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

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 amplicon (250 bp). Sequencing of these amplicons showed that mgrB was disrupted by the insertion of ISKpn14, a IS element belonging to the ISF 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 ISKpn14 inserted at different sites, responsible for colistin resistance.

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

Correspondence: Dr. Anil Kumar MD, Clinical Professor, Department of Microbiology, Amrita Institute of Medical Sciences, Amrita Vishwa Vidyapeetham, Cochin, Kerala, India. Phone: +91484 2801234 (Extn: 8015). Fax: +91484 2802020. E-mail: anilkumar@aim.edu

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 susceptible). Se realizó la amplificación del gen mgrB por PCR empleando cebadores específicos. La presencia de los genes mcr-1 y mcr-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 salvage (250 pb). La secuenciación de estos 3 amplicones mostró que el gen mgrB estaba alterado por la inserción de ISKpn14, un elemento IS que pertenece a la familia ISF. 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
Colistin resistance due to insertional inactivation of the \textit{mgrB} in \textit{Klebsiella pneumoniae} of clinical origin: First report from India

A. Kumar, et al.

Rev Esp Quimioter 2018;31(5): 406-410

INTRODUCTION

Due to high rates of infections due to ESBL producing \textit{Enterobacteriaceae} in India, carbapenems are extensively used for their treatment [1]. This lead to the emergence of carbapenem-resistant \textit{Enterobacteriaceae} (CRE) due to plasmid mediated NDM-1 metallo-beta lactamase 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 beta lactam 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 \textit{pmrHFIJKLM} operon which in turn is regulated by the \textit{phoP/phoQ} two component system [5]. A small transmembrane protein \textit{mgrB} negatively regulates the \textit{phoP/phoQ} system preventing activation of \textit{pmrHFIJKLM} operon in \textit{K. pneumoniae}. Previous studies have reported that insertional inactivation of \textit{mgrB} gene in \textit{K. pneumoniae} lead to upregulation of the \textit{phoP/phoQ} system, causing overexpression of the \textit{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 \textit{mgrB} gene lead to its inactivation [5,6]. Mutations in \textit{mgrB}, \textit{phoP/phoQ}, \textit{pmrA}, \textit{pmrB}, \textit{pmrC}, and \textit{crrABC} regulatory systems have also been found responsible for colistin resistance [7]. Plasmid mediated colistin resistance due to \textit{mcr-1} to \textit{mcr-5} encoding for phosphoethanolamine transferase are also being increasingly reported from all over the world [8]. Though colistin resistant \textit{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) \textit{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 (\(>\ 2\ mg/L\) resistant; and \(\leq 2\ mg/L\) susceptible) [11]. Resistance to colistin in these 17 isolates was confirmed by CLSI recommended broth microdilution method [12]. Colistin-susceptible \textit{K. pneumoniae} ATCC 70063 was used as control. They were also tested for \textit{bla}\textsubscript{CTX-M-1+}, \textit{CTX-M-2+}, and \textit{CTX-M-9-like-encoding genes} and \textit{bla}\textsubscript{NDM-1} genes by PCR [2,13].To determine the mechanism for colistin resistance PCR amplification and sequencing of the specific for \textit{mgrB} gene was performed using specific primers (\textit{mgrB-extF}: 5'-TTAAGAA-GGCCGTGCTATCC-3’ and \textit{mgrB-extR}: 5'-'AGGCGGTCATTCTAC-CACC-3') [7]. Plasmid mediated colistin resistance due \textit{mcr-1} and \textit{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 \textit{mgrB} gene of the 17 \textit{K. pneumoniae} isolates revealed a larger~1000bp) ampiclon in three (CR8, 12, & 28) when compared with the wild type \textit{mgrB} amplicon (250 bp). Sequencing of these amplicons showed that \textit{mgrB} was disrupted by the insertion of \textit{ISKpn14}, a IS element belonging to the IS \textit{I} 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

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Schematic representation of different insertion events identified in the \textit{mgrB} gene. (a) isolate CR8, (b) isolates CR 28, (c) isolate CR 12}
\end{figure}
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 \textit{mcr-1} and \textit{mcr-2} by PCR.

**DISCUSSION**

Reports on molecular characterization of colistin resistant isolates from India are scarce. Only two publications from India reported ten \textit{K. pneumoniae} isolates with mutations in the \textit{mgrB}, \textit{phoP/phoQ}, \textit{pmrA}, \textit{pmrB}, \textit{pmrC}, and \textit{crrABC} regulatory systems [10]. To our knowledge there have been no previous reports of colistin resistance in \textit{K. pneumoniae} due to insertion inactivation of the \textit{mgrB} 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 IS5 family [14]. The most common IS causing truncation of the *mgrB* gene belong to the IS5 family [15]. ISKpn14 is a 768 bp IS belonging to the IS5 family. Insertional inactivation by ISKpn14 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 ISKpn14 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 ISKpn14 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 ISKpn14 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.

