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Short Communication

Detection of chromosomal and plasmid-mediated mechanisms of colistin resistance in *Escherichia coli* and *Klebsiella pneumoniae* from Indian food samples

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ABSTRACT

Objectives: Numerous previous publications on the detection of bacterial isolates harbouring the *mcr-1* gene from animals and humans strongly suggest an underlying route of transmission of colistin resistance via the food chain. The aim of this study was to investigate the presence of colistin-resistant (Col-R) bacteria in Indian food samples and to identify the underlying mechanisms conferring colistin resistance.

Methods: Raw food material, including poultry meat, mutton meat, fish, fruit and vegetables, collected from food outlets in Chennai, India, were processed to identify Col-R bacteria using eosin methylene blue agar supplemented with colistin. Colistin minimum inhibitory concentrations (MICs) were determined by the broth microdilution method. PCR for the *mcr-1* and *mcr-3* genes was performed on Col-R *Escherichia coli* and *Klebsiella pneumoniae* isolates. Mutations in the *mgrB* gene were analysed in *K. pneumoniae* isolates. One representative *mcr-1*-positive *E. coli* was subjected to whole-genome sequencing.

Results: Of 110 food samples tested, 51 (46.4%) were positive for non-intrinsic Col-R Gram-negative bacteria. Three *E. coli* isolates were found to harbour *mcr-1*, whereas none were positive for *mcr-3*. Ten *K. pneumoniae* isolates had alterations in *mgrB*, with mutations in four and insertional inactivation in six.

Conclusion: The presence of Col-R bacteria and the *mcr-1* gene in raw food samples further complicates the antimicrobial resistance scenario in India. To the best of our knowledge, this is the first report in the global literature on *mgrB* mutation and its insertional inactivation conferring Col-R in *K. pneumoniae* from food samples.

1. Introduction

Colistin is the last-resort antibiotic for the treatment of patients with infections by extensively drug-resistant Gram-negative bacteria. The increasing rate of colistin resistance is a serious challenge in clinical practice with major public-health implications [1,2].

Traditionally, colistin resistance in *Klebsiella pneumoniae* was known to be chromosomally mediated involving mutations in the *mgrB*, *phoP*, *phoQ*, *pmrA*, *pmrB*, *pmrC and crrABC* genes [1,3]. A large number of reports on the plasmid-mediated colistin resistance gene *mcr-1* and its variants are now available from many countries [3,4]. Use of colistin as a growth promoter on livestock farms is a well-known contributing factor to the spread of colistin resistance between animals and the environment and its subsequent transmission to humans [4,5].

There are published reports of colistin resistance in Indian hospitals, with *K. pneumoniae* dominating the picture and mutations in the *mgrB* gene being the most common mechanism conferring colistin resistance [2,6]. Isolated reports of *mcr-1* positivity in Indian clinical isolates are also available [7]. A recent environmental study from an Indian city detected the presence of the *mcr-1* gene in a single river water sample [8]. Although colistin is used as a growth promoter in poultry farming in India, there are no published data on colistin-resistant (Col-R) bacteria in Indian raw food samples [9]. The aim of this study was to investigate the

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presence of Col-R bacteria in poultry and other raw food materials in India and to determine the possible molecular mechanisms of resistance.

2. Materials and methods

2.1. Sample collection

Raw food samples, including poultry meat, fish, mutton meat, fruit and vegetables, were collected from 22 sources (14 shops and 8 households) in Chennai, a major metropolitan city in India, in the period October–November 2017. Although the samples were collected in a non-systematic manner, outlets and homes were selected from geographically different parts of the city. Household samples were collected before washing and storing in the refrigerator to avoid the possibility of cross-contamination in the kitchen. Of the 14 shops, 10 were supermarkets from which vegetable, fruit, poultry meat and fish samples were collected. Four shops were stand-alone meat shops selling poultry meat, mutton meat and fish. Of the eight households, three provided only vegetable samples and the rest provided vegetable and poultry meat samples. All of the samples were collected in sterile containers. Clinical samples or samples from live animals were not included in the study.

