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A Cross Sectional Study on Prevalence of Antibiotic Resistance and Role of Efflux Pumps in Fluoroquinolone Resistance by using Efflux Pump Inhibitors in Isolated Cultures from Poultry, Dairy Farms and MTCC Strains from Reservoirs

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Authors' contributions

This work was carried out in collaboration between all authors. Author SD designed the study. Author LS performed the experiments, wrote the protocol and wrote the first and final draft of manuscript. Authors PB, SB and AM managed the analyses of the study. Authors PS and RK managed the literature searches. Author SD read and approved the final manuscript.

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ABSTRACT

Aims: Emergence of antibiotic resistance in bacterial strains has always remained a crucial concern. Mutations in antibiotic target sites, over expression of efflux pump are the major modes of development of bacterial antibiotic resistances. The present study was conducted to determine antibiotic resistance and role of efflux pumps in fluoroquinolone resistance by using efflux pump

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inhibitors.
Place and Duration of Study: The Research was conducted during June 2011 to March 2012 at Department of Biotechnology, Kurukshetra University, Kurukshetra, Haryana – India.
Methodology: Out of 57 bacterial strains 19 were procured from collection centres (reservoirs) and 38 were isolated from dairy (n = 10) and poultry farms (n = 28) and screened against 12 antibiotics of different groups by well assay. Further, fluoroquinolone sensitive/resistant strains were tested to observe the decline in minimum inhibitory concentration (MIC) levels in the presence/absence of efflux pump inhibitors.
Results: Antibiotic resistance in tested strains was higher against nitrofurantoin, lincomycin, cefixime and chloramphenicol. Majority of the bacterial strains (94.74%) showed resistance to two or more antibiotics. Isolated bacterial strains were exhibiting more antibiotic resistance than reservoir strains indicating their exposure to antibiotics. Reduction in MIC (2-4 folds) was observed when Piperine (28.07%) or Plumbagin (19.29%) was used in combination with fluoroquinolones. The findings emphasized that majority of efflux pump inhibitors are active against Gram-positive bacteria. Overexpression of efflux pump and higher antibiotic resistance was also observed in subgroup III exhibiting resistance to combination of cefixime/nitrofurantoin.
Conclusion: Efflux mediated resistance appears to contribute significantly to fluoroquinolone resistance and multidrug resistance in organisms, which may be due to involvement of active efflux pumps of both MFS and RND Family.

Keywords: Antibiotic; resistance; Efflux pump inhibitors; fluoroquinolones; MIC.

ABBREVIATIONS

MIC: Minimum inhibitory concentration; EPI: Efflux pump inhibitor; CFU: Colony forming unit ℃: Degree Celsius; h: Hour; DMSO: Dimethyl sulfoxide.

1. INTRODUCTION

Infectious diseases caused by microorganisms are causing approximately 17 million deaths each year, worldwide [1]. Recently, WHO report showed that in non-industrialized countries, 45% of adult deaths as well as 63% of early deaths in children deaths are caused by infectious diseases. Emergence of such new, rare or known infectious diseases has stimulated interest to develop new drugs against antibiotic resistant strains [2]. Various socio-medical factors (non-judicious, improper and misuse of antibiotics) contributes high in generating these antibiotic resistant bacterial strains. Besides direct consumption, agricultural practices account for over 60% antibiotic usage [3-5].

Microbes are attaining antibiotic-resistance, which present a challenge to researchers and threat to patients. Bacteria have evolved several mechanisms to acquire antibiotic resistance. Antibiotic-resistance has also been enhanced by mutation, clonal evolution, and horizontal gene or plasmid transfer [6-9]. The bacterial strains differ in their resistance to antibiotics by various modes such as, metabolic pathway alteration, target site alteration of ribosome, efflux pumps and enzymatic cleavage of antibiotics (β-lactams, aminoglycosides, chloramphenicol) [10-15].

