Research Article

Identification and characterization of colistin-resistant *E. coli and K. pneumoniae* isolated from Lower Himalayan Region of India

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Abstract
Multidrug resistance is one of the worldwide public health concerns. Water represents the most suitable environment, for the exchange of antibiotic resistance genes among pathogenic to non-pathogenic bacteria. Therefore, we aimed to screen the presence of bla_{NDM-1}, bla_{TEM}, bla_{SHV}, bla_{CTX-M} and *mcr1–5* genes among water samples from different locations of Lower Himachal Pradesh. We examined the genotypic incidences of bla_{NDM-1}, bla_{TEM}, bla_{SHV}, bla_{CTXM} and *mcr1–5* by polymerase chain reaction. Survivability assay, fitness cost assay and biofilm assay were performed for phenotypic characterization. The presence of bla_{NDM-1} and its related variants were analysed and confirmed by sequencing-based approaches. A total of 73 bacterial strains were identified on M-lauryl sulphate agar medium. Out of 73 colistin-resistant isolates, 34 were *E. coli* and 39 were *K. pneumoniae*. Out of 34 samples, 2 (5.8%), 2 (5.8%), 5 (14.7%), 5 (14.7%) and 4 (11.76%) *E. coli* were bla_{TEM}, $bla_{SHV,}$ bla_{CTXM-1}, bla_{CTXM-2} and $bla_{CTXM-15}$ positive, respectively. Among 39 K. *pneumoniae*, 15 (38.4%), 6 (15.3%), 10 (25.6%), 9 (23.07%) and 10 (25.6%) were bla_{TEM}, bla_{SHV}, bla_{CTXM-1}, bla_{CTXM-2} and $bla_{CTXM-15}$ positive, respectively. Interestingly, we observed one *E. coli (HG4)* isolate with both bla_{NDM-1} and *mcr-1* gene. Further analysis showed HG4 isolate has lesser survivability on the cotton swab, long lag phase and less biofilm production compared to colistin-sensitive isolates. Detection of *E. coli* with bla_{NDM-1} and *mcr-1* in this geographical region is an alarming signal for tourists, community, health workers and policymakers. Hence, it is utmost important to take appropriate measures to control the dissemination of antibiotic resistance gene in such pristine locations.

Keywords bla_{NDM-1} · *mcr-1* · *E. coli* · *K. pneumoniae* · Water sources · Himachal Pradesh

1 Introduction

Overuse of antimicrobial agents promotes the spread of antibiotic resistance genes worldwide [1]. An environment containing bacteria with a trace of antibiotic resistance genes (ARGs) can increase the possibility of acquiring antibiotic resistance in human pathogens [2]. In addition, the population dynamics of natural microbial populations can also be challenged by spreading the ARGs in the natural ecosystem [3]. The increasing incidences of antimicrobial resistance in human pathogens apply an incredible effect on the global human health care system and are assessed to cause thousands of deaths annually [4].

Avinash Singh and Anand Kumar Keshri have contributed equally to this work

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In 2017, a report on major rivers of India showed most of the bacterial population of Indian rivers have high levels of resistance to broad-spectrum antibiotics (i.e. third-generation Cephalosporin [5]). A study on the River Cauvery in Karnataka showed 100% (283/283) of *E. coli* isolates were resistant to Cephalosporin [6]. In 2016 study, 17.4% (40/230) of Gram-negative bacteria of River Yamuna were found to be extended spectrum β-lactamase producers [7]. Interestingly, 17% of *E. coli* and 13% of *Klebsiella* species were found to be resistant to Cephalosporin in potable water sources apart from rivers from Uttar Pradesh, India [8]. Similarly, a report from East Sikkim showed 50% of *E. coli* and 72% of *Klebsiella* species were resistant to cephalosporin in natural water bodies [9]. Colistin, a last-resort antibiotic, is used for the treatment of infections caused by carbapenem-resistant *E. coli* and *K. pneumoniae* bacterium. **In China, *mcr-1*-mediated colistin resistance among *E. coli* was isolated from pigs, chickens and humans** [10]. Recently, colistin resistance among GNBs has been reported in India [11, 12].