2.2. Screening for colistin resistance with eosin methylene blue (EMB) agar

Minced samples (ca. 5 g) were suspended in 10 mL of brain–heart infusion broth. Following overnight incubation, a loopful of suspension was inoculated on a modified SuperPolymyxin plate without adding amphotericin B or daptomycin [10]. Plates were prepared using EMB agar (code M317; HiMedia, Mumbai, India) supplemented with 2, 3 and 4 μg/mL of colistin, respectively (colistin sulphate, code 51681; Sisco Research Laboratories Pvt. Ltd., Mumbai, India). Grown bacterial colonies were subcultured on MacConkey agar without antibiotics to isolate and identify the micro-organism. Isolates were identified using standard biochemical methods. All Gram-negative bacterial isolates obtained were suspended in 10 mL of 0.9% NaCl to obtain a 0.5 McFarland standard. Then, 10 μL of the suspension was streaked on EMB agar containing 3.5 μg/mL of colistin using a three-phase streaking pattern and was incubated at 37 °C for 24–48 h. An isolate was considered Col-R if there was growth on the second and third frame of the streak line, whereas it was considered to be susceptible to colistin if there was no growth or if the growth was limited to the first frame of the streak line (due to inoculum effect). *Escherichia coli* ATCC 25922 was used as a negative control, and *Proteus mirabilis* ATCC 25933 was used as a positive control. Organisms with intrinsic Col-R such as *Proteus* spp., *Morganella* spp., *Providencia* spp. and *Serratia* spp. were excluded.

2.3. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) for colistin were determined by the broth microdilution method in cation-adjusted Muller–Hinton broth and the results were interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints for *Enterobacteriaceae* and *Pseudomonas* (susceptible, ≤2 μg/mL and resistant, >2 μg/mL) [11]. Known *mcr-1*-positive *E. coli* (courtesy of Dr Olga Perovic, National Institute for Communicable Diseases, Johannesburg, South Africa) was used as a positive control. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality controls. Antimicrobial susceptibility testing of the *Enterobacteriaceae* isolates for other routinely used antibiotics was performed by the Kirby–Bauer disk diffusion method using disks manufactured by HiMedia, India, including imipenem (10 μg), meropenem (10 μg), cefotaxime (30 μg), ceftazidime (30 μg), cefpodoxime (10 μg), amoxicillin/clavulanic acid (AMC) (20/10 μg), aztreonam (30 μg), amikacin (30 μg), gentamicin (10 μg), ciprofloxacin (5 μg), trimethoprim/sulfamethoxazole (SXT) (1.25/23.75 μg), chloramphenicol (30 μg) and tigecycline (15 μg). The results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines [12], except tigecycline which was interpreted using the US Food and Drug Administration (FDA) breakpoint. Col-R *Pseudomonas* spp. isolates were not analysed further.

2.4. Molecular characterisation

Molecular analysis was performed on all Col-R *E. coli* and *Klebsiella* spp. isolates. DNA was extracted by boiling lysis of a turbid bacterial suspension in distilled water for 20 min. The lysate was centrifuged at 15 000 rpm for 10 min and the supernatant was stored at −20 °C and was used for PCR. For sequencing, DNA was extracted using a QIAsymphony DSP Virus/Pathogen Mini Kit (QIAGEN, Hilden, Germany) as per the manufacturer's instructions.

