Colistin Resistance among Human Clinical Isolates from a Tertiary Care Hospital in Nepal

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ABSTRACT

Background

Colistin is increasingly being used for treatment of multidrug resistant (MDR) bacterial infections in human. The plasmid mediated *mcr-1* gene was detected recently among the poultry in Nepal. Plasmid mediated colistin resistance among human isolates would severely compromise the treatment of MDR bacterial infections.

Objective

To find out the prevalence of colistin resistance among the gram-negative bacteria isolated from clinical specimen.

Method

A descriptive cross-sectional study was conducted in a tertiary care hospital in Kathmandu. Colistin resistance among all the gram negative bacilli (GNB), isolated in a year, from all clinical specimen subjected for aerobic bacterial isolation, was detected by disc diffusion and agar dilution methods. Those with a minimum inhibitory concentration (MIC) > 2 mcg/ml were subjected to polymerase chain reaction for detection of *mcr-1* gene.

Result

A total of 1993 aerobic GNB were isolated from clinical samples received from February 2018 to January 2019. By the agar dilution screening method, 2.1% of the GNBs isolated had MIC of colistin > 2 mcg/ml. None of these possessed the *mcr*-1 gene.

Conclusion

Given the occurrence of increased MIC of colistin in the clinical isolates in our set up, nationwide active surveillance would generate data for Nepal. The study for the presence of other genes (chromosomal or plasmid mediated) responsible for the increased MIC of colistin would further guide the control measures.

KEY WORDS

Colistin, MCR-1, Multi-drug resistance

INTRODUCTION

Infections due to multidrug resistant (MDR) bacteria have become a global problem. Gram positive and gram negative bacteria with resistance to multiple classes of antibiotics pose a difficult clinical situation. Antibiotics which were shelved earlier due to their adverse effects have once again been brought to use since these are the only options left for the treatment of infections by these MDR pathogens. Colistin is one such antibiotic being used as the last resort antibiotic against most of MDR Gram negative bacteria.[1] With the rise in MDR in Nepalese health care set ups, colistin is increasingly being used for treatment of human cases. Its use in the veterinary sector as growth promoter and for therapeutics far exceeds the consumption in human health-care.

Reports of plasmid mediated colistin resistant Enterobacteriaceae from cloaca of hen and chicken meat from Nepal have emerged.[2] It is now known from the global distribution and spread of the plasmid mediated colistin resistant gene (*mcr* gene), that food animals were the sources for humans.[3] However, the prevalence of colistin resistance and the incidence of the mobile colistin resistance (*mcr*) gene in clinical isolates is not known.

Therefore, this research was carried out to gauge the magnitude of the problem of colistin resistance in human health and establish the role of *mcr-1* gene in its spread. All gram negative isolates from clinical specimen were screened for colistin resistance using two different methods and attempt was made to detect the plasmid mediated *mcr-1* gene among the phenotypically resistant isolates.

METHODS

A descriptive cross-sectional study was conducted on gram-negative bacilli (GNB) isolated from all clinical samples of patients attending Nepal Medical College Teaching Hospital (NMCTH), Gokarneshwor-8, Kathmandu, Nepal from 1st February 2018 to 30th January, 2019. Since the local prevalence of colistin resistance has not been established, all the GNB isolated from clinical specimen during the study duration were included in the study. Repeat isolates from the same patient from repeat specimen were excluded from study to avoid duplication of isolate. Colistin non-susceptible GNB, including Proteus group, *Serratia* spp., *Burkholderia* spp., *Brucella* spp., *Chromobacterium* spp were also excluded.[3]