The Indian pharmaceutical industry has an estimated US$15 billion in revenue in 2014 which is approximately 20% of the global demand for generic drugs (Nordea Asset Management 2015). Eighty per cent of antibiotics sold by multinational pharmaceutical companies are being manufactured in India or China. The effluents from manufacturing units contain significant levels of antibiotic residues, leading to waterways and lakes in India being contaminated [1, 13]. The Central Pollution Control Board (CPCB) sets effluent standards for the pharmaceutical industry in India, which is also applicable to all state pollution control boards. Unfortunately, antibiotics are not included in the current standards and therefore they are not monitored in pharmaceuticals effluent discharge (CPCB Effluent Standards 2013). Himachal Pradesh is a major touristic place in India; its economy is majorly dependent on tourism and agriculture. The Baddi town is one of the Asia's largest pharmaceutical hubs; this is home of around 270 pharmaceutical companies. These manufacturing units are discharging their effluent with residual antibiotics in local water bodies and contaminating them in the absence of any strict guidelines. Traces of antibodies can change the microbial ecosystem of the surrounding environment by putting selection pressure on the microbial population for antibiotic resistance. Therefore, **we aimed for the presence of bla_{NDM-1}, bla_{TEM}, bla_{SHV}, bla_{CTX-M} and *mcr1–5* genes among** water samples from different locations of Lower Himachal Pradesh.

2 Materials and methods

2.1 Sampling sites

Water samples were collected from different locations of Manali to Baddi (Himachal Pradesh, India) in sterile water collection bottles (Fig. 1) following the standard **method in the month of January 2019** [1]. Briefly, 500 mL of water sample was collected in autoclaved polypropylene bottles from 60 cm beneath the water surface and bottles were immediately kept on ice and stored overnight at 4 °C, and bacteriological analysis was undertaken on the following day.

2.2 Bacteria isolation

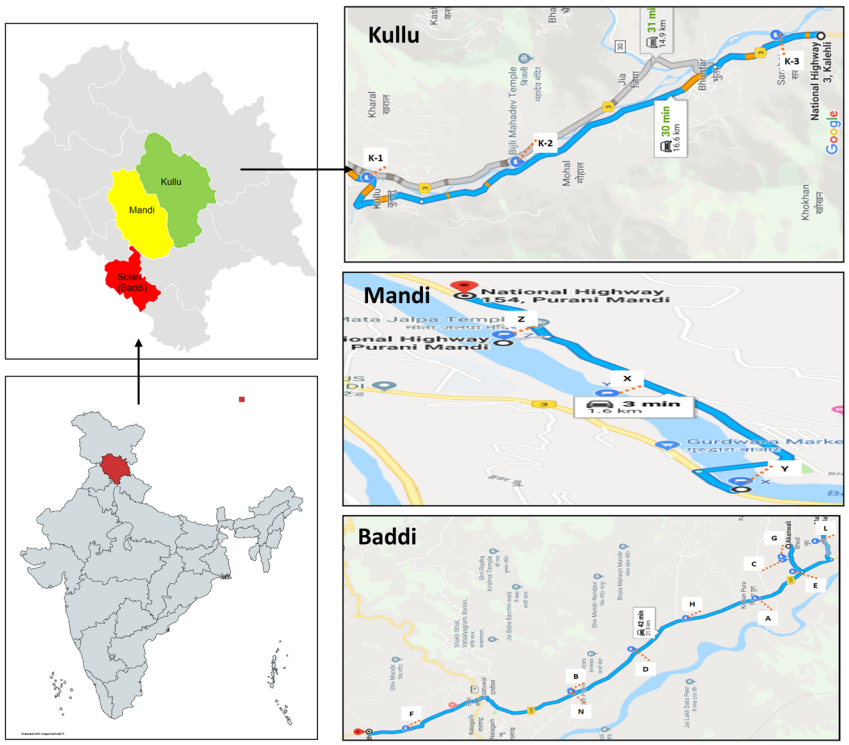
Screening of colistin-resistant bacteria was done on M-lauryl sulphate agar media (Hi-Media, Mumbai, India) with 2 µg per mL colistin. Bluish-green coloured colonies on the media were *E. coli and* yellowish colonies on media were *K. pneumoniae.* We further characterized these isolates by standard biochemical tests by using Hi25™ Enterobacteriaceae Identification Kit (Hi-Media, India). Bacterial isolates were preserved in 20% glycerol stocks and stored at −80 °C.