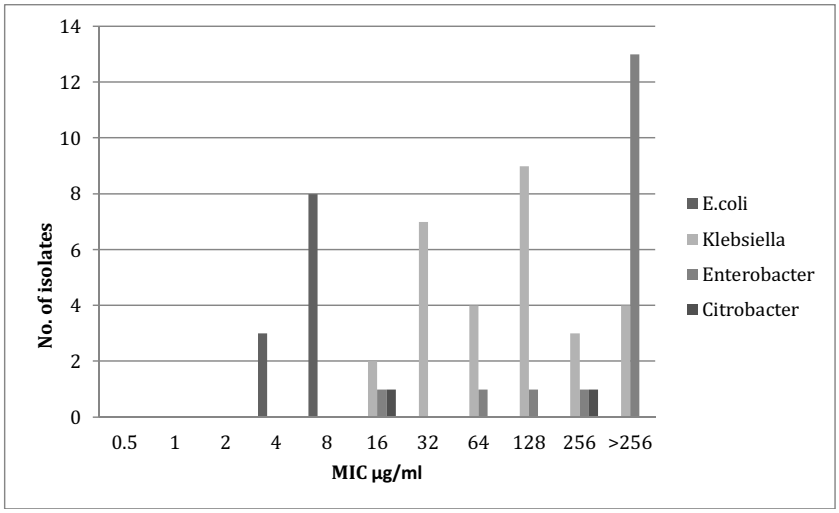

Fig. 1. Colistin minimum inhibitory concentration (MIC) distribution of bacterial isolates from food samples in Chennai, India.

2.4.1. PCR for the mcr-1 *and* mcr-3 *genes*

Multiplex PCR for the *mcr-1* and *mcr-3* genes was performed with 2 μM each of the *mcr-1* and *mcr-3* primers according to the method described by Liu et al. and Yin et al. [4,13]. The *mcr-1*-positive control strain was provided by Dr Olga Perovic, and an in-house *mcr-3*-positive control strain was used. The *mcr-3* control strain was identified as *mcr-3.11* (unpublished data).

2.4.2. PCR and sequencing of the mgrB *gene*

The *mgrB* gene was amplified with the primers *mgrB* ext F and *mgrB* ext R, which amplify the flanking region along with *mgrB* as described by Cannatelli et al. [14]. Sequencing of the *mgrB* gene was performed using an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The position of the insertion in *mgrB* was determined by aligning with the reference *mgrB* gene of *K. pneumoniae* ATCC 35657.

2.4.3. Whole-genome sequencing (WGS)

One representative *mcr-1*-harbouring *E. coli* isolated from a poultry sample was subjected to WGS using an Ion Torrent PGM platform (Life Technologies, Carlsbad, CA) with 400-bp read chemistry as described by the manufacturer, with 12.6× coverage. Annotation of the genomes was performed with Rapid Annotation using Subsystem Technology (RAST) (http://rast.nmpdr.org/) and PATRIC (https://www.patricbrc.org/). ResFinder, PlasmidFinder, VirulenceFinder and Multi-Locus Sequence Typing (MLST) tools (https://cge.cbs.dtu.dk/) were used for further analysis.

3. Results

A total of 110 food samples (vegetables, $n = 63$; fish, $n = 21$; poultry, $n = 19$; mutton, $n = 4$; and fruits, $n = 3$) were screened, of which 51 (46.4%) were found to contain Col-R organisms, including 23 vegetable samples, 11 fish samples, 12 poultry samples, 3 mutton samples and 2 fruit samples. At least one sample was positive from all 22 sources, including households that provided only vegetable samples. From the 51 positive samples, 71 bacterial isolates were identified, including 11 *E. coli*, 29 *Klebsiella* spp., 17 *Enterobacter* spp., 2 *Citrobacter* spp. and 12 *Pseudomonas* spp. Sixteen samples had more than one Col-R isolate. Colistin MICs of the 11 *E. coli* isolates were 4–8 μg/mL and all of these isolates were susceptible to third-generation cephalosporins. Most of the *E. coli* isolates were resistant to SXT (10/11) and chloramphenicol (9/11) and a few were resistant to ciprofloxacin (5/11) and tigecycline (1/11). The 29 *Klebsiella* spp. had high colistin MICs ranging from 16 μg/mL to >256 μg/mL (Fig. 1), and only a few isolates were resistant to cefotaxime (5/29), ceftazidime (4/29), cefpodoxime (4/