Among these mechanisms, resistance in bacteria acquired by efflux pump remains crucial. Efflux pumps play a vital role in the development of multidrug resistance as they export different substances including various types of antibiotics and chemicals such as dyes, organic solvents and detergents, molecules needed for the cell-cell communication, biocides and metabolic products [16-18]. In addition, efflux pumps are found to export several unrelated substances including molecules produced by the host organism (such as bile), indicating that these systems also have a role in allowing bacteria to survive in their ecological niche. Many of the efflux pumps are of clinical relevance because they can render bacterial infection untreatable by the agent(s) of choice. The genetic component of efflux pumps reside on chromosomes or on transmissible genetic elements, *i.e.* plasmids. Efflux pumps are classified on the basis of the number of components that the pump has (single or multiple) , the number of transmembrane-spanning regions that the transporter protein has, the energy source that the pump uses and the types of substrate that the pump exports. Antibiotics may act as inducers and regulators of the expression of some efflux pumps. A single efflux pump can confer resistance to a wide range of antimicrobials. There are five families of efflux pump proteins that are associated with

multi drug resistance (MDR): the ATP binding cassette (ABC) super family, the major facilitator super family (MFS), the multidrug and toxic-compound extrusion (MATE) family, the small multidrug resistance (SMR) family and the resistance nodulation division (RND) family. A single organism can express more than one type of MDR efflux pumps which may belong to the same family or different families of efflux pumps. Such type of expression can be found in organisms such as *Pseudomonas aeruginosa* which can express more than one type of Mex efflux pump, and *Escherichia coli* that can express more than one type of Acr Efflux pump (both of which are pumps belonging to the RND family). For Gram negative bacteria, the efflux pumps that are associated with clinically significant resistance to drugs belong to the RND family, whereas for Gram positive bacteria, the clinically significant efflux pumps are members of the MFS [19]. Over expression of the *Staphylococcus aureus* MFS efflux pump NorA confers resistance to fluoroquinolones, even though the fluoroquinolone ciprofloxacin is a substrate of NorA [20-23]. The present study was aimed to check the prevalence of bacterial resistance to various classes of antibiotics alone or in combination among random samples of bacteria, the role of efflux pumps in fluoroquinolone resistance by using efflux pump inhibitors and comparing the results obtained with reference to isolated and reservoir bacterial strains.

2. MATERIALS AND METHODS

2.1 Bacterial Strains Tested

A total of 57 bacterial strains from various sources were tested in this study. Thirteen Gram-positive {*Bacillus cereus* (MTCC 430), *Bacillus polymyxa* (NCDC 68), *Bacillus pumilus* (MTCC 7411), *Bacillus stearothermophilus* (MTCC 8505), *Bacillus subtilis* (MTCC 8509 and MTCC 121), *Lactobacillus brevis* (NCDC 371), *Lactobacillus plantarum* (NCDC 20), *Staphylococcus aureus* (MTCC 3160 and MTCC 109), *Staphylococcus epidermidis* (MTCC 3086 and MTCC 435), *Staphylococcus hominis* (MTCC 4435)} and Six Gram-negative {*Escherichia coli* (MTCC 1885), *Klebsiella pneumoniae* (MTCC 4030), *Pediococcus acidilactici* (NCDC 252), *Proteus vulgaris* (MTCC 426), *Pseudomonas aeruginosa* (MTCC 424 and MTCC 7453)} bacterial strains were procured from Microbial Type Culture Collection Institute of Microbial Technology, Chandigarh (MTCC) and National Dairy Research Institute, Karnal (NCDC) India, and were termed as reservoir bacterial strains. The remaining 38 bacterial strains were isolated from various nasal samples of chicken and milk samples of buffaloes and were termed as isolated bacterial strains.

2.2 Isolation and Identification of Bacteria

The different bacterial strains were isolated and cultured by standard methods of NCCLS [24,25] using spreading and streaking technique. Selective media like Baird Parker agar and Slanetz and Bartley Agar media (Hi-media Pvt. Ltd. Mumbai, India) were used to isolate *Staphylococcus* sp. and *Enterococcus* sp. respectively from the nasal samples of chicken and milk samples of buffaloes. The bacterial strains were grown on respective media for 48 to 72h at 37°C. The bacterial isolates were further identified on the basis of their morphological and biochemical characteristics [26]. MTCC and NCDC samples were cultured on Nutrient agar media at 37°C for 24h.

