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Brief communication (Original)

Colistin susceptibility of gram-negative clinical isolates from Tamil Nadu, India

Nachimuthu Ramesh[a], Manohar Prasanth[a], Subramani Ramkumar[b], Maray Suresh[c], Ashok J. Tamhankar[e,f] Gothandam K. M[a], Sivashanmugam Karthikeyan[a], Bulent Bozdogan[a,d]

[a]*School of Bio Sciences and Technology, VIT University Vellore, Tamil Nadu 632014, India*
[b]*Department of Microbiology, Hitech Diagnostic Centre, Chennai, Tamil Nadu 600010, India*
[c]*Department of Medical Laboratories, College of Science, Majmaah University 15361, Saudi Arabia*
[d]*Medical Microbiology Department, Adnan Menderes University, Aydin 09100, Turkey*
[e]*Department of Public Health Sciences, Karolinska Institutet, Stockholm SE-171 77, Sweden*
[f]*Department of Environmental Medicine, R.D. Gardi Medical College, Ujjain 456006, India*

Background: Colistin is one of the oldest antibiotics in the polymyxin group, and is used mostly against gram-negative bacteria. Because of developing resistance among clinical isolates colistin has become an alternative drug for multidrug resistant bacteria.

Objectives: To determine colistin resistance among isolates from Tamil Nadu, India.

Methods: We included 94 gram-negative isolates from two centers in Tamil Nadu in the present study. Isolates were identified by 16S rRNA sequencing. Minimal inhibitory concentrations (MICs) were determined by agar dilution.

Results: The isolates identified at species level included 48 *Escherichia coli*, 9 *Klebsiella pneumoniae*, 10 *Pseudomonas aeruginosa*, 5 *Proteus mirabilis*, 4 *Salmonella enterica*, 3 *Enterobacter hormaechei*, 3 *Enterobacter cloacae*, 2 *Achromobacter xylosoxidans*, 2 *Acinetobacter baumannii*, 1 *Providencia vermicola*, 1 *Acinetobacter towneri*, 1 *Enterobacter gergoviae*, 2 *Providencia rettgeri*, 1 *Enterobacter asburiae*, 1 *Pseudomonas stutzeri*, and 1 *Salmonella typhi*. The MIC of colistin ranged from 0.12 μg/ml to 128 μg/ml. The MIC_{50} was 1 μg/mL and MIC_{90} was >128 μg/ml. The MIC ≥ 8 μg/mL was resistant breakpoint for all the species. A total of 27 isolates were resistant to colistin. Colistin resistant isolates included *E. coli* (9/48), *K. pneumoniae* (6/9), *P. aeruginosa* (3/10), *A. baumannii* (1/2), *P. mirabilis* (4/5), *E. cloacae* (1/3), *P. rettgeri* (2/2), and *S. enterica* (1/4). Carbapenem susceptibility of colistin resistant isolates was tested and 14 were found to be resistant to meropenem.

Conclusions: Our study indicates the emergence of colistin resistant isolates from clinical samples among different groups of gram-negative organisms. Resistance to both carbapenem and colistin occurs. Developing new antibiotics and programs to reduce nosocomial infections is necessary especially for multidrug resistant isolates.

Keywords: Antimicrobial agents, gram negative, multi-drug resistant, pathogenesis, polymyxin

Bacterial resistance towards antibiotics is a clinical threat because it increases the problem of infectious disease. Concern regarding multidrug resistant (MDR) bacteria, especially nosocomial pathogens is attracting more interest because new drugs to overcome resistant bacteria in the drug development pipeline are not readily available [1]. Morbidity and mortality because of gram-negative MDR nosocomial pathogens is high [2, 3]. Because of irrational use of antibiotics pathogens can develop and share resistance to common antimicrobials and the development of new drugs appears distant [4]. This growing resistance has rekindled interest in colistin, one of the oldest antibiotics [5]. Colistin is in the polymyxin group of antibiotics, and was available for clinical use from 1959, although it was not always the first preferred drug for many years [6, 7]. The use of colistin against panresistant nosocomial infections caused especially by *Pseudomonas* and *Acinetobacter* spp. has been reported recently [8-12]. The mechanism of action for this bactericidal drug involves the disruption of the outer cell membrane by completely displacing divalent