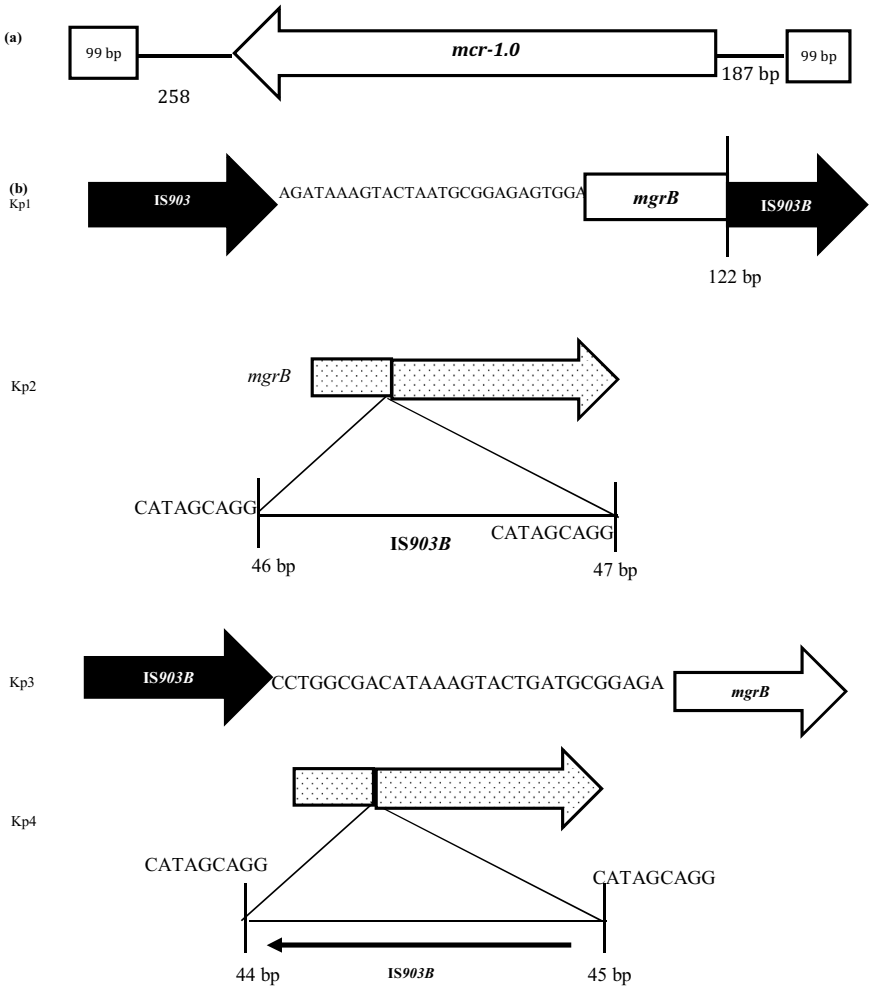

Fig. 2. Schematic representation of *mcr-1* and *mgrB* gene alterations. (a) Contig with the *mcr-1* gene. The flanking region of *mcr-1* contains inverted repeats similar to insertion sequence IS*Apl1*. (b) Insertion sequences associated with the *mgrB* gene. Strain Kp1: IS*903* (605 nt) is separated from *mgrB* by 27 nucleotides (nt) on one end. *mgrB* is intact up to 41 amino acids and is then disrupted by IS*903B* (377 nt) on the other end. Strain Kp2: IS*903B* is inserted between the 46th and 47th nt of the *mgrB* gene. There is also a repeat region of 9 bases. Strain Kp3: IS*903B* is separated from *mgrB* by 29 nt, and the *mgrB* gene is intact without mutation. Strain Kp4: IS*903B* is in the opposite orientation to the *mgrB* between the 44th and 45th nt.