2.3 Antimicrobial susceptibility testing

The overnight grown culture of *E. coli* and *K. pneumoniae* was adjusted to the McFarland standard of 0.5 (equal to 1.5×10^8 CFU/mL) (Hi-Media, Mumbai, India). Then, both *E. coli* and *K. pneumoniae* were subjected to antibiotic susceptibility test (AST) by E test and broth microdilution test for colistin, using Mueller Hinton media in micro-titter plate (Hi-media, Mumbai, India) (Bauer, et al. 1966: 493-6). The detection of colistin-resistant isolates by micro-broth dilution method was done as per CLSI guidelines M07-A9 [14] The *E. coli* (ATCC25922) (Pan sensitive) was taken as negative control [15]. ESBL's presence was confirmed by a double-disc synergy test utilizing ceftazidime (30 µg) and a mix of ceftazidime (30 µg) and clavulanic acid (10 µg) as per standard guideline [16]. Phenotypic detection of Metallo beta-lactamases was done by using Imipenem with & without EDTA Ezy MIC™ Strips test (IPM+EDTA/IPM (Hi-Media India).

2.4 Molecular characterization by PCR

Overnight culture of colistin-resistant *E. coli* and *Klebsiella* was centrifuged at 10,000 × g for 5 min. DNA was extracted from pellet using PureLink® Genomic DNA Kit (Invitrogen,



Code	Geographical coordinates
A	30.963400972204436, 76.76077928462149
B	31.015551839837524, 76.71984703981774
C	30.955784839384542, 76.77763620913629
D	30.998469485231734, 76.73820967678421
E	30.952837411002033, 76.77262171836904
F	31.062078740680583, 76.70314708466837
G	30.956090,76.779096
H	30.983000,76.751984
I	31.151271828642667, 76.66962573499463
K-1	31.958965236315233, 77.11565189473602
K-2	31.92524405473658, 77.12024375698051
K-3	31.86639878646084, 77.15729439967664
X	31.699693075648923, 76.94388848840289
Y	31.704739789398786, 76.9411524793996
Z	31.702655874238253, 76.94673600398318
L	30.946457559075053, 76.78594066194685
M	30.793643374457787, 76.91388180617997
N	31.01541216754136, 76.71935432637663

Fig. 1 Water sample collection sites and their geographical coordinates

CA, USA). The DNA integrity was confirmed by 1% agarose gel electrophoresis and quantitated using Nano-Drop 2000 (Thermo Fisher Scientific, MA, USA) at 260 and 280 nm. Primers for bla_{NDM-1}, bla_{TEM}, bla_{SHV}, bla_{CTXM} and *mcr1–5*, bla_{CTXM-1}, bla_{CTXM-2}, bla_{CTXM-8} and $bla_{CTXM-15}$ were used; the sequence of these primers is given in Supplementary File: Table S1. Polymerase chain reaction (PCR) was performed using Master Cycler Gradient (Bio-Rad, USA) and amplified products were analysed on 1.5% agarose gel electrophoresis (Figure 2a, b) stained with EtBr and analysed on gel doc Bio-Plex 200TM (Bio-Rad, USA).

2.5 Sanger sequencing and sequence alignment

Sanger sequencing was performed by using an ABI 3730xl DNA Analyzer (Applied Biosystems, India) at the Eurofins Genomics India Pvt. Ltd, Bengaluru. Bio-edit software was used for sequence alignment and cluster analysis; 100% similarity with referral gene was deemed as gene match. Mega software was used for the construction of the phylogenetic tree.

2.6 Biofilm assay

Biofilm assay was performed as previously published protocol [17]. Biofilm assay was performed in 3 isolates: HG4 (environmental isolate), positive control of colistin-resistant isolate (Gifted by Prof. Kashi Nath Prasad, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow) and sensitive strain *E. coli* ATCC25922.

2.7 Survivability assay on a dry cotton swab

Survivability assay was done as our previously published protocol [18].

2.8 Fitness cost

The persistence of an ARG mutation in bacterial populations is determined in part by its fitness; mutations with little or no fitness cost are considered to be more likely to persist if there is no antibiotic selection pressure. The fitness cost of hVISA isolates was studied by comparing the length of the lag phase during the growth curve [18]. The lag phase duration was taken to be the beginning of the maximum growth rate.