Correspondence to: Ramesh Nachimuthu, School of Bio Sciences and Technology, VIT University, Vellore, Tamil Nadu 632014, India. E-mail: ramesh.n@vit.ac.in, drpnramesh@gmail.com

cations from the phosphate group of membrane lipids through binding to the lipopolysaccharides and phospholipids of gram-negative organisms [13, 14]. Colistin can also prevent the pathophysiological effects of endotoxin in the circulation by neutralizing lipopolysaccharides [15, 16]. Currently, colistin is increasingly being used against multidrug resistant gram-negative bacteria which include *Pseudomonas aeruginosa* [11, 17], *Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae,* and *Salmonella enterica*. The dosage and frequency of colistin administration to effect its bactericidal activity against multiple drug resistant bacteria is the biggest concern, although there are differing breakpoint levels assigned against each species [3, 18].

Colistin resistant organisms are reported in various parts of the world, including resistance of *Pseudomonas aeruginosa* in cystic fibrosis from UK [17], carbapenemase-producing *Klebsiella pneumoniae* resistant to colistin [19, 20, 22], *Acinetobacter baumannii* [21,24], and polymyxin resistant *Escherichia coli* [22, 23]. Increasing numbers of reports regarding colistin-resistant bacteria indicates a developing threat to future treatment options for diseases caused by gram-negative bacteria. In this study, we report the prevalence of colistin resistant bacteria from clinical isolates in Tamil Nadu.

Methods

Collection of isolates

All 94 clinical isolates included in the present study were from two different regions in Tamil Nadu; Chennai and Trichy, and were collected between March and July 2014. All the isolates identified to be multidrug resistant using a disk diffusion method in clinical centers were received in vials from both Chennai (Hitech Diagnostic Centre) and Trichy (Doctor's Diagnostic Centre). Anonymized isolates (unlinked to patient data) were received at the Antimicrobial Resistant Laboratory, School of Bio Sciences and Technology in VIT University, Vellore, India. All isolates were subcultured onto brain-heart infusion agar (HiMedia Laboratories, Mumbai, India) and stored at –80°C for further analysis.

Minimal inhibitory concentration

Minimal inhibitory concentrations (MICs) for colistin and meropenem were determined using a microbroth dilution method. Briefly, Mueller Hinton (MH) broth No. 2 with controlled cations (HiMedia Laboratories) was prepared and 100 µl was aliquoted into each of the 96 wells in a microtiter plate. A total of 100 µl of broth with antibiotic with concentrations ranging from 0.25 µg/ml to 256 µg/ml were made and a single well in each row was allocated as a growth control without antibiotic. Then 100 µl of MH broth with bacteria (to have final count of 5×10^4 ml) prepared from an overnight culture of a single colony inoculated into MH broth No. 2 with controlled cations was added. Microtiter plates were incubated at 37°C for 18 h. An MIC value ≥ 8 µg/mL was considered to be the resistance breakpoint.

Molecular studies

DNA was extracted from all the isolates using a boiling method. Briefly, 500 µL bacteria grown overnight were centrifuged at 10,000 rpm for 5 min, and to the pellet 100 µL of sterile distilled water was added and heated for 10 min at 95°C. The supernatant was then used as a template. The 16S rRNA gene from each isolate was amplified and the amplicons were sent to Macrogen, South Korea for sequencing. Sequence homologies were determined using a nucleotide BLAST analysis at www.ncbi.nlm.nih.gov/BLAST and identified at a species level.