29), tigecycline (6/29), SXT (2/29), AMC (4/29), chloramphenicol (1/29) and ciprofloxacin (1/29). The *Enterobacter* spp. were non-susceptible to AMC, cephalosporins and tigecycline and had colistin MICs ranging from 16 μg/mL to >256 μg/mL. Of the two *Citrobacter* spp. isolates, one isolate was resistant to tigecycline and both were susceptible to the other tested antibiotics. All Col-R isolates were susceptible to imipenem, meropenem, amikacin, gentamicin and aztreonam. The colistin MICs of *Pseudomonas* spp. ranged from 4–32 μg/mL.

3.1. Analysis of the mcr-1 gene

Of the 71 isolates screened, 3 *E. coli* (one mutton and two poultry meat samples) were found to harbour *mcr-1.0*. WGS of a representative *mcr-1*-positive *E. coli* (from poultry meat) showed that the isolate belonged to sequence type 50 (ST50) and harboured IncX1 and IncHI2/HI2A plasmids. The flanking region of *mcr-1* contained inverted repeats similar to insertion sequence IS*Apl1* (Fig. 2a). The virulence genes *iss* (increased serum survival) and *gad* (glutamate decarboxylase) were present. The isolate harboured antimicrobial resistance genes such as *aadA1*, *aadA2*, *aph(6)-ld* and *aph(3″)-lb* (aminoglycoside resistance), $bla_{\text{TEM-1B}}$ (β-lactam resistance), *qnrS1* (fluoroquinolone resistance), *tetA* (tetracycline resistance), *dfrA14* and *dfrA15* (trimethoprim resistance), *mcr-1* (colistin resistance), *floR* (phenicol resistance) and *sul3* (sulphonamide resistance). WGS data of the *mcr-1*-positive *E. coli* isolate were deposited in GenBank with accession nos. **PPXA00000000** and **PPXA01000133.**

3.2. Analysis of the mgrB gene

All 29 Col-R *K. pneumoniae* isolates were sequenced to determine *mgrB* mutations (Table 1). Thirteen isolates lacked a mutation in *mgrB*. Three isolates had premature termination in *mgrB*, and one isolate was found to contain phenylalanine in place of isoleucine at the 45th amino acid. Six isolates were negative for *mgrB* PCR, suggesting partial or complete deletion of the gene. Six isolates were associated with IS*903* belonging to the IS*5* family. Fig. 2b shows details of the association of IS*903* and IS*903B* with *mgrB* in four *K. pneumoniae* isolates. IS*903* was inserted at different locations and in different orientations in the *mgrB* gene. In isolate Kp1, IS*903* [605 nucleotides (nt)] was separated from *mgrB* by 27 nt and the isolate also had IS*903B* (377 nt) disrupting the *mgrB* gene from the 123rd nucleotide. Hence, *mgrB* was intact until 41 amino acids and was then disrupted by IS*903B*. The IS*903* showed 99% identity with the reference, and IS*903B* showed 97% identity. In isolate Kp2, the *mgrB* gene was disrupted by IS*903B* insertion between the 46th and 47th nt. In this isolate, IS*903B* showed 97% identity with the reference. In isolate Kp3, IS*903B* was seen flanking *mgrB* without disrupting the *mgrB* gene. The IS*903B* was separated from *mgrB* by 29 nt. IS*903B* in the opposite orientation to *mgrB* was seen between the 44th and 45th nt in isolate Kp4. Sequences of the *mgrB* gene with insertion sequences of strains Kp1 to Kp4 were deposited in GenBank with accession nos. **MH337362**, **MH337363**, **MH337361** and **MH000621**, respectively.

4. Discussion

Consistent with the global trend, various Indian centres have reported an increasing prevalence of Col-R bacteria in hospitalised patients [2,15,16]. Although there are many publications on the prevalence of multidrug-resistant Gram-negative bacteria in raw food samples in India, we could not track any study reporting the presence of Col-R bacteria [17].