3 Results

Water samples were collected from the low pharma waste region (Lower Himalayas) to the high pharma waste region (plains). Total, 73 bacterial strains were identified on the M-lauryl sulphate agar medium. Out

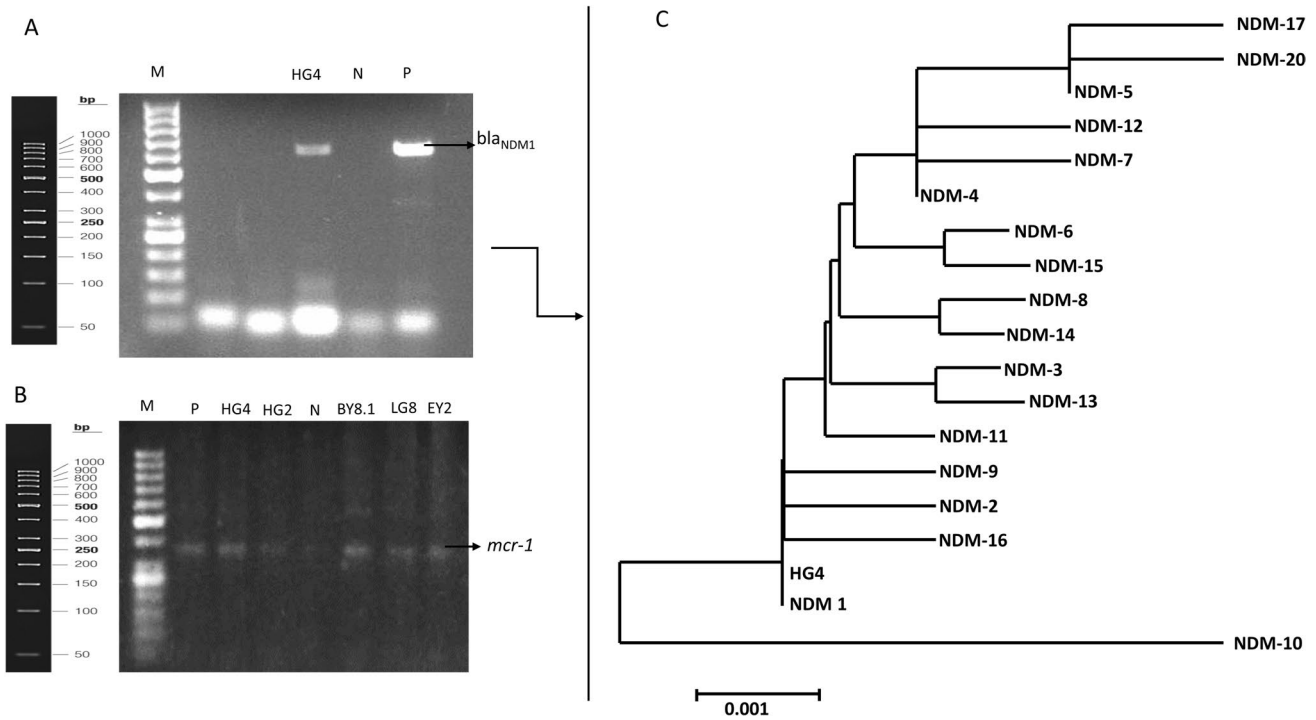


Fig. 2 **a** Gel Image of NDM-1 Positive HG4 isolate (M: 50 bp ladder, HG4 sample, P positive control, N negative control. **b** Gel Image of *mcr-1* positive 5 isolates (M 50 bp ladder, P positive control, HG4, HG2, BY8.1, LG8, EY2 *mcr-1* positive isolates, N negative control). **c** The evolutionary history was inferred using the neighbour-joining method. The optimal tree with the sum of branch length = 0.02409398 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The analysis involved 19 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 813 positions in the final dataset. Evolutionary analyses were conducted in MEGA6

of 73 colistin-resistant isolates, 34 were *E. coli* and 39 were *K. pneumoniae*. Five isolates: 2/34 (5.8%) *E. coli* and 3/39 (7.6%) *K. pneumoniae* were *mcr-1* positive, but most interestingly, one *E. coli* had both bla_{NDM-1} and *mcr-1* gene. The E test result showed 11 (32.35%) *E. coli* and 13 (33.33%) *K. pneumoniae* had MIC more than 2µg/ml (Table 1). Similarly, BMD (broth microdilution assay) result showed 8 (23.5%) of *E. coli* and 12 (30.7%) *K. pneumoniae* have MIC more than 2µg/ml (Table 1). No colistin-resistant *E. coli* or *K. pneumoniae* were found in Kullu and Manali valley and underground water (Near Baddi) during the screen (Table 2). ESBL detection kit showed a total of 14.7% (5/34) *E. coli* and 38.46% (15/39) *K. pneumoniae* were ESBL positive phenotypically (Table 3). The molecular analysis for the *mcr-1* gene by PCR showed 5 isolates: 2 (5.8%) *E. coli* and 3 (7.6%) *K. pneumoniae* were *mcr-1* positive (Table 4). In this study, we noticed the presence of both *mcr-1* and NDM-1 in an isolate of *E. coli* (HG4); this was further confirmed by DNA sequencing and the sequence of NDM-1 was submitted to the gene bank (accession number **MT367572**). This is the first report from Himachal Pradesh where an environmental sample has both *mcr-1* and NDM-1 positive gene in an isolate. The antibiotic sensitivity pattern of the HG4 isolate showed that this is resistant to all selected antibiotics except meropenem (Table 4). Out of 34 isolates, 2 (5.8%), 2 (5.8%), 5 (14.7%), 5 (14.7%) and 4 (11.76%) *E. coli* were bla_{TEM}, bla_{SHV}, bla_{CTXM-1}, bla_{CTXM-2} and $bla_{CTXM-15}$ positive, respectively. Among 39 *K. pneumoniae*, 15 (38.4%), 6 (15.3%), 10 (25.6%), 9 (23.07%) and 10 (25.6%) were bla_{TEM}, bla_{SHV}, bla_{CTXM-1}, bla_{CTXM-2} and $bla_{CTXM-15}$ positive, respectively (Table 4).