Results

Identification of isolates

Identification of all 94 isolates included in the present study was determined by 16S rRNA gene sequencing. The results showed that among 94 isolates, 48 were *E. coli*, 9 *K. pneumoniae*, 10 *P. aeruginosa*, 5 *Proteus mirabilis*, 4 *Salmonella enterica*, 3 *Enterobacter hormaechei*, 3 *Enterobacter cloacae*, 2 *Achromobacter xylosoxidans*, 2 *Acinetobacter baumannii*, 1 *Providencia vermicola*, 1 *Acinetobacter towneri*, 1 *Enterobacter gergoviae*, 2 *Providencia rettgeri*, 1 *Enterobacter asburiae*, 1 *Pseudomonas stutzeri*, and 1 *Salmonella typhi*.

Colistin susceptibility testing

All the isolates sent to our laboratory were multidrug resistant as determined by disk diffusion. All multidrug resistant isolates tested against colistin with concentrations ranging from 0.12 µg/mL to 128 µg/mL showed MIC_{50} 1 µg/mL and MIC_{90} >128 µg/mL, respectively. Colistin resistant isolates (**Figure 1**) included *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *A. baumannii*, *P. mirabilis*, *E. cloacae*, *P. rettgeri*, *S. enterica*.

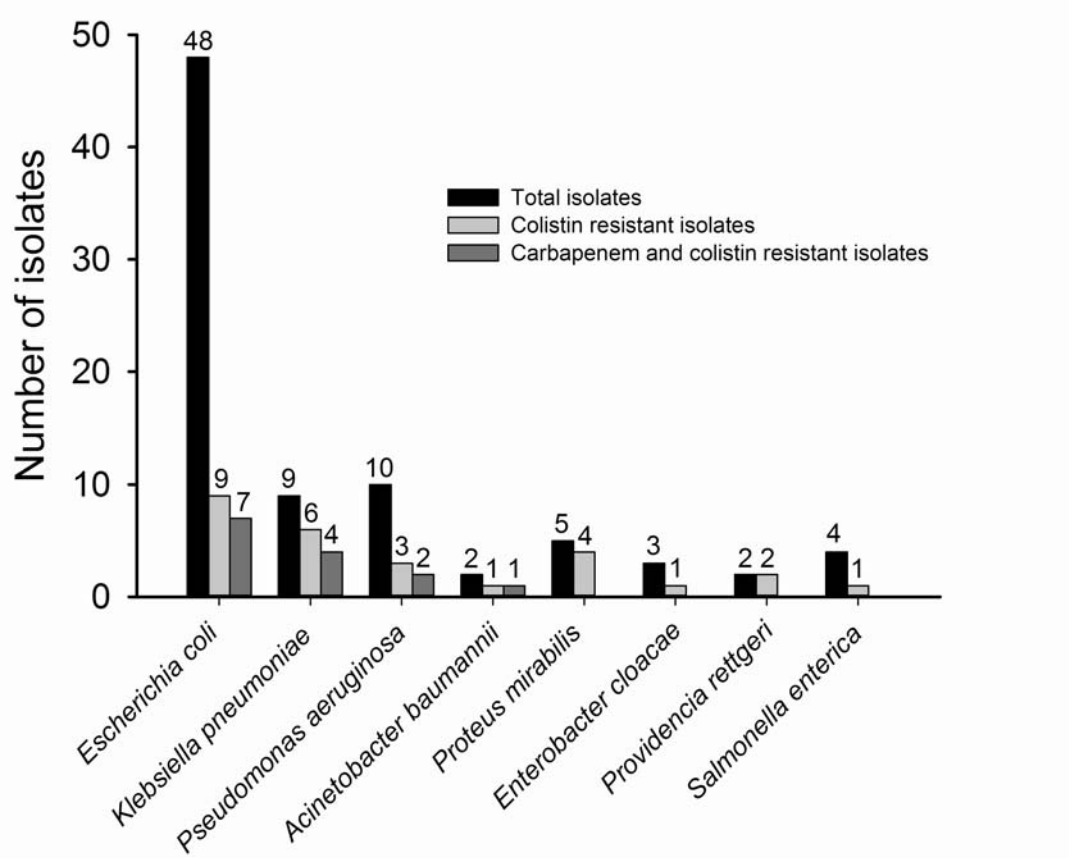

Figure 1. Comparison of the total number of isolates from each bacterial species with colistin resistance, and carbapenem and colistin resistant isolates. Numbers at the top the bars indicate the number of isolates.