2.3 Susceptibility Test

All the fifty seven bacterial strains from various sources were screened against the twelve antibiotics of different classes acting on different sites of bacteria. The antibiotics belonging to different classes used in this study were amikacin (AMK), ampicillin (AMP), cefixime (CFM), chloramphenicol (CHL), ciprofloxacin (CIP), erythromycin (ERY), lincomycin (LCM), nitrofurantoin (NIT), norfloxacin (NOR), ofloxacin (OFX), tetracycline (TET), trimethoprim (TMP). Bacterial strains were tested against all these antibiotics (10µg/mL in 10% DMSO) belonging to different classes. Susceptibility of antibiotics was tested at 10µg/mL as according to CLSI Performance Standard for Antimicrobial Susceptibility Testing, using the modified Kirby–Bauer diffusion technique [27]. One hundred microlitre (100µL) of the inoculum of tested organism (1.5×10^6 CFU/mL) were cultured on nutrient agar by using spread plate technique and wells of 8mm diameter were made for loading the antibiotics. Each of the bored wells was filled with 50µL of antibiotics. The plates were allowed to stand for 1 h at room temperature for diffusion of the antibiotic into agar and incubated at 37°C for 24h [28]. Sterile DMSO (10%) served as the negative control. The diameter of the zones of inhibition produced were measured and interpreted using the CLSI zone

diameter interpretative standards [29]. The tests were conducted in triplicate.

2.4 Prevalence of Efflux Pumps in Bacterial Samples

2.4.1 EPI Inhibitors

Efflux Pump Inhibitors (EPI) are compounds that have ability to reduce MIC (2 to 8 times) or reverse antibiotic resistance [30]. Piperine and Plumbagin are reported EPIs of the antibiotics ciprofloxacin and ofloxacin respectively [31,32]. Piperine was purchased from Natural remedies Pvt. Ltd. Bangalore. Plumbagin was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.4.2 Minimum Inhibitory Concentration (MIC) determination

MIC of 57 bacterial strains against ciprofloxacin and ofloxacin (10µg/mL in 10% DMSO) was determined by micro dilution technique using 96-well microtiter plates as described by the National Committee for Clinical Laboratories standards [24]. The MIC of antibiotics was determined by making serial dilution of the antibiotics. The final inoculum of 10^6 CFU/mL was prepared in 5 ml nutrient broth. Positive controls were without the antibiotics. Tubes were incubated at 37ºC for 24h. The activity was measured as a function of turbidity at 660 nm. Lack of turbidity was further confirmed by pouring suspension aliquot of 0.1mL into pre-sterilized Petri dishes with respective medium [24]. The tests were conducted in triplicate. The MIC is defined as the lowest concentration of compound that inhibits visible growth, MIC breakpoint for most of the antibiotics is less than 10µg/mL. [29].

2.4.3 Effect of EPI on MIC levels of antibiotics

To determine the extent of the efflux pump mediated antibiotic resistance in various bacterial strains, MIC levels for ciprofloxacin and ofloxacin were determined in the presence and absence of Piperine (30µg/mL in 10% DMSO) and Plumbagin (30µg/mL in10% DMSO) respectively [30]. Potentiation for efflux pump inhibition experiment was performed by testing synergism of EPIs (Piperine and Plumbagin) with ciprofloxacin and ofloxacin respectively against bacterial strains. [50µL of Piperine/Plumbagin (30µg/mL) + 50µL of Ciprofloxacin/Ofloxacin at its MIC, 1/2 MIC, 1/4 MIC]. Per cent Efflux Pump prevalence was determined.

2.5 Statistical Analysis

Frequencies and proportions of strains resistant to the different classes of antibiotics were calculated.

