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Screening of Multi-drug resistant Colistin Resistant gram-negative Bacteria in Environmental and Clinical Samples from Central India.

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SUMMARY

Objective: Colistin is said to be the last resort antibiotic against multi drug resistant gram-negative bacterial infections both in humans and animals. Emergence of colistin resistant bacteria gram-negative bacilli represent a global public health threat that limits therapeutic options for hospitalized patients. In the present study, we describe the occurrence of colistin resistant bacteria in both hospital and in environmental samples in the part of Central India with special reference to Jabalpur area.

Methods: For analysis, 16 samples were collected including urine samples from hospital and soil samples from different environmental conditions. Collected isolates were identified using biochemical characterization and tested against different antibiotic susceptibility to observe multi drug resistance.

Results: Total of 2 (12.5%) colistin resistant bacteria were isolated out of 16 samples i.e E.coli and Pseudomonas from environment and clinical samples respectedly. They are resistant to different antibiotics showing multi-drug resistance.

Conclusions: Presence of multi- drug resistant colistin resistant gram negative bacteria in Central India was observed. The prevalence of colistin resistance in Central India as per our study is low but not negligible. We have to study more about the resistant bacteria and the reason of emergence in our locality.

Keywords: Antibiotic resistance, multi drug resistance, colistin, gram-negative bacterial infection, central india.

INTRODUCTION

Antibiotic resistance has been defined as *"the silent tsunami facing modern medicine"* [1]. The development of new antibiotics is not common today [2]. Due to the decrease in the development of newer antibiotics and increasing antibiotic resistance, we must rely on old antibiotics such as colistin [3].

Colistin is a branched cyclic decapeptide with a fatty acid tail and is also called polymixin E [4]. It plays an important role in tackling multidrug resistant (MDR) gram-negative pathogens, particularly *Pseudomonas aeruginosa, Acinetobacter baumannii, and Enterobacteriaceae* [5]. Colistin was first described in the 1950s, but due to its nephrotoxicity and neurotoxicity, its popularity began to decrease in the 1970s [6]. As MDR strains became more prevalent among gram-negative bacteria, they were reintroduced as old and valuable antibiotics as a last-resort treatment option [7]. Colistin is used in both humans and animals for the treatment of gram-negative bacterial infections [8]. The mechanism involves colistin attachment to the outer membrane of the cell, cell lysis and ultimately cell death [9].

The World Health Organization (WHO) has classified colistin as the "highest priority critically important antimicrobial for human medicine" [10]. However, due to the unfortunately inappropriate and irrational use of colistin in humans and animals (mostly as growth promoters in animals), resistance against colistin has emerged [11]. This is a global concern, as colistin is said to be the "last man standing" for the treatment of MDR [12]. Before 2015, colistin resistance was assumed to be chromosomally acquired when the bacteria showed modifications in the outer membrane, leading to a lack of interaction between colistin and the bacterial membrane [13]. In 2015, the first plasmid-mediated mobile colistin resistance (MCR) gene was discovered, named MCR 1 [14]. The rapid emergence of colistin resistance worldwide is due to horizontal gene transfer. To date, there are 10 variants (MCR 1-10) of the gene [15]. Colistin resistance is more prevalent in middle- and low-income countries such as Europe, Southeast Asia, the USA, and Brazil [16]. It is very important to identify the bacteria that are highly harmful and contagious to humans and animals. A full infectious cycle can occur because colistin resistance can be found in food animals, human discharge, and the aqua-environment [17].

Colistin resistance has emerged across the globe [18] and researchers worldwide are trying to overcome this situation. In the context of India, there are few reports on this topic. There are data on colistin-resistant organisms, *such as MDR*

Enterobacteriaceae, Acinetobacter and *Pseudomonas*, in southern, eastern and northern India [19-25]. These organisms are isolated from hospital wastewater, food products, food animals and, most importantly, clinical samples, including bloodstreams, urine, pus, sputum, etc.

Resistance against colistin is disseminated throughout the country rapidly. It is also necessary to perform a surveillance study in central India for the prevalence of colistin-resistant organisms since there are no data reported from this region. In this context, we isolated colistin-resistant bacteria from clinical samples collected at a local hospital and from environmental samples collected from local poultry and cattle farms in the Jabalpur area, M.P.

MATERIAL AND METHODS

1. Sample collection

The present research is based on the study of two types of samples (clinical and environmental). Clinical urine samples (08) were collected from the Microbiology and Clinical Pathology Department as part of routine care for hospitalized infected patients from Marble City Hospital, Jabalpur. Soil and water samples from poultry farms and cattle fields in the Jamtara region, Jabalpur, were taken as environmental samples (08). A total of 16 samples were collected between January and February 2020. No additional clinical specimens were obtained for purposes of research; therefore, informed consent was not needed. The samples were collected aseptically in sterile plastic vials and polybags. The samples were transported under ice-cold conditions from the collection point to the research station. The details are given in Table 1.