Sample collection and processing

Prior to commencing the study, ethical clearance was obtained from the Institutional Review Committee of NMCTH. All clinical specimens which included pus, endotracheal aspirates, sputum, urine, blood, and body fluids like cerebrospinal fluid, ascitic fluid, peritoneal fluid, pleural fluid, synovial fluid, pericardial fluid, tissue, swabs and catheter tips (except Foley's catheter tip) were processed for aerobic bacterial culture following the standard microbiological procedures.[4] Briefly, except for blood and urine specimens, Gram stained smears from all specimens were read and recorded. The specimens were inoculated in a set of appropriate culture media. Adequate volume of blood was inoculated into blood culture bottles with Brain Heart Infusion (BHI) broth, incubated overnight and subcultured on blood agar and Mc Conkey agar every alternate day. Any colony appearing on the solid media were identified by gram staining and a panel of biochemical tests. Blood cultures were considered sterile after incubation for five days. Similarly, tissue and body fluids were inoculated onto both solid media (blood agar, Mac Conkey agar and chocolate agar) and BHI broth. Urine was inoculated on CLED medium for semi-quantitative culture. Colonies grown after overnight incubation aerobically at 35^0C were identified by Gram staining and biochemical tests. Antimicrobial susceptibility tests for each were done by Kirby Bauer Disc Diffusion method and were interpreted according to CLSI guidelines.[4]

For antibiotic sensitivity testing by Kirby Bauer disc diffusion method, 4-5 similar looking colonies of isolate were suspended in peptone water and incubated at 37^0C for 2 hours. The growth in the tube was then compared with and adjusted to 0.5 McFarland's standard. A sterile cotton swab was dipped into the suspension and squeezed against the side of the tube and then used to inoculate Mueller Hinton agar plate. Antibiotic discs were placed without delay and incubated at 37^0C for 18-24 hours. Quality control was performed using *E coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 strains. The antibiotic discs manufactured by Hi-media, Mumbai, India of following concentrations were used (ampicillin (10 µg), cefixime (5 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), co-trimoxazole (1.25 µg trimethoprim + 23.75 µg sulphamethoxazole), amikacin (30 µg), piperacillin-tazobactam (100/10 µg), meropenem (10 µg), colistin (10 µg), nitrofurantoin (300 µg for urinary isolates only). Isolates with zone of inhibition (ZOI) around colistin disc less than 11 mm were subjected to MIC detection using agar dilution method. A loopful of bacteria in log phase were inoculated onto Mueller-Hinton agar (Hi-media, Mumbai, India), supplemented with different concentrations of colistin (Sigma-Aldrich, Inc.) and incubated overnight at 37^0C for detection of minimum inhibitory concentration (MIC).[5]

Those isolates with MIC > 2 mcg/ml were tested for presence of *mcr*-1 plasmid gene by PCR. The PCR was performed at Decode, Sinamangal, Kathmandu, Nepal. PCR master mix was subjected for PCR using the following primer pair:

Forward primer: CLR5-F 5'CGGTCAGTCCGTTTGTTC 3'

Reverse primer: CLR5-R 5'CTTGGTCGGTCTGTAGGG 3'

Controls: *Escherichia coli* NTCC 13846 (*mcr*-1 positive)

Escherichia coli ATCC 25922 (*mcr*-1 negative)

Data was analyzed using SPSS 16.0 software. Chi-square test was applied to analyze the data. A p value of < 0.05 was considered statistically significant.

RESULTS

During the study period, 19498 samples were processed. Urine was the most common sample received for aerobic bacterial culture. Blood and other body fluids (exudates) and tissues were the other types of samples processed. A total of 1993 aerobic GNB were isolated from clinical samples. Urine sample yielded the highest number of GNB (n=1,373; 68.89% of the total GNB isolates). Thirty belonged to the genera which are intrinsically nonsusceptible to polymixins. Ninety-eight of the remaining 1963 i.e 4.9% of the GNBs showed ZOI of less than 11 mm around colistin disc (considered resistant according to the disc manufacturer's reference chart). All 1963 GNBs including the 98 isolates with decreased ZOI were subjected to MIC test for colistin by agar dilution. Forty-five isolates (out of 98 isolates showing a reduced ZOI) had a MIC of > 2 mcg/ml. These 45 isolates were studied for the detection of *mcr-1* gene. None of the isolates showed the presence of this gene.

Analysis of isolates with MIC > 2 mcg/ml

Out of the 45 isolates showing MIC of > 2 mcg/ml, 28(62%) and 17(38%) respectively were from inpatients and outpatients. Most of the isolates were from urine (n=23; 51%) but no statistically significant association (p value > 0.05) was seen with the type of sample they were isolated from. Neither was there any significant association with gender and age of patients from whom these were isolated.