Antibiotic resistance % = Number of resistant bacteria/Total number of bacteria × 100

% Efflux prevalence=Number of bacterial strains in which MIC was declined in presence of EPI/Total number of bacterial strains × 100

Prevalence of efflux pump overexpression, cross-resistance, and magnitude of MIC decrease with EPI were compared by chi-square.

3. RESULTS

3.1 Isolation and Identification of Bacteria

Morphologically distinct colonies of *Staphylococcus* sp. as black coloured colonies were obtained on Baird parker agar media. Mehroon coloured cocci of *Enterococcus* sp. were distinctly obtained on Slanetz and Bartley Agar media. The isolated *Staphylococcus* sp. were confirmed by positive catalase test. A total of 38 bacterial strains were isolated from various nasal samples of chicken and milk samples of buffaloes. Out of these 38 strains, eleven strains of *Staphylococcus* sp. (SA-1 to SA-10, SA-21) and seventeen strains of *Enterococcus* sp. (E-1 to E-17) were isolated from chicken nasal samples. Ten strains of *Staphylococcus* sp. (SA-11 to SA-20) were obtained from buffalo milk samples.

3.2 Resistance to Single and Multiple Antibiotics

Only, 8.77% (5/57) strains were resistant to all tested antibiotics and not a single bacterial strain was sensitive to any antibiotic, which supports the recent trend of increasing antibiotic resistance among different bacteria. The studied strains showed high resistance to nitrofurantoin (98.25%), cefixime (96.49%), lincomycin (94.74%), chloramphenicol (89.47%), trimethoprim (85.96%) and norfloxacin (84.21%). Resistance to multiple antibiotics was very common with lincomycin and nitrofurantoin (94.74%), cefixime and nitrofurantoin (92.98%). Additionally, 82.45% bacteria showed multiple resistance to cefixime, chloramphenicol and lincomycin. Similar, percentage was shown with cefixime, chloramphenicol, lincomycin and nitrofurantoin. While 80.70% were resistant to

combination of cefixime, chloramphenicol, lincomycin, nitrofurantoin and norfloxacin. Combined resistance to nitrofurantoin, cefixime, lincomycin, chloramphenicol, trimethoprim and norfloxacin was found in 31.58% bacterial strains. The detailed explanation of antibiotic resistance are presented in Table 1.

Among MTCC bacterial strains, only 42.10% possessed resistance against 7 to 12 antibiotics while among isolated bacterial strains the percentage was found to be 89.47%, indicating that exposure to antibiotics in cattle and poultry lead to more multidrug resistance as compared to the old MTCC bacterial strains from reservoirs.

3.3 Effect of EPIs on Antibiotic MICs

MICs of antibiotics determined in presence and absence of efflux pump inhibitors and the results were compared. Two fold or more reduction in MIC levels in the presence of EPIs was considered as an indication of active efflux pumps in antibiotic resistant bacterial strains [30]. It was observed that the MIC levels of ciprofloxacin were lowered in 16 of 57 bacteria in the presence of Piperine (Table 2). The results indicate that 28.07% of bacterial strains attained antibiotic resistance due to active efflux pump of ciprofloxacin. A total of 16 bacterial strains declined the ciprofloxacin MIC to two fold out of which 11 were Gram-positive and 5 were Gram-negative.

Similarly, MIC levels of ofloxacin were declined in 11 of 57 strains in the presence of Plumbagin (Table 2). In the presence of Plumbagin the MIC values for ofloxacin were found to decrease up to 4 fold in one strain whereas the decrease was only upto 2 folds for the remaining 10 strains. Out of 11 bacterial strains, 10 were Gram-positive and 1 was Gram-negative.

MTCC bacterial strains have high prevalence (75%) of efflux pump activity towards ciprofloxacin as compared to isolated bacterial strains. Similar results were observed with the efflux pump of ofloxacin but with a lower percentage (45.45%). Overall, the efflux pump over expressed phenotype in fluoroquinolones (ciprofloxacin and ofloxacin) was observed in a significantly greater number (62.5%) in MTCC bacterial strains than isolated bacterial strains (37.5%).

