colistin resistance due to *mcr-1* to *mcr-5* encoding for phosphoethanolamine transferase are also being increasingly reported from all over the world [8]. Though colistin resistant *K. pneumoniae* have often been reported in India there are only two recent articles characterizing the underlying mechanism [9,10].

MATERIALS AND METHODS

The study was conducted from January to June 2017 in a 1,200 bedded tertiary care teaching hospital in South India. Colistin-resistant non-duplicate (one isolate per patient) *K. pneumoniae* isolates encountered during routine susceptibility testing using VITEK 2 (bioMerieux) automated system were included in the study. Out of 932 isolates, 17 were found to be resistant to colistin using breakpoints recommended by EUCAST (> 2mg/L resistant; and ≤ 2mg/L susceptible) [11]. Resistance to colistin in these 17 isolates was confirmed by CLSI recommended broth microdilution method [12]. Colistin-susceptible *K. pneumoniae* ATCC 70063 was used as control. They were also tested for $bla_{\text{CTX-M}}$ (detects CTX-M-1-, CTX-M-2-, and CTX-M-9-like-encoding genes) and $bla_{\text{NDM-1}}$ genes by PCR [2,13]. To determine the mechanism for colistin resistance PCR amplification and sequencing of the specific for *mgrB* gene was performed using specific primers (*mgr*B-extF:5′-TTAAGAAGGCCGTGCTATCC-3′ and *mgr*B-extR:5′-AAGGCGTTCATTCTACCACC-3′) [7]. Plasmid mediated colistin resistance due *mcr-1* and *mcr-2* to was investigated by PCR [8]. The study was approved by the Institutional Ethics Committee of our institute. (IEC-AIMS 2017-MICROB-117)

RESULTS

PCR amplification of the *mgrB* gene of the 17 *K. pneumoniae* isolates revealed a larger~1000bp) amplicon in three (CR8, 12, & 28) when compared with the wild type *mgrB* ampiclon (250 bp). Sequencing of these amplicons showed that *mgrB* was disrupted by the insertion of IS*Kpn14*, a IS element belonging to the IS*1* family (figure 1). In CR8 the insertion occurred between the nucleotides 123 and 124 and was bracketed by 7 bp target site duplication (CACTATT). In the CR28 the insertion occurred between the nucleotides 51 and 92 and was

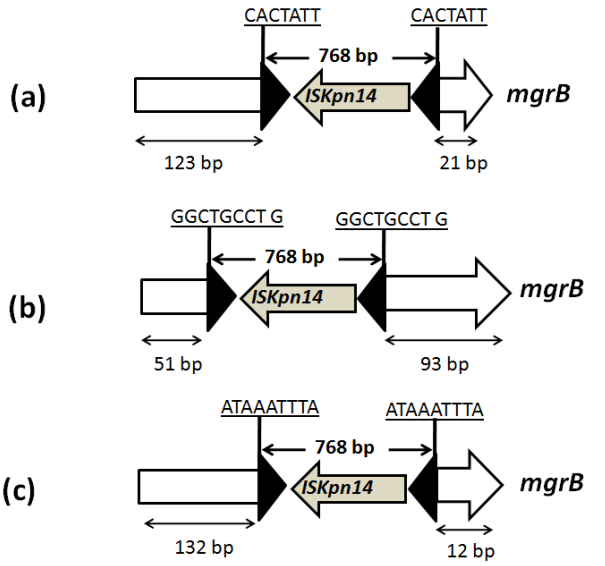

Figure 1 Schematic representation of different insertion events identified in the mgrB gene. (a) isolate CR8, (b) isolates CR 28,(c) isolate CR 12

Table 1 **Demographics, susceptibility, and molecular characteristics of colistin-resistant *K. pneumoniae* isolates.**

Sample ID	CR28	CR12	CR8	CR6
Age/Sex	58/M	44/M	68/M	49/M
Underlying condition	DM,CLD, Child B, Left above knee amputation	Asymptomatic bacterinuria	CLD, Child B, subdural haematoma, 2 months hospitalization	Motor neuron disease, bronchopneumonia, sepsis.
Outcome	Survived	Asymptomatic	Expired	Survived
Sample	Tissue	Urine	Urine	Sputum
Ticarcillin/clavulanic acid	R	S	R	R
Piperacillin/tazobactam	R	S	R	R
Ceftazidime	R	S	R	R
Cefoperazone/sulbactam	R	S	R	R
Cefepime	R	S	R	R
Aztreonam	R	S	R	R
Doripenem	S	S	S	R
Imipenem	S	S	S	R
Meropenem	S	S	S	R
Amikacin	R	S	R	R
Gentamicin	R	S	R	R
Ciprofloxacin	R	S	R	R
Minocycline	R	S	R	R
Tigecycline	I	S	R	I
Colistin MIC broth microdilution (VITEK)	32 mg/L (16 mg/L)	16 mg/L (8 mg/L)	1,024 mg/L (16 mg/L)	4 mg/L (8 mg/L)
Cotrimoxazole	S	S	S	R
PCR				
NDM-1	Neg	Neg	Neg	Neg
CTX-M	Pos	Neg	Pos	Neg
mcr-1	Neg	Neg	Neg	Neg
mcr-2	Neg	Neg	Neg	Neg
mgrB truncated by IS	Kpn14	Kpn14	Kpn14	Premature stop codon
Insertion site between	+51 and +92	+132 and +133	+123 and +124	NA