REFERENCES

1. Ah Y, Kum AJ, Lee JY. Colistin resistance in *Klebsiella pneumoniae*. Int J Antimicrob Agents. 2014; 44(8-15), doi: 10.1128/AAC.04763-14
2. Kumarasamy KK, Toleman MA, Walsh TR, Bagaría J, Butt F et al. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. Lancet Infect Dis. 2010; 10(9):597-602. doi: 10.1016/S1473-3099(10)70143-2.
3. Carmeli Y, Akova M, Cornaglia G, Daikos GL, Garau J, et al. Controlling the spread of carbapenemase-producing Gram-negatives: therapeutic approach and infection control. Clin. Microbiol Infect. 2010; 16(10):112–111. doi: 10.1111/j.1469-0691.2009.03115.x.
4. Zusman O, Avni T, Leibovici L, Adler A, Friberg L, Stergiopoulou T, Carmeli Y, Paul M. Systematic review and meta-analysis of in vitro synergy of polymyxins and carbapenems. Antimicrob Agents Chemother, 2013; 57(10):514–60. doi: 10.1128/AAC.01230-13.
5. Poirel L, Jayol A, Bontron S, Villegas MV, Ozdamar M et al. The *mgrB* gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*. J Antimicrob Chemother. 2015;70:75–80. doi: 10.1093/jac/dku323.
6. Cannatelli A, D’Andrea MM, Giani T, Di Pilato V, Arena F et al. In vivo emergence of colistin resistance in *Klebsiella pneumoniae* producing KPC-type carbapenemases mediated by insertional inactivation of the PhoQ/PhoP mgrB regulator. Antimicrob Agents Chemother. 2013; 57: 5521–6. doi: 10.1128/AAC.01480-13.
7. Olaitan AO, Diene SM, Kempf M, Berrazeg M, Bakour S et al. Worldwide emergence of colistin resistance in *Klebsiella pneumoniae* from healthy humans and patients in Lao PDR, Thailand, Israel, Nigeria and France owing to inactivation of the PhoP/PhoQ regulator *mgrB*: an epidemiological and molecular study. Int J Antimicrob Agents. 2014; 44(6):500–7. doi: 10.1016/j.ijantimicag.2014.07.020.
8. Xavier BB, Lammers C, Ruhal R, Kumar-Singh S, Butaye P et al. Identification of a novel plasmid-mediated colistin-resistance gene, *mcr-2*, in *Escherichia coli*, Belgium. Euro Surveill 2016; 7:21(27). doi: 10.2807/1560-7917.ES.2016.21.27.30280.
9. Pragasam AK, Shankar C, Veeraaghavan B, Biswas I, Nabarro LE et al. Molecular Mechanisms of Colistin Resistance in *Klebsiella pneumoniae* Causing Bacteremia from India—A First Report. Front. Microbiol. 2017;7:2135. doi: 10.3389/fmicb.2016.02135.
10. Veeraaghavan B, Perumalla SK, Devanga Ragupathi NK, Pragasam AK, Muthuriandri Sethuvel DP, Inian S, et al. Coexistence of Fosfomycin and Colistin Resistance in *Klebsiella pneumoniae*: Whole-Genome Shotgun Sequencing. Genome Announc. 2016;4:6(6). doi: 10.1128/genomeA.01303-16.
11. European Committee on Antimicrobial Susceptibility Testing. 2015. Breakpoint tables for interpretation of MICs and zone diameters. Version 5.0. http://www.eucast.org/.
12. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard–Tenth Edition. CLSI document M7-A10. Wayne, PA: Clinical and Laboratory Standards Institute; 2015.
13. Mathai D, Kumar VA, Paul B, Sugumar M, John KR, Manoharan A, et al. Fecal carriage rates of extended-spectrum β-lactamase-producing Escherichia coli among antibiotic naïve healthy human volunteers. Microb Drug Resist 2015;21:59–64. doi: 10.1089/ mdr.2014.0031.
14. Kieffer N, Poirel L, Nordmann P, Madec JY, Haenni M. Emergence of colistin resistance in Klebsiella pneumoniae from veterinary medicine. J Antimicrob Chemother. 2015; 70(4):1265-7. doi: 10.1093/ jac/dku485.

15. Cannatelli A, Giani T, D’Andrea MM, Di Pilato V, Arena F et al. MgrB inactivation is a common mechanism of colistin resistance in KPC-producing Klebsiella pneumoniae of clinical origin. Antimicrob Agents Chemother. 2014; 58(10):5696-703. doi: 10.1128/AAC.03110-14.

16. Nordmann P, Jayol A, Poirel L. Rapid detection of polymyxin resistance in Enterobacteriaceae. Emerg Infect Dis. 2016; 22:1038-1043. doi: 10.3201/eid2206.151840.