The magnitude of MIC didn't vary much and the range of MIC in case of ofloxacin-Plumbagin combination was 5 to 2.5µg/mL (two fold) except in one from 2.5 to 0.625µg/mL (four fold) while in case of ciprofloxacin-Piperine combination MIC was declined to half (Table 3).

Table 1. Prevalence and percent of antibiotic resistance among 57 bacterial strains

Antibiotics	Percentage resistance% (Number of resistant bacterial strains)
NIT	98.25 (56)
LCM	96.49 (55)
CFM	94.74 (54)
CHL	89.47 (51)
TMP	85.96 (49)
NOR	84.21 (48)
ERY	57.89 (33)
TET	45.61 (26)
CIP	42.11 (24)
OFX	33.33 (19)
AMP	31.58 (18)
AMK	24.56 (14)
LCM + NIT	94.74 (54)
CFM + NIT	92.98 (53)
CFM + LCM & CHL + NIT*	89.47 (51)
CHL + LCM	87.71 (50)
CFM + CHL & LCM + NOR & NIT + NOR*	84.21 (48)
CFM + NOR & CHL + NOR*	82.45 (47)
CFM + TMP & NIT + TMP*	33.33 (19)
CHL + TMP & LCM + TMP & NOR + TMP*	31.58 (18)
CFM + CHL + LCM & CFM + CHL + LCM + NIT*	82.45 (47)
CFM + CHL + LCM + NIT + NOR	80.70 (46)
CFM + CHL + LCM + NIT + NOR + TMP	31.58 (18)

**Combinations (two or three) in the same column indicating similar percentage of resistance*

Table 2. Decline in MIC among 57 bacterial strains in presence of efflux inhibitors

Efflux inhibitors	No. of bacterial strains (%)	
	2 fold MIC decline	4 fold MIC decline
Piperine (ciprofloxacin)	16 (28.07%)	-
Plumbagin (ofloxacin)	10 (17.54%)	1(1.75%)

Table 3. List of bacterial strains possessing decline in MIC of ciprofloxacin and ofloxacin in presence of piperine and plumbagin respectively

Sr. No.	Bacterial strains	Ci MIC (µg/mL)	Decline in Ci MIC(µg/mL) Ci+Piperine	Of MIC (µg/mL)	Decline in Of MIC(µg/mL) Of+Plumbagin
1	*Bacillus cereus* (MTCC 430)	5	2.5	NE	NE
2	*Bacillus polymyxa* (NCDC 68)	NE	NE	5	2.5
3	*Bacillus stearothermophilus* (MTCC 8505)	2.5	1.25	NE	NE
4	*Bacillus subtilis* (MTCC 8509)	NE	NE	5	2.5
5	*Bacillus subtilis* (MTCC 121)	5	2.5	5	2.5
6	*Staphylococcus aureus* (MTCC 3160)	2.5	1.25	NE	NE
7	*Staphylococcus aureus* (MTCC 109)	5	2.5	NE	NE
8	*Staphylococcus epidermidis* (MTCC 3086)	5	2.5	NE	NE
9	*Staphylococcus epidermidis* (MTCC 435)	2.5	1.25	NE	NE
10	*Staphylococcus hominis* (MTCC 4435)	NE	NE	2.5	1.25
11	*Escherichia coli* (MTCC 1885)	2.5	1.25	5	2.5
12	*Klebsiella pneumoniae* (MTCC 4030)	1.25	0.625	NE	NE
13	*Proteus vulgaris* (MTCC 426)	0.312	0.156	NE	NE
14	*Pseudomonas aeruginosa* (MTCC 424)	1.25	0.625	NE	NE
15	*Pseudomonas aeruginosa* (MTCC 7453)	1.25	0.625	NE	NE
16	SA-2	NE	NE	5	2.5
17	SA-3	NE	NE	NE	NE
18	SA-4	NE	NE	NE	NE
19	SA-5	NE	NE	NE	NE
20	SA-6	NE	NE	NE	NE
21	SA-7	5	2.5	NE	NE
22	SA-8	NE	NE	5	2.5
23	SA-10	NE	NE	Resistant	10
24	SA-13	1.25	0.625	NE	NE
25	SA-16	2.5	1.25	5	2.5
26	SA-20	NE	NE	2.5	0.625
27	SA-21	NE	NE	5	2.5
28	E-5	5	2.5	NE	NE