Most of the *E. coli* were isolated from urine from patients attending the outpatient department. The rest of the other GNBs were isolated from samples from patients admitted in the hospital. Most (n=23) of the isolates showing an increased MIC for colistin were from cases of urinary tract infection; *E. coli* being the most common. Seventeen were isolated from body fluids and respiratory samples. Only 5 were isolated from blood, out of which 3 were *K. pneumoniae*. The highest rate of colistin resistance (4.7%) was observed among the *Acinetobacter baumannii* complex (ABC) and the lowest (1.4%) among the *P. aeruginosa* (table 1).

Antibiotic susceptibility pattern for the colistin resistant isolates were studied. Most were resistant to the commonly prescribed beta lactams, fluoroquinolones and aminoglycosides. More than half the isolates were resistant to carbapenems (table 2 and table 3).

Table 1. **Distribution of GNB in different samples**

	E.coli	*Klebsiella pneumoniae*	*Citrobacter fruendii*	*Pseudomonas aeruginosa*	ABC (n=7)
Blood	32	18	26	44	14
Urine	848	307	61	32	72
Exudate	112	128	34	72	58
Total	992	453	121	148	144
Colistin MIC > 2 mcg/ml	17	16	3	7	2
Percentage	1.7	3.5	2.4	4.7	1.4

Table 2. **Overall antibiotic sensitivity pattern for 45 isolates with colistin MIC > 2 mcg/ml**

Antibiotic	*Sensitive (n)*	*Sensitive (%)*
Ampicillin	1	5.8
Cefexime	10	22.2
Ceftriaxone	11	25.6
Ceftazidime	00	00
Ciprofloxacin	14	31.1
Cotrimoxazole	11	24.4
Amikacin	12	26.7
Piperacillin-tazobactam	23	51.1
Meropenem	22	48.9
Tigecycline	41	91.1

DISCUSSION

There are various in vitro methods proposed for the detection of colistin resistance. The simple method of antimicrobial susceptibility test (AST) by disc diffusion however is not recommended for the susceptibility testing against colistin. It is noted that the colistin molecule from the filter paper disc does not diffuse well through the agar medium. This allows the bacteria to grow very near the antibiotic disc thereby giving a small ZOI and therefore a false interpretation of the isolate being resistant.[6] The disc diffusion and gradient diffusion methods are not recommended for the reporting of resistance against colistin.[7] Our study supports this claim. A very large number of isolates (n=98) (4.9%) of the GNB isolated had a ZOI of less than 11 mm around the colistin disc. According to the interpretation chart provided by the manufacturer (HiMedia, India), these isolates are interpreted as resistant to colistin.

From the results of the MIC test by agar dilution in this study, it can be extrapolated that the disc diffusion test can be utilized to screen for GNB for further MIC testing. The 58 isolates which grew in the 1 mcg/ml colistin incorporated plate all belonged to the 98 isolates showing a decreased ZOI for colistin. Thus, we deduce that the disc diffusion method can be used to screen for those isolates which are truly sensitive to colistin. (This inference needs reconsideration. Perhaps it can be said that isolates showing ZOI >11 mm can be considered sensitive but less than 11 mm need to

Table 3. Antibiotics susceptibility pattern of the 45 isolates having MIC for colistin > 2 mcg/ml.