Phylogenetic analysis of NDM sequences showed maximum similarity with NDM type 1 isolates by using MEGA6 software (Fig. 2c). Fitness cost result showed in the presence with half MIC of Col^R HG4 isolate have a higher lag phase (3 hours) compared to the control strain (1 hour) (Fig. 4a). Survivability study showed environmental isolate Col^R HG4 has lesser survivability (14 days) on dry cotton swab compared to positive (28 days) and negative control (35 days) (Fig. 4b). Results of biofilm assay also showed HG4 has laser biofilm production

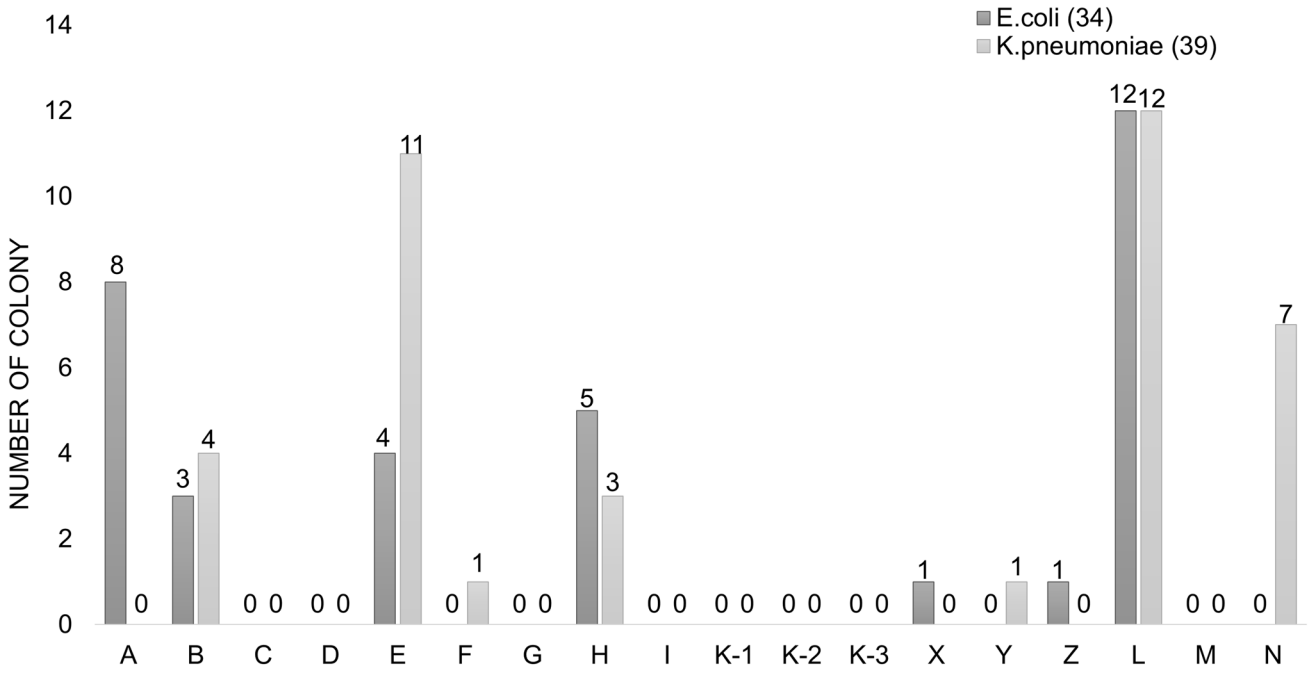


Fig. 3 Distribution of *E. coli* and *K. pneumoniae* strain based on bacterial growth on M-lauryl sulphate selective media in different samples