SA: Staphylococcus aureus; E: Enterococcus; Ci: Ciprofloxacin; Of: Ofloxacin; NE: No effect

3.4 Subgroupings

The results of above study revealed that bacterial strains could be sub grouped into the seven categories on the basis of their combinational resistance (Table 4). We compared the subgroup results covering both objectives of our study: 1) Antibiotic resistant (AbR) prevalence, 2) Efflux pump over expression (EPO) prevalence (Table 4).

The III subgroup *i.e* CFM+NIT showed maximum antibiotic resistance prevalence (92.98%) and highest prevalence of the EPO phenotype in ciprofloxacin (75%). Similarly, the subgroup which demonstrated the highest prevalence of

the EPO phenotype in ofloxacin was II subgroup (100%). Overall comparison showed that (CFM + NIT) resistant strains subgroup showed maximum antibiotic resistance prevalence and EPO prevalence. The ciprofloxacin Efflux pump over expression (EPO) strains possessed resistance against antibiotics like cefixime and nitrofurantoin (75%), while ofloxacin EPO, possessed resistance against cefixime and lincomycin in all eleven strains (Table 4).

4. DISCUSSION

Antibiotic-resistance in microorganisms acquired by active efflux pumps is key target for novel drug researchers. Surveillances on such aspects provide the vital information. The findings of present study have revealed the prevalence of resistance (100%) to antibiotics studied *e.g.* chloramphenicol, quinolones, nitrofurans, lincosamides, etc. (Table 1). The present study showed an alarming resistance potential against nitrofurantoin (98.25%) and a recent, third generation antibiotic of cephalosporin class *i.e.* cefixime (94.74%). Resistance against two or more of antibiotics (LCM+NIT) which was observed in this study was higher than other earlier investigations conducted by Addis [33] who reported 83.3% resistance in *Salmonella* isolated from lactating cows. 29.82% bacterial strains were possessing resistance against fluoroquinolones (ciprofloxacin, norfloxacin and ofloxacin) in the present study. MTCC bacterial samples showed lower antibiotic resistance prevalence as they are old samples preserved in reservoirs and not recently exposed to antibiotic doses, which might explain the lower antibiotic resistance prevalence. Antibiotics are routinely used along with growth promoters in poultry and cattle feed [34,35]. The increased prevalence of antibiotic resistance as a result of individual antibiotic use highlight the increasing threat posed by antibiotic resistance and suggest further evidence for the need towards a commitment to ensure antibiotics are used in a rational manner [4].

Efflux mechanisms have become recently recognized as major components of resistance to many classes of antibiotics. Many of the efflux pumps are of clinical relevance as they can render a bacterial infection untreatable by the agent(s) of choice [19].

Data from the present study revealed that the MICs of 19.29 % and 28.07 % bacterial strains were affected in the presence of Plumbagin and Piperine respectively suggesting the importance of efflux pumps in ofloxacin and ciprofloxacin resistant bacterial strains respectively. Among all studied strains (except one) the MIC decline potential was only up to half fold with Piperine and Plumbagin. Overall inhibitory effect of efflux inhibitors on fluoroquinolones MICs was observed in 42.10% bacterial strains showing a good contribution of active efflux pumps in the development of fluoroquinolones resistance in the tested strains. It is notable that many of the fluoroquinolone-resistant strains are associated with cross-resistance to structurally unrelated antimicrobial agents [36].