	E. coli (n=17)			*Klebsiella pneumoniae* (n=16)			*Citrobacter fruendii* (n=3)			*Pseudomonas aeruginosa* (n=2)			ABC (n=7)		
	OPD n=11	IPD n=6	Total %	OPD n= 4	IPD n=12	Total %	OPD n= 1	IPD n= 2	Total %	OPD n= 0	IPD n= 2	Total %	OPD n= 1	IPD n= 6	Total %
AMP	1	0	5.8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
CFM	4	1	29.4	2	1	18.7	1	0	33.3	-	0	0	1	0	16.7
CTX	3	2	29.4	1	2	18.7	2	0	66.7	NA	NA	NA	1	0	16.7
CAZ	NA	NA	NA	NA	NA	NA	NA	NA	NA	-	0	0	NA	NA	NA
CIP	3	0	17.6	3	5	50	2	0	66.7	0	0	0	0	1	16.7
COT	6	1	41.2	0	3	18.7	0	1	33.3	0	0	0	0	0	0
AK	6	1	41.2	3	2	31.2	0	0	0	0	0	0	0	0	0
PIT	8	1	55	4	3	43.7	1	2	100	0	2	100	1	1	33.3
MRP	8	1	52.9	3	3	37.5	1	1	66.7	0	2	100	1	2	42.8
TG	11	5	94.1	4	10	87.5	1	1	66.7	0	2	100	1	6	100

AMP=Ampicillin; CFM=Cefexime; CTR=Ceftriaxone; CAZ=Ceftazidime; CIP=Ciprofloxacin; COT=Co-trimoxazole; AK= Amikacin; PIT= Piperacillin/Tazobactam; MRP= Meropenem; TG= Tigecycline, ABC= *Acinetobacter baumanii* complex

be further investigated by MIC. This reconfirms that colistin resistance can not be confirmed by disc diffusion method.)

The joint polymyxin breakpoint working group of CLSI and EUCAST recommends the broth dilution method to calculate the MIC.[8] Agar based screening method for bacteria suspected to be colistin resistant, coupled with confirmation by broth microdilution and/or molecular methods is described as one of the promising method for detection of colistin resistance.[9] The results from the MIC by agar dilution method in this study showed that the MICs ranged from ≤ 1 mcg/ml to ≥ 4 mcg/ml. Out of the 1993 GNB tested, MIC of colistin for 1948 isolates was ≤ 2 mcg/ml and for 45 isolates it was > 2 mcg/ml. The CLSI defines the resistance to colistin as MIC ≥ 4 mcg/ml for *Pseudomonas aeruginosa* and *Acinetobacter* spp. For *E. coli, K. pneumoniae, K. aerogenes,* the epidemiological cut off value for wild type is ≤ 2 mcg/ml and for non-wild type (NWT) is ≥ 4 mcg/ml (CLSI 2019). For clinical isolates, the EUCAST describes the breakpoint resistance of colistin as MIC > 2 mcg/ml for *Enterobacterales, Pseudomonas* and *Acinetobacter*.[10] The 45 clinical isolates in this study which had a MIC of > 2 mcg/ml comprised of Pseudomonas *aeruginosa* (n=2; 1.4%), *Acinetobacter baumannii* complex (n=7; 4.7%), *E. coli* (n=17; 1.7%), *K. pneumoniae* (n=16; 3.4%) and *C. fruendii* (n=3; 2.4%). Overall, by the agar dilution screening method, 2.1% of the GNB isolated had MIC of colistin > 2 mcg/ml. None of these possessed the *mcr-1* gene.

A systematic review and meta-analysis of colistin resistant bacteria with *mcr* genes study from 47 countries across 6 continents between 1980-2018 published in July 2019 showed that the highest occurrence of *mcr* gene was among the non- pathogenic *E. coli*, followed by Aeromonas, pathogenic *E. coli, Citrobacter, Klebsiella, Enterobacter, Salmonella, Shigella* in that order.[11] Among all the Gram negative isolates in our study, the highest number of isolates (4.7%) showing an increased MIC for colistin was found among the *Acinetobacter baumanii* complex (ABC). Resistance to colistin is increasingly being reported among ABC.[12-14] In a study from Korea, 27.9% of ABC isolates were found to be colistin resistant.[13]

The plasmid borne *mcr-1* gene is the most often responsible for the acquisition of colistin resistance by bacteria. Since this is plasmid borne, it is very efficiently transferred horizontally.[15] The *mcr-1* gene initially spread from the bacteria in animal population to bacteria in the human population through food or contact with animals and poultry.[15,16] This was first detected in *E. coli* isolated from pigs and meat in China in 2015.[15] After this first report, more reports of *mcr-1* detection from human clinical samples started emerging from different parts of the world. The occurrence of the plasmid bearing this gene in either the human or the animal/poultry population is worrisome since they are easily transferred within and outside the population.[15]