Table 1 Minimum Inhibitory Concentration of Colistin against *E. coli* and *K. pneumoniae* by *E. Test* and broth microdilution method (BMD)

Colistin MIC result					
E. Test			BMD		
MIC (µg/ml)	*E. coli*	*K. pneumoniae*	MIC (µg/ml)	*E. coli*	*K. pneumoniae*
≤ 2	23	26	≤ 2	26	27
> 2	11	13	> 2	8	12

Table 2 Antibiotic Sensitivity Pattern of HG4, *Escherichia coli* ATCC25922, positive control

Antibiotic	HG4	*E. coli* ATCC	Positive control
Cefoxitin	13 (R)	24 (S)	11 (R)
Ceftazidime	14 (R)	28 (S)	12 (R)
Cefepime	12 (R)	24 (S)	11 (R)
Cefoperazone/sulbactam	64 (R)	0.5 (S)	64 (R)
Imipenem	4 (R)	0.03 (S)	12 (R)
Meropenem	24 (S)	29 (S)	12 (R)

R Resistant, S Sensitive

Table 3 ESBL producers

ESBL producers		
Strain	ESBL positive	ESBL negative
Escherichia coli (34)	5 (14.7%)	29 (85.29%)
Klebsiella pneumoniae (39)	15 (38.46%)	24 (61.53%)

Data presented in total no/%

compared to colistin-sensitive *E. coli* ATCC 25922 strain (Fig. 4c).

4 Discussion

The presence of (ARBs) in natural water sources is a matter of serious concern for a healthy community as they may transfer ARGs to human commensals [19, 20]. The presence of ARBs and ARGs in water sources, water treatment plants and drinking water distribution systems and their effects on community and environment have been previously reported [21, 22]. The discharge of pharmaceuticals waste (mainly antibiotics) in natural water sources creates a selection pressure for the spread of antibiotic resistance [23]. The overuse of antibiotics

Table 4 Genotypic characterization

Genotypic characterization									
Strain	*mcr-1 positive*	*bla*NDM-1 *positive*	*bla*TEM positive	*bla*SHV positive	*bla*CTXM positive	*bla*CTXM-1 positive	*bla*CTXM-2 positive	*bla*CTXM-8 positive	*bla*CTXM-15 positive
Escherichia coli (34)	2 (5.8%)	1 (2.9%)	2 (5.8%)	2 (5.8%)	0	5 (14.7%)	5 (14.7)	0	4 (11.76%)
Klebsiella pneumoniae (39)	3 (7.6%)	0	15(38.4%)	6 (15.3)	0	10 (25.6%)	9 (23.07%)	0	10 (25.6%)