A very high proportion (46.4%) of food samples analysed in the current study had the presence of Col-R bacteria. Extensive use of colistin as a growth promoter in the poultry and aquaculture industries as well as use of poultry litter as manure in agriculture

Table 1
mgrB gene sequencing results of *Klebsiella* isolates.

Lab. no.	Source	Colistin MIC (μg/mL)	*mgrB* alteration
1	Fish	32	Inactivation by IS*903* and IS*903B*
2	Poultry	128	Inactivation by IS*903B* in coding region (between nt +46 and +47)
3	Vegetable	256	IS*903B* flanking *mgrB*
4	Vegetable	128	Inactivation by IS*903B* in coding region (between nt +44 and +45)
5	Vegetable	64	Inactivation by IS*903B* (between nt +69 and +70)
6	Poultry	256	IS*903B* flanking *mgrB*
7	Fish	16	Wild-type
8	Fruit	32	Premature termination (20th aa)
9	Vegetable	32	Wild-type
10	Vegetable	32	45th aa changed from isoleucine to phenylalanine
11	Vegetable	128	Wild-type
12	Poultry	16	Partial or complete deletion (no PCR amplification)
13	Vegetable	128	Wild-type
14	Vegetable	128	Wild-type
15	Vegetable	64	Wild-type
16	Vegetable	64	Wild-type
17	Vegetable	32	Wild-type
18	Poultry	>256	Premature termination (20th aa)
19	Fish	32	Wild-type
20	Vegetable	32	Wild-type
21	Vegetable	>256	Partial or complete deletion (no PCR amplification)
22	Vegetable	128	Wild-type
23	Poultry	256	Partial or complete deletion (no PCR amplification)
24	Fish	128	Partial or complete deletion (no PCR amplification)
25	Vegetable	>256	Partial or complete deletion (no PCR amplification)
26	Vegetable	128	Wild-type
27	Poultry	128	Premature termination (30th aa)
28	Fish	64	Wild-type
29	Fish	>256	Partial or complete deletion (no PCR amplification)

MIC, minimum inhibitory concentration; nt, nucleotide; aa, amino acid.

could be the reason behind this. Cross-contamination of raw food samples by outlet professionals could be another potential reason for such a high rate.

The *mcr-1.0* gene was identified in three *E. coli* isolates. To the best of our knowledge, this is the first Indian study reporting *mcr-1* in Indian food samples. Detection of *mgrB* alterations in *K. pneumoniae* of food origin is a very significant finding. The presence of insertional inactivation in the *mgrB* gene could offer an alternative explanation for the extensive dissemination of colistin resistance in *K. pneumoniae* in food samples. The finding that IS*903* inserted in multiple different locations in *mgrB* suggests that this is not a rare event that is clonally amplified, but rather multiple independent disruption events.

Samples were collected from one major metropolitan Indian city in a random fashion. Hence, the percentage positivity does not provide a true prevalence of colistin resistance in various food samples. The study also does not provide data on the prevalence at a national level. Colistin is used extensively as a growth promoter and India currently has no regulations to prevent this practice [9,17]. The Chennai Declaration of medical societies in India and National Action Plan on Antimicrobial Resistance plan have made clear-cut recommendations to ban the use of antibiotics as growth promoters [18].

5. Conclusions

The presence of Col-R bacteria and the *mcr-1* gene in raw food samples further complicates the antimicrobial resistance scenario in India. To the best of our knowledge, this is the first report in the global literature on mutations and insertional inactivation of the *mgrB* gene conferring acquired colistin resistance in *K. pneumoniae* of food origin.

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Competing interests

AG has received advisory fees, lecture fees or consultancy fees from Cipla, bioMérieux, Glenmark, Astellas, Sun Pharma, Pfizer, MSD and Abbott. All other authors declare no competing interests.

Ethical approval

Not required.

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