Wide difference in prevalence colistin resistance in GNB have been described from across the globe. Few reports of colistin resistance among human clinical isolates from Nepal exist. However, the test methods utilized were disc diffusion method, which is not recommended either by CLSI or EUCAST. This is the first study from Nepal where human clinical isolates were tested for colistin resistance by determining MIC by agar dilution and confirmation by PCR. However, colistin resistance with detection of plasmid mediated *mcr-1* gene was reported from poultry from Nepal in 2019. Out of the 118 *E. coli* isolated from 324 cloacal swabs, 27 (22.8%) were colistin resistant and harbored *mcr*-1 gene.[2] It is therefore a matter of time for the resistance bearing pathogen/plasmid to reach the human population from poultry. The other case, involving a Nepalese national, is from a hospital in Qatar, reported in 2018. Respiratory secretions of a 58 years old Nepali

male with subarachnoid hemorrhage, admitted to the ICU in Qatar, yielded a highly virulent *E. coli* lineage with *mcr-1* mediated colistin resistance.[17]

There are few studies from India which showed the presence of colistin resistance in human clinical isolates. Shankar et al. reported from Christian Medical College Vellore, that none of the 65 *K. pneumoniae* isolated in 2016-2017 were resistant to colistin by microbroth dilution technique, harbored *mcr-1* or *mcr*-3 genes.[18] Their MIC ranged from 4 to 256 mcg/ml. Mutations in chromosomal genes were responsible for colistin resistance in these isolates. In a cohort of patients with complicated UTI in an Indian tertiary care hospital, 8 out of the 224 i.e 3.5% isolates of ABC were resistant to colistin. The MIC ranged from 0.016 to 256 mcg/ml for colistin.[19] In a study at the Post Graduate Institute, Chandigargh, India, 250 MDR clinical isolates of *E. coli* (142) and *K. pneumoniae* (108) were studied for colistin resistance. Twenty-five isolates (20 *K. pneumoniae* and 5 *E. coli*) were resistant to colistin, with a prevalence of 3.52 % in *E. coli* and 18.5% in *K. pneumoniae* among the MDR isolates. PCR for the *mcr-1* and mcr-2 genes was negative.[20] Outbreak of colistin resistant *K. pneumoniae* was reported from ICU of a trauma centre in India. All 7 isolates were colistin resistant by MIC determination. They were negative for *mcr-1* gene but possessed chromosomal mutations.[21] Eight *K. pneumoniae* isolated from blood stream infection at CMC Vellore, India during 2013-2014, were resistant to colistin, (MIC ranged from 4 to 1024mcg/ml); none possessed *mcr-1* or *mcr-2* genes. The resistance was attributed to chromosomal mutations.[22]

The prevalence of colistin resistance is lower in Europe and the Americas. The plasmid mediated resistance however seems to occur more in these regions. A study from Lombardy Italy in 2016 showed a prevalence rate of 0.5% (18/3902) for colistin resistance in *E. coli* (MIC ranged from 4-8 mcg/ml) in clinical isolates from 6 different hospitals. The *mcr-1* gene was responsible for resistance in 10 out of 18 isolates.[23] The *mcr-1* possessing isolates were mostly obtained from urine of both outpatients (n=7/12) and inpatients (n=3/6). In our study, more number of isolates from inpatient source had an increased MIC of colistin compared to those from the outpatient.