2. Screening of colistin-resistant bacteria

For bacterial enrichment, 1 ml of each sample was added to 100 ml of pre-sterilized colistin enrichment broth medium (4 µg/ml concentration of colistin in Luria Bertani broth). All the samples were incubated in an orbital shaking incubator (Remi C-24 BL) at 37°C for 24 h at 180 rpm.

Screening was performed with the help of the disk diffusion method. The bacterial inoculum was prepared as described by Lalitha (2004) with slight modifications [26]. Briefly, the bacterial inoculum of the bacterial isolates was prepared in normal saline with turbidity equivalent to 0.5 McFarland standards. Inocula were spread onto sterile Muller Hinton agar (MHA) plates, 2 colistin (10 mcg) disks were placed on each plate, and the plates were incubated at 37°C for 24 h.

3. Morphological characteristics

The bacterial isolates exhibiting resistance were identified on the basis of morphological and cultural characteristics. Briefly, Gram staining was performed as per standard protocols, and different culture media, such as EMB, MacConkey, and XLD, were used to predict resistant organisms (gram-negative).

4. Biochemical characterization of colistin-resistant isolates

Different isolates representing resistance were picked from culture plates, and the isolates were identified using the tests described by Cowan and Garrity [27-28]. These included biochemical tests. The biochemical tests included catalase, oxidase, indole, methyl red, VP, citrate utilization, H_2S production, TSI, sugar fermentation and urease tests.

5. Antibiotic susceptibility test

Using the Kirby–Bauer disk diffusion method, AST was performed to explore antibiotic resistance phenotypes. Antibiotic disks diffuse a specific concentration of antibiotics. The bacterial suspension in NaCl must have definite turbidity (0.5 McFarland). Seventeen antibiotics, namely, penicillin G (10mcg), amoxycillin (500mcg), cefoxitin (30mcg), cefazolin (30mcg), ampicillin (10mcg), norfloxacin (10mcg), Ciprofloxcin (5mcg), cotrimoxazole (25mcg), levofloxacin (5mcg), nitrofurantoin (300mcg), nystatin (50mcg), ceftazidime (30mcg), amphotericin B (750cg), streptomycin (1mcg), cefixime (200mcg) and oflaxacin (200mcg), were used against the isolated bacteria. The antibiotic disk diffused into the agar plate, preventing bacterial growth, and this dispersion zone was transparent around the disk and is called the inhibition zone. However, strains were considered MDR if they were resistant to more than three different antibiotics.

RESULTS

1. Isolation of colistin-resistant bacteria from collected samples

Bacterial colony formation on media (LB broth) supplemented with colistin (4 mg/L) was used as a measure of bacterial resistance to colistin. Among the 16 samples collected, no bacterial colonies formed from the 6 inoculated samples. These 6 samples were considered to be susceptible to colistin. The remaining 10 samples showed wild bacterial growth against colistin at the same concentration. Primary screening (with colistin discs) of the above 10 samples revealed that out of 10 bacterial samples, 4 contained bacterial strains resistant to colistin. No clear zone formed around the discs. Among the 10 strains, 6 had colistin-susceptible bacteria, as inhibitory zones of different diameters around the discs were formed, and 4 of the isolated strains were colistin resistant, with no inhibitory zone. Out of the 4 samples, 3 were collected from the environment, and 1 was from the hospital. The details are shown in Table 2.

It is important to note that AST tests in agar plates (E-test and disk diffusion test methods) are limited to the screening of colistin resistance and may have missed some isolates.

2. **Identification of isolated bacterial strains**

The isolated resistant bacteria were identified via morphological and biochemical tests. In our study, the composition of microorganisms in the samples was described in terms of naturally colistin-resistant bacteria and those with acquired colistin resistance. Identification of colistin-resistant bacteria in the isolates revealed the presence of two gram-positive bacteria and two gram-negative bacteria. The gram-positive bacterial isolates (50%) were *Bacillus subtilis* and *Streptococcus* sp. The dominant strains were naturally colistin resistant for all GPB strains. In contrast, the GNB strains that were isolated were believed to have acquired resistance to colistin via the mcr gene or other resistance mechanisms. Here, 50% of the colistin-resistant GNB were screened as *E. coli* or *Pseudomonas* sp. The *E. coli* strain was isolated from an environmental sample, and colistin-resistant *Pseudomonas* sp. was detected in hospital samples (Table 3).

3. **Antibiotic susceptibility test against different antibiotics**

The isolated gram-negative colistin-resistant bacteria exhibited cross-resistance to different antibiotics. According to the susceptibility tests to antimicrobial agents, 100% of the isolated bacterial strains were resistant to ampicillin, nalidixic acid, cotrimoxazole, nystatin, levofloxacin and cefoxitin.