Data presented in total no/%

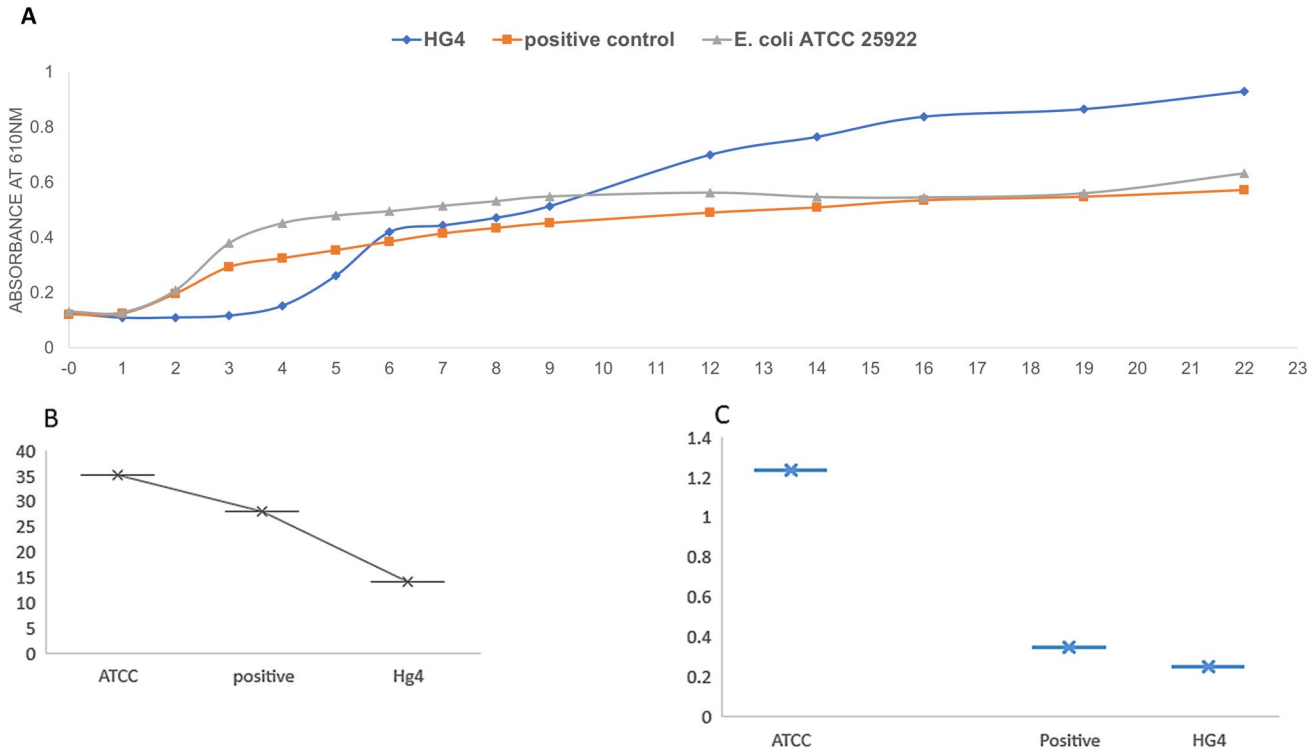


Fig. 4 **a** Growth kinetics of HG4, positive control and *E. coli* ATCC 25,922 sensitive strain to check the lag phase duration. **b** Box and whisker graph shows survivability of Hg4, positive control and *E. coli* ATCC 21,922 sensitive strain (survivability is given in number of days). **c** Biofilm production by HG4, positive control and *E. coli* ATCC 25,922 sensitive strain

among livestock animals also favours the distribution of antibiotic resistance in the aquatic environment [24, 25]. In this study, we have reported the first case of colistin-resistant *E. coli* with *mcr-1* and bla_{NDM-1} gene from the water samples of Himachal Pradesh, India. Himachal Pradesh is situated in the Western Himalaya part of India, and tourism and agriculture are two important constituents of this state's economy. Baddi is the biggest and only industrial town in this state (Fig. 3).

The *mcr-1* and NDM-1 genes are mostly located on plasmids of bacteria and can be easily transferred to sensitive strains by horizontal transfer of genes. The presence of such superbugs (Col^R HG4) in environment is of serious concern for the general community, agriculture and human health. A 2013 study described the presence of antimicrobial resistance microbes as "serious threats to the environment" to low-labour cost Asian countries like India, Bangladesh, China and Pakistan. Most of these manufacturing sites did not follow standard environmental regulation and guidelines, and effluent is directly discharged into the domestic sewage network without any prior treatment, thereby exposing microbes to drugs and drug-resistant microbes to humans and animals subsequently [26]. In this study, we have reported that most