The *mcr* genes have been detected from the gut flora as well. A study on gut colonization by colistin resistant GNB among critically ill patients in Greece, revealed 52% carrying the resistant bacteria; 20% had colistin resistant *K. pneumoniae* while the rest were carrying intrinsically colistin resistant enterobacterioceae. Seven cases had infection with colistin resistant *K. pneumoniae* and 12 from intrinsically colistin resistant strains.[24] Most of these patients had a history of being treated with colistin. Similar study on Singaporean patients with diarrhoea showed a lower carriage rate (8%) of colistin resistant (*mcr-1* possessing) enterobactericeae in their stool. Noteworthy however, is that these were patients without a history of prior exposure to colistin. These studies highlight the potential risk of infection and transmission of *mcr-1* Enterobacteriaceae from patients carrying the bacteria.[25]

Some plasmids carrying the *mcr-1* gene co-possess genes responsible for resistance to other classes of antibiotics like beta lactams, quinolones, aminoglycosides, sulfonamides, tetracyclines and fosfomycin.[26] and some co-possess the NDM carbapenamase genes.[27-29] Although none of our isolates possessed the plasmid, antibiograms of the isolates with higher MIC for colistin were recorded. Multidrug resistance was frequent (n=28) and pan drug resistance was seen in 4 isolates. Only around half of these isolates were susceptible to carbapenems (48.8%) and piperacillin-tazobactam (51%). All of the *P. aeruginosa* isolates in our study showing an increased MIC for colistin were sensitive to carbapenems while others showed variable resistance. Among the *mcr-1* positive *E. coli* isolates from cloaca of broiler chicken from 7 different poultry farms in Nepal, > 80% were resistant to ciprofloxacin, tetracycline, and sulfamethoxazole-trimethoprim. Three of the 27 *mcr-1* encoding isolates produced extended-spectrum β-lactamase (ESBL) and carried bla_{CTX-M} gene.[2]

The first *E. coli*, possessing *mcr-1* gene, isolated from human blood stream infection outside from China was MDR and was sensitive only to carbapenem.[30] The 12 Enterobacteriaceae with *mcr-1* gene, isolated from stool of patients attending a Singaporean hospital, showed variable antibiogram and none were resistant to carbapenems.[2] Between 2000 and 2016, most of the *E. coli* possessing *mcr-1* gene isolated from food animals in Brazil co-possessed CTX-M ESBLs. None of the human isolates had *mcr-1* gene.[31] All the five colistin resistant *E. coli* strains, from different origins in China, coproducing ESBL also showed resistance to chloramphenicol, ciprofloxacin and tetracycline; while all were susceptible to aztreonam, imipenem and cefepime.[32] In two multicentric analysis of carbapenemase producing *K. pneumoniae* clinical isolates from the US hospitals, 13-16% were colistin resistant.[6,33] Another multicentric study from Italy showed an alarming 43% of carbapenmase producing *K. pneumoniae* to be colistin resistant as well.[34] In both the studies, colistin resistance was chromosomally mediated and not plasmid borne.

Co-production of ESBLs or carbapenemases by *mcr-1* harbouring Enterobacteriaceae has now been documented.[35-37] Studies from India showed a varying resistance rates to carbapenems among *P. aeruginosa* which were resistant to colistin.[38] Plasmid mediated carbapenem and colistin resistance in *E. coli* isolated from a case of urinary tract infection was described from Switzerland in 2015. Its origin was speculated to be a veterinary source. The spread of plasmid borne colistin resistance to ESBL or carbapenemase producing Enterobacteriaceae is inevitable to produce pandrug resistant pathogens.[36]

Besides the *mcr-1* gene, other mobile genes which confer bacteria resistance to colistin have been discovered.[39-42]

However, the other types of *mcr* are not as widespread as *mcr-1*. These have been reported at different frequencies from different places worldwide. Detection of these genes was beyond the scope of this study. Since none of the isolates with raised MIC for colistin possessed the commonly occurring *mcr-1* gene, the resistance could have been mediated by chromosomal or other *mcr* genes.

CONCLUSION

An increased MIC of colistin in human clinical isolates was observed. Since the common plasmid mediated *mcr-1* gene was not detected, other genes (chromosomal or plasmid mediated) should be sought for. A nationwide study based on WHO's recommendations for screening for colistin resistance in isolates resistant to carbapenem (since these may be pan drug resistant strains) would be able to correctly reflect the status of colistin in Nepal.[9] Since *mcr* is already documented in poultry from Nepal, the human health sector should be alert for any occurrence of plasmid mediated colistin resistant bacteria from human specimen. Robust infection control practices and antimicrobial stewardship would help prevent further spread of colistin resistant bacteria.

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