Table 4. Comparison of Antibiotic resistant (AbR) prevalence and Efflux Pump Over expression (EPO) prevalence within the subgroups of specific antibiotics

Sr. No	Sub groups	Antibiotic Resistant Subgroups	AbR prevalence %	EPO prevalence %	
				ciprofloxacin+ Piperine	ofloxacin+Piperine
1.	I	CFM + CHL	84.21 (48/57)	56.25 (9/16)	90.90 (10/11)
2.	II	CFM + LCM	89.47 (51/57)	68.75 (11/16)	100 (11/11)
3.	III	CFM + NIT	92.98 (53/57)	75.0 (12/16)	90.90 (10/11)
4.	IV	CFM + CHL + LCM	82.45 (47/57)	50.0 (8/16)	81.81 (9/11)
5.	V	CFM + LCM + NIT	85.96 (49/57)	56.25 (9/16)	90.90 (10/11)
6.	VI	CHL + LCM + NIT	87.71 (50/57)	56.25 (9/16)	81.81 (9/11)
7.	II	CFM + CHL + LCM + NIT	82.45 (47/57)	43.75 (7/16)	81.81 (9/11)

85.96% bacterial strains were resistant to one or more fluoroquinolones (ciprofloxacin, ofloxacin and norfloxacin) tested out of which 48.97% bacterial strains seemed to possess Major Facilitator Super Family and Resistance Nodulation Division Family efflux transporters. Reduction in MIC level in presence of Plumbagin and Piperine inhibitors provide evidence for the presence of both proton motive force and ATP dependent extrusion system involved in fluoroquinolone resistance [37].

The present study is also in accordance with the earlier reports, that the vast majority of EPI, are active against Gram-positive bacteria and particularly in *Staphylococcus* strain [32]. One of the major antibiotic resistance mechanisms utilized by more than 15 species of Gram-negative bacterial cells is the Resistance Nodulation Division efflux pump, which eliminates several classes of antibiotics such as penicillins, cephalosporins, macrolides, aminoglycosides, fluoroquinolones and tetracyclines [38].

Singh and co-workers, [37] have also reported the reversal of resistance to ofloxacin in presence of efflux pump inhibitors {(CCCP (35.5%), DNP (46.6%) and verapamil (53.3%)} in *M. tuberculosis* isolates. The present study is also in accordance to them with variable bacterial strains.

Khan et al. in 2006 [31] reported the EPI effect of piperine with ciprofloxacin in *in vitro* combination studies against *S. aureus* and suggested its role as an EPI in *S. aureus*. These result motivated the current study to use Piperine and Plumbagin as an EPI in various bacterial strains.
In the first half of the study, the isolated bacterial strains were possessing more antibiotic resistance prevalence than MTCC reservoir strains, indicating that they are more exposed to antibiotic but in the second half of the study it is vice versa in case of ciprofloxacin-Piperine combination and almost equal in case of ofloxacin-Plumbagin combination. This implies EPO is irrelevant of source and age of bacterial strains whether isolated strains or reservoir strains.

Efflux mediated resistance appears to contribute significantly to fluoroquinolone resistance and multi drug resistance in organisms, our data support the fact that increased fluoroquinolone usage can negatively impact susceptibility of organisms to multiple classes of antibiotics.

5. CONCLUSION

The findings of present study have indicated high prevalence of resistance to antibiotics studied such as chloramphenicol, quinolones, nitrofurans and lincosamides. Decline in MIC of 19.29% and 28.07% bacterial strains in the presence of Plumbagin and Piperine respectively suggest the importance of efflux pumps in ofloxacin and ciprofloxacin resistant bacterial strains. The current study suggests the wise use of antibiotics in combating alarming problem of antibiotic resistance. The data from the present study indicates that efflux pumps are a reason for antibiotic resistance. So, there is a necessity to search new EPI from natural sources. There are numerous potentially beneficial consequences of the inhibition of efflux pumps in improving the clinical performance of various antibiotics. The search of potential efflux pump inhibitors provides an approach to generate therapy by interaction between different mechanisms of resistance. This kind of approach decreases the frequency of emergence of resistant strains also. The present study offers the support for the diagnosis of a possible resistance mechanism due to active efflux pump.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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