Carbapenem resistance among colistin resistant isolates

Among 27 colistin resistant isolates 14 (52%) were resistant also to meropenem. A total of 15% isolates were found to be resistant to both meropenem and colistin. Isolates with dual resistance were *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii*. For colistin MIC_{50} was 1 μg/mL and MIC_{90} was >128 μg/mL, for meropenem MIC_{50} < 0.06 μg/mL and MIC_{90} > 128 μg/mL (**Table 1**).

Discussion

Knowledge of antibiotics that bacteria are susceptible to is necessary to overcome the problem of developing bacterial resistance towards common antibiotics. Interest in colistin has reemerged because of its antibacterial activity that finds use against many carbapenem resistant bacteria [8-10, 12]. However, the development of resistance is becoming a problem again. Colistin is used mainly against gram-negative bacteria including *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*. Susceptibility and resistant breakpoints and dosage are another problem because they differ geographically.

Reports on colistin resistant bacteria from various parts of the world suggest that there is a developing resistance towards colistin among gram-negative bacteria, although the mechanism of resistance is not clear. A study of *Acinetobacter baumannii* from Spain suggests that among 115 isolates, 19% were resistant [25], in Korea 27.9% of 214 isolates were resistant [26], and in Australia 93.8% of 16 isolates were heteroresistant to colistin [24]. *Klebsiella pneumoniae* studies indicate that 18 isolates obtained from patients in Greece [27], 55 (6.8%) of 221 from South Korea [28], and 6 (27%) of 22 from Australia [29] were resistant to colistin. *Pseudomonas aeruginosa* from patients with cystic fibrosis may have resistance to colistin [14]. Although the number of isolates examined in the present study was relatively small, especially for *K. pneumoniae*, we detected a high level of resistance to colistin. The present study

Table 1. MICs of 27 colistin resistant isolates including 14 isolates resistant to both colistin and meropenem

Strain No.	Organism	MIC (μg/ml)		Strain No.	Organism	MIC (μg/ml)	
		Colistin	Meropenem			Colistin	Meropenem
1.	*E. coli*	>128	32	19.	*P. mirabilis*	>128	0.12
2.	*E. coli*	>128	0.12	20.	*P. aeruginosa*	16	64
3.	*E. coli*	>128	<0.06	21.	*P. aeruginosa*	16	0.25
4.	*E. coli*	32	>128	22.	*P. aeruginosa*	8	32
5.	*E. coli*	16	16	23.	*P. rettgeri*	>128	0.12
6.	*E. coli*	16	16	24.	*P. rettgeri*	>128	0.12
7.	*E. coli*	8	16	25.	*S. enterica*	8	<0.06
8.	*E. coli*	8	16	26.	*A. baumannii*	16	32
9.	*E. coli*	16	16	27.	*E. cloacae*	>128	0.12
10.	*K. pneumoniae*	>128	8				
11.	*K. pneumoniae*	16	16				
12.	*K. pneumoniae*	16	8				
13.	*K. pneumoniae*	16	0.12				
14.	*K. pneumoniae*	16	<0.06				
15.	*K. pneumoniae*	8	16				
16.	*P. mirabilis*	>128	0.25				
17.	*P. mirabilis*	>128	0.25				
18.	*P. mirabilis*	>128	0.12				

MIC, minimal inhibitory concentration

includes a large set of gram-negative organisms isolated in clinical centers from different patients showing a high level of resistance to colistin. In addition, resistance to both meropenem and colistin occurs in some strains. This study shows the emergence of colistin resistance among gram-negative bacteria, including carbapenem resistant isolates in India. Surveys and measures are necessary to limit dissemination of bacteria that accumulate resistance to both colistin and carbapenem.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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