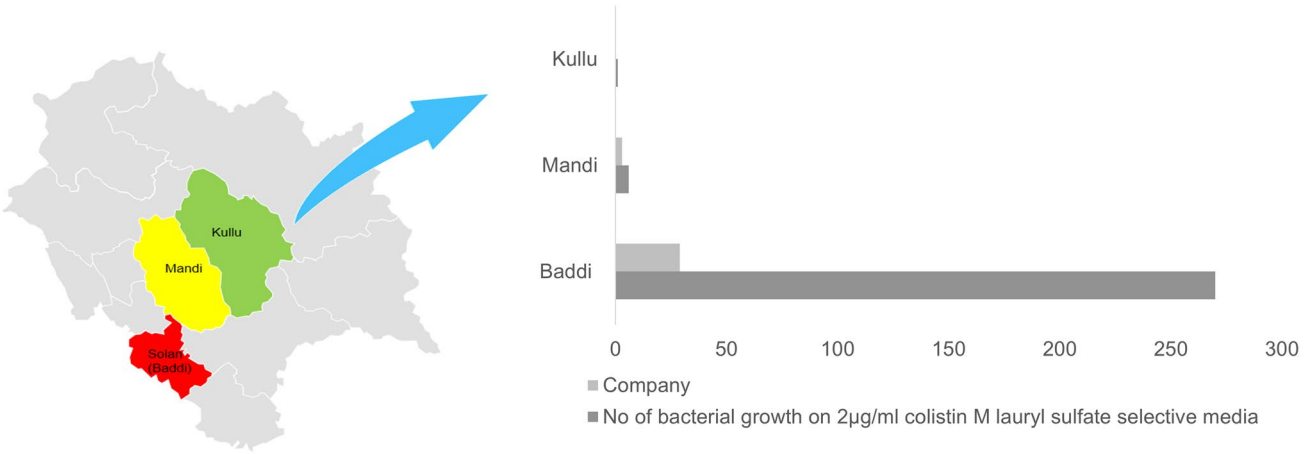


Fig. 5 Impact of pharma companies waste on colistin resistance in water. Maximum phenotypic colistin resistance observed in Baddi followed by Mandi. One NDM-1 and *mcr-1* positive isolate found by PCR

of the colistin-resistant isolates and NDM-1 and *mcr-1* positive *E. coli* isolates were found to be from Baddi area, compared to other non-industrial sites (Kullu and Mandi) (Fig. 5). Probably, significant quantitative levels of antibiotics released from manufacturing units, that combine with runoff from farms, human wastewater, give an ideal rearing ground for drug-resistant bacteria. Bacteria in these environments are ready to share or exchange genetic material within intra- or inter-species.

The genotypic characterization showed Col^{R}HG4 isolate was $bla_{\text{NDM-1}}$, bla_{TEM}, bla_{SHV} and *mcr-1* positive. The Sanger sequencing and sequence alignment further confirmed that this isolate was $bla_{\text{NDM-1}}$. The presence of *mcr-1* positive isolate in animal and human-borne Enterobacteriaceae with co-occurrence of other resistance genes such as ESBL and MBL has been reported worldwide [10, 27, 28]. Like our study, a report from Switzerland showed co-occurrence of *mcr-1* gene with ESBLs in Enterobacteriaceae from rivers and lakes [29]. In 2017, another report from China had identified 23 *mcr-1* positive isolates from the environmental waters in Hangzhou, China, which is another industrial city [30]. Similarly, in 2018, bla_{KPC}, bla_{NDM} and *vanA* gene along with 18 *mcr-1* positive strains and six *mcr-3* positive strains were found from Fauna River in China. They concluded it might be due to the urban activities and presence of antibiotic-resistant bacteria in water [31]. In another study, the first case of the colistin-resistant *mcr-1* gene in two *E. coli* strain from seawater of Algerian coasts was reported by Drali R. et al. 2018 [32]. Noteworthy, ARGs such as bla_{CTXM}, $bla_{\text{NDM-1}}$ and the *mcr-1* genes that provide resistance to broad-spectrum antibiotics, including last-resort agents, were reported in all major rivers of India [33, 34]. But it was not reported from this geographical region of India.

Biofilm formation is one of the most important virulence factors exhibited by *E. coli* among other virulence factors. In this study, we found Col^{R}HG4 as less biofilm former than Col^{S} *E. coli* ATCC 25922. This suggests that the Col^{S}-type *E. coli* tend to form more biofilm than resistant ones. In 2016, Qi et al. studied the relationship between biofilm production and resistance among *Acinetobacter baumannii* and they also found that MDR and XDR isolates tend to form thin biofilms than non-MDR strains [35]. However, in contrast to our results, other previous studies had shown a direct relationship between antimicrobial resistance and biofilm production [36].

The HG4 also has less survivability than the clinical isolates and Col^{S} *E. coli* ATCC 25922, which suggests that the presence of resistance gene in resistant strain may lead to less survival. Similar to our study, Pettibone et al. also observed antibiotic sensitive bacteria can survive longer than resistant bacteria [36]. The growth kinetic analysis showed a higher lag phase of Col^{R}HG4 (3 hours) compared to Col^{S} *E. coli* ATCC 25922(1 hour). This result indicates fitness cost associated with Col^{R} HG4 isolate but for a conclusion validation study on more samples is required.

5 Conclusions

In conclusion, incidences of $bla_{\text{NDM-1}}$ and *mcr-1* isolate in single bacteria in this geographical region are an alarming signal for tourists, the community, health workers and policymakers. Hence, it is utmost important to think about strict regulation and monitoring of pharma waste management in such an industrial area. Further research is needed on a bigger sample size to know the relationship between anthropogenic activities and antibiotic resistance and the underlying genetic basis of colistin resistance and

the accompanying biological features including fitness, growth rate, biofilm formation ability and other virulence characteristics.

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Declarations

Conflict of interest None of the funders has any role in deciding/influencing the outcome of this study.

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