S = susceptible; I = intermediate; R= resistant; Neg = negative; Pos = positive

bracketed by 9 bp target site duplication (GGCTGCCTG) while in CR12 insertion was observed between the nucleotides 132 and 133 and was bracketed by 9 bp target site duplication (ATAAATTTA). In all the three cases transposon was inserted in the reverse orientation. Sequencing of the remaining 14 isolates with wild type *mgrB* ampiclon (250 bp) showed mutation only in one isolate (CR6). The CR6 isolate had a frame-shift mutation after the second codon leading to six alternative amino acids followed by a premature stop codon.

The clinical and demographic details and the susceptibility patterns are given in table 1. All isolates were resistant to colistin (> 16 mg/L) by Vitek 2 system. MICs for colistin by broth-microdilution ranged from 4-1,024 mg/L. None of the isolates were positive for NDM-1 by PCR (table 1). Isolate CR12 was a non-ESBL susceptible to all antibiotics except for colistin. Isolate CR28 and CR8 were ESBL producer. None of the isolates were positive for plasmid mediated colistin resistance genes *mcr-1* and *mcr-2* by PCR .

DISCUSSION

Reports on molecular characterization of colistin resistant isolates from India are scarce. Only two publications from India reported ten *K. pneumoniae* isolates with mutations in the *mgrB*, *phoP/phoQ*, *pmrA*, *pmrB*, *pmrC*, and *crrABC* regulatory systems [10]. To our knowledge there have been no previous reports of colistin resistance in *K. pneumoniae* due to insertional inactivation of the *mgr*B from India. Here we report

three cases of infection with *K. pneumoniae* resistant to colistin due to insertional inactivation of *mgrB* gene.

Isolate CR8 was surprisingly susceptible to most of the antibiotics and was resistant only to colistin. Further it was isolated from a patient with asymptomatic bacterinuria. Similarly, Kieffer *et al.* had also reported a susceptible *K. pneumoniae* isolate recovered from a case of bovine mastitis with resistance to colistin due to insertional inactivation by IS *903B* element belonging to IS*5* family [14]. The most common IS causing truncation of the *mgrB* gene belong to the IS5 family [15]. IS*Kpn14* is a 768 bp IS belonging to the IS*1* family. Insertional inactivation by IS*Kpn14* has been previously reported from a solitary isolate from Colombia and France, three isolates from Turkey and two from Italy [7,14-16]. In all our three isolates the IS*Kpn14* was inserted at different sites.

The study showed that colistin resistance in 23.5% (4/17) *K. pneumoniae* isolates was due to loss of function of *mgrB* which is not in agreement with a recent study from India which reported a rate of 50% [9]. We describe for the first-time from India, insertional inactivation of *mgrB* by IS*Kpn14* inserted at different sites, responsible for colistin resistance. Frame shift mutation of *mgrB* resulting in colistin resistance has been described earlier in a solitary study from India [10]. The resistance in the remaining 13 isolates may be due to mutations in *phoP/phoQ, pmrA, pmrB, pmrC,* and *crrABC* regulatory systems as reported in a previous study from India. As opposed to previous reports of pan resistant isolates from India, two of our isolates were not carbapenemase producer among them one was also susceptible to most of the antibiotics. Our study was limited by the fact that mutations in *phoP/phoQ, pmrA, pmrB, pmrC,* and *crrABC* regulatory systems which are also responsible for colistin resistance were not investigated and *K. pneumoniae* strain typing was also not done to determine clonality.

The study showed that colistin resistance in 20% of the *K. pneumoniae* isolates was due to loss of function of *mgrB*. We describe for the first-time from India, insertional inactivation of *mgrB* by IS*Kpn14* inserted at different sites, responsible for colistin resistance. Plasmid mediated colistin resistance due to *mcr-1* and *mcr-2* was not identified in *K. pneumoniae* from India.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest

FUNDING

None to declare

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