Sample S2, i.e., *E. coli*, was resistant to cefoxitin, cefazolin, ampicillin, nalidixic acid, ciprofloxacin, levofloxacin, nystatin, streptomycin and cefixime. Sample U14, i.e., *Pseudomonas sp*., was resistant to cefoxitin, ampicillin, nalidixic acid, cotrimoxazole, levofloxacin and nystatin. See Table 4 and Fig. 1.

As mentioned above, if the isolates were resistant to more than 3 antibiotics, they were assumed to be multidrug resistant. Therefore, both the isolates exhibited multidrug resistance.

DISCUSSION

The dissemination of antibiotic-resistant bacteria in medical settings is worrying [24]. The most disturbing is MDR gram-negative bacteria, as they can spread nosocomial infections that may lead to death [29]. Severe infections triggered by bacteria showing multiple resistances to most commonly and commercially existing antibiotics, such as fluoroquinolones, aminoglycosides, carbapenem, and ß-lactams, have been increasing in recent years. We only have to rely on the old antibiotic colistin as a last resort pharmacon against these MDR gram-negative bacteria [30]. The uncontrollable and illegal use of colistin accelerated the dissemination of colistin resistance in animals and, consequently, humans [31]. Infection due to colistin-resistant gram-negative bacterial pathogens is a menace, as there are limited options available to treat such infections. There is repeated usage of colistin in infections where there is still susceptibility to lower antibiotics, and colistin is occasionally used at suboptimal doses. This resistance transmission cycle has never ended, as colistin is frequently used in poultry and pig farms, dairy and pisciculture, possibly leading to the discharge of colistin residues into the atmosphere in small doses and the induction of colistin resistance in saprophytic organisms, which subsequently come in contact with the human body in different ways [32]. In 2016, the European Medicines Agency (EMA) updated its guidelines to minimize the use of colistin in animals to reduce its impact on human health. The Health Organization (WHO) is a critically important antimicrobial of highest priority [33]. According to a study from India, the overall prevalence of colistin resistance (19.6%) of the acquired type was much greater than the reported prevalence in Western countries [32]. In one of the first studies from a tertiary care setting in South India, eight colistin-resistant *Klebsiella pneumoniae* strains were isolated from blood cultures between 2013 and 2014. In a study from Northeast India, 2.5% colistin resistance was detected by the broth microdilution method among the gram-negative isolates [34]. A recent study from South India reported the emergence of colistin resistance (2.5% isolates) among MDR GNB isolates from cancer patients [35]. In one additional study, twenty-four colistin-resistant isolates were reported over 18 months. Urine was the most common source of the isolate, followed by blood and respiratory samples [22]. According to a study from Western India, 9.98% of 359 samples were colistin-resistant gram-negative bacilli [20]. Wattal et al. found 8% bacterial resistance to colistin in intensive care unit (ICU) samples via surveillance of multidrug-resistant organisms in a tertiary health care setting in North India [21]. Another study from North India reported the emergence of tigecycline- and colistin-resistant *A. baumannii* during the UTI [36]. Ramesh et al. reported colistin resistance in almost all gram-negative organisms in a study across two centers in South India [37-38].

The present study is the first report of colistin resistance in Central India. This study has several limitations, as does the relatively small sample size of a small area of Central India. The present study may lead to the pioneering idea of the present situation of colistin resistance in Central India and lead to further detailed studies of societal welfare. Our study covered one of the areas of central India, i.e., Jabalpur, and 2 (12.5%) of the 16 samples were colistin-resistant gram-negative bacteria (8 from the environment and 8 from the clinic).

The prevalence of colistin resistance in Central India in our study was low but not negligible. We have to study more about the resistant bacteria and the reason for emergence at our locality. The resistant isolates in our study were susceptible to lower-class antibiotics but still resistant to the (priority antibiotic) colistin. The reason behind the resistance in our study is assumed to be the irrational use of colistin at low doses in animals as well as humans [39].

On the basis of the findings of this study, we recommend multicenter studies to use better techniques for obtaining a better understanding of local molecular epidemiology and for detecting area-wide resistance to colistin in Central India.

CONCLUSION

Prevalence of colistin resistance in Central India is present. The percentage of emergence is low but not negligible and cannot be unnoticed. In conclusion, our overview of colistin resistance suggested that the transmission of such multidrug-resistant organisms can be controlled by strict infection control measures and proper hand hygiene practices. Surveys and actions are needed to limit the dissemination of bacteria that accumulate resistance to colistin. To track the emergence of colistin resistance in central India, prospective studies will be carried out in other locations.

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CONFLICT OF INTERESTS

The authors report no conflict of interest.

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Table 1: Details of the collected samples

Sample	No. of Samples
Marble City Hospital	08
Cattle Field 1	02
Cattle Field 2	02
Poultry 1	02
Poultry 2	02
Total	16

Table 2: Details of the collected samples showing colistin resistance.

Subject	Sample	Location	Resistant/Susceptible
Poultry 1	Fecal Soil	Jamtara	Susceptible
Poultry 1	Waste Water	Jamtara	Susceptible
Cattle Field 1	Waste Water	Jamtara	Resistant(U1)
Cattle Field 1	Soil	Jamtara	Susceptible
Cattle Field 2	Waste Water	Jamtara	Resistant (U2)
Cattle Field 2	Soil	Jamtara	Susceptible
Poultry 2	Fecal Soil	Jamtara	Resistant (S2)
Poultry 2	Waste Water	Jamtara	Susceptible
Hospital	Urine	Marble City	Susceptible
Hospital	Urine	Marble City	Susceptible
Hospital	Urine	Marble City	Susceptible
Hospital	Urine	Marble city	Susceptible
Hospital	Urine	Marble City	Susceptible
Hospital	Urine	Marble City	Resistant (U14)
Hospital	Urine	Marble City	Susceptible
Hospital	Urine	Marble City	Susceptible

Table 3: Identification of Colistin-Resistant Bacteria

Samples and isolated site	No. of resistant samples	% of resistant Samples isolated	Isolated strains
U1 (cattle field 1)	1	25%	*Bacillus subtilis*
U2 (cattle field 2)	1	25%	Streptococcus sp.
S2 (poultry 2)	1	25%	*E. coli*
U14 (Marble City Hospital)	1	25%	Pseudomonas sp.
Total No. of samples	4	100%	-

Table 4: Isolated samples against different antibiotics. S3: *E.coli*, S14: *Pseudomonas sp.*

ANTIBIOTICS	S2	U14
Ampicillin (AMP)	Resistant	Resistant
Nalidixic acid (NA)	Resistant	Resistant
Norfloxacin (NX)	Susceptible	Susceptible
Ciprofloxacin (CIP)	Resistant	Susceptible
Cotrimoxazole (COT)	Susceptible	Resistant
Levofloxacin (LE)	Resistant	Resistant
Nitrofurantoin (NIT)	Susceptible	Susceptible
Nystatin (NS)	Resistant	Resistant
Cefoxitin (CX)	Resistant	Resistant
Ceftazidime (CAZ)	Susceptible	Susceptible
Amphotericin B	Susceptible	Susceptible

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Penicillin	Susceptible	Susceptible
Streptomycin	Resistant	Susceptible
Cefixime	Resistant	Susceptible
Amoxycillin	Susceptible	Susceptible
Oflaxacin	Susceptible	Susceptible
Gentamycin	Susceptible	Susceptible
Amikacin	Susceptible	Susceptible
Cefazolin	Resistant	Susceptible

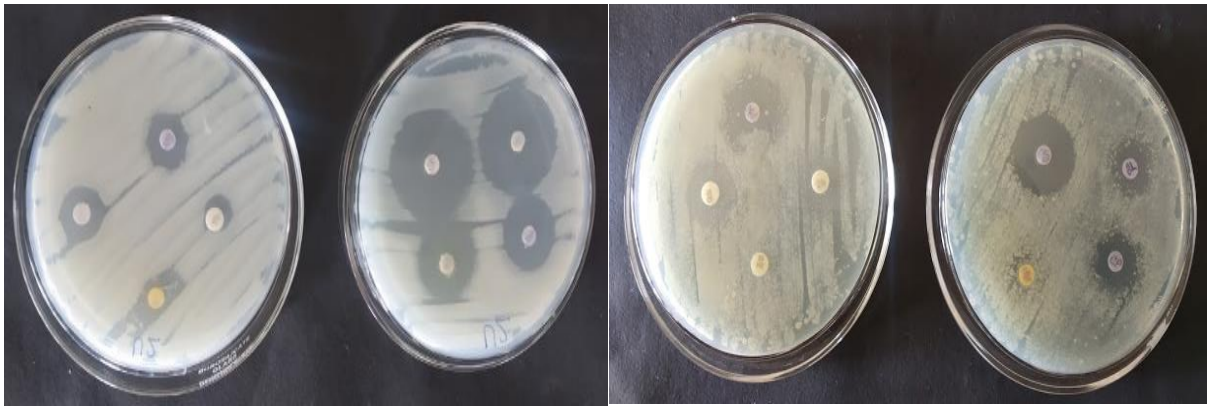

Figure 1: Observation of inhibition zone of different antibiotics against isolated bacteria – Antibiotic Susceptibility Assay