

Original Research Article

A cross sectional study on prevalence of antibiotic resistance and efflux pump inhibitor of isolated cultures from poultry, dairy farms and MTCC strains from reservoirs

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 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 centre (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 presence/absence of efflux pump inhibitors.

Results: Antibiotic resistance in tested strains was higher against nitrofurantoin, lincomycin, cefixime and chloramphenicol. Maximum bacterial strains (94.74%) showed resistant to two or more antibiotics. Isolated bacterial strains were possessing 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 inhibitor are active against Gram-positive bacteria. Further, overexpression of efflux pump and higher antibiotic resistance was 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

⁰C: Degree Celcius

h: Hour

DMSO: Dimethylsulfoxide

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% and 63% of population and early children deaths, respectively, are caused by infectious diseases. Emergence of such new, rare or known infectious diseases has stimulated interest to generate new drugs against antibiotic resistant strains. 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 [2,3,4].

Microbes are attaining the antibiotic-resistance, which present a challenge to researchers and threat to mankind. Several mechanisms have evolved to confer antibiotic resistance in bacteria. The antibiotic-resistance can be enhanced by mutation, clonal evolution, and horizontal gene or plasmid transfer [5,6,7,8]. The bacterial strains diversify in 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) [9,10,11,12,13,14].

Among these mechanisms, resistance in bacteria acquired by efflux pump remains crucial. Efflux pumps play a vital role to build the multidrug resistance as they export several unrelated substances including molecules produced by host organisms as well as various types of antibiotics and chemicals such as

dyes, organic solvents and detergents, molecules needed for the cell-cell communication, biocides and metabolic products [15,16,17]. The pumps that effect such efflux typically 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 chromosome 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) superfamily, 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 [18]. The present study was aimed to check the availability of resistance to various classes of antibiotics alone or in combination among random samples of bacteria, in the second part of study was, also determined the prevalence of antibiotic resistances due to efflux pump.

2. MATERIALS AND METHODS

2.1 Bacterial strains tested

A total of 57 bacterial strains from various sources were tested in the 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. A total of 38 bacterial strains were isolated from various nasal and milk samples of chicken and buffaloes respectively 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 [19] using spreading and streaking technique. Selective media like Baird Parker agar and Slantez 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 72 h at 37⁰C. The bacterial isolates were further identified on the basis of their morphological and biochemical characteristics [20]. MTCC and NCDC samples were cultured on Nutrient agar media at 37⁰C for 24 h.

2.3 Susceptibility test

The antibiotics belonging to different classes used in the study were amikacin (Ak), ampicillin (Am), cefixime (Ce), chloramphenicol (Ch), ciprofloxacin (Ci), erythromycin (Er), lincomycin (Li), nitrofurantoin (Ni), norfloxacin (No), ofloxacin (Of), tetracycline (Te), trimethoprim (Tr). Bacterial strains were tested against all these antibiotics belonging to different classes (10µg/ml in 10% DMSO). Disc diffusion method was performed according to Bauer Kirby and NCCLS methods [19,21] with an inoculum of 10⁶ CFU (Macfar Land standard). After incubation for 24 h at 37⁰ C on nutrient agar media, the plates were observed and the antibiotic susceptibility/resistance was evaluated by presence or absence of zone of inhibition (diameter in mm). The tests were conducted in triplicate. 10% DMSO was used as negative control.

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 (2 to 8 times) or reverse antibiotic resistance [22]. Piperine and Plumbagin are reported EPI of ciprofloxacin and ofloxacin antibiotics [23,24]. Piperine was purchased from Natural remedies Pvt. Ltd. Bangalore. Plumbagin was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

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112 **2.4.2 Minimum Inhibitory Concentration (MIC) Determination**

113 MIC of 57 bacterial strains against ciprofloxacin and ofloxacin (10µg/ml in 10% DMSO) was determined
114 by dilution technique. The final inoculum of 10⁶ CFU/ml was prepared in 5 ml nutrient broth. Positive
115 controls were without the antibiotics. Tubes were incubated at 37⁰ C for 24 h. The activity was measured
116 as a function of turbidity at 660 nm. Lack of turbidity was further confirmed by pouring suspension aliquot
117 of 0.1 ml into pre-sterilized Petri dishes with respective medium [19]. The tests were conducted in
118 triplicate. The MIC is defined as the lowest concentration of compound that inhibits visible growth.

119 **2.4.3 Effect of EPI on MIC levels of Antibiotics**

120 To determine the extent of the efflux pump mediated antibiotic resistance in various bacterial isolates,
121 MIC levels for ciprofloxacin and ofloxacin were determined in the presence and absence of piperine (30
122 µg/ml in 10% DMSO) and plumbagin (30 µg/ml in 10% DMSO) respectively [22]. Per cent Efflux Pump
123 prevalence was determined.

124 **2.5 Statistical Analysis**

125 Frequencies and proportions of strains resistant to the different classes of antibiotics were calculated.
126 Antibiotic resistance %= Number of resistant bacteria/Total number of bacteria × 100
127 Prevalence of efflux pump overexpression, cross-resistance, and magnitude of MIC decrease with EPI
128 were compared by chi-square.

129 **3. RESULTS**

130 **3.1 Isolation and Identification of Bacteria**

131 Morphologically distinct colonies of *Staphylococcus* sp. as black coloured cocci were obtained on Baird
132 parker agar media. Mehroon coloured cocci of *Enterococcus* sp. were distinctly obtained on Slantez and
133 Bartley Agar media. The isolated *Staphylococcus* sp. gave catalase positive test. A total of 38 bacterial
134 strains were isolated from various nasal and milk samples of chicken and buffaloes. Among them eleven
135 were *Staphylococcus* sp. (SA-1 to SA-10, SA-21) and Seventeen *Enterococcus* sp. (E-1 to E-17) were
136 isolated from chicken nasal samples. Ten *Staphylococcus* sp. (SA-11 to SA-20) were obtained from
137 buffaloes milk samples.

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139

140 **3.2 Resistance to Single and Multiple Antibiotics**

141 All the fifty seven bacterial strains from various sources were screened against the twelve antibiotics of
 142 different classes acting on different sites of bacteria. Only, 8.77% (5/57) strains were resistant to all tested
 143 antibiotics and not a single bacterial strain was sensitive to any antibiotic, indicating 100% potency of
 144 antibiotic resistance trait. The studied strains showed high resistance to nitrofurantoin (98.25%), cefixime
 145 (96.49%), lincomycin (94.74%), chloramphenicol (89.47%), trimethoprim (85.96%) and norfloxacin
 146 (84.21%). Resistance to multiple antibiotics was very common with lincomycin and nitrofurantoin
 147 (94.74%), cefixime and nitrofurantoin (92.98%). Additionally, 82.45% bacteria showed multiple resistance
 148 to cefixime, chloramphenicol and lincomycin. Similar, percentage was shown with cefixime,
 149 chloramphenicol, lincomycin and nitrofurantoin. While 80.70% were resistant to combination of cefixime,
 150 chloramphenicol, lincomycin, nitrofurantoin and norfloxacin. Combined resistance to nitrofurantoin,
 151 cefixime, lincomycin, chloramphenicol, trimethoprim and norfloxacin was found in 31.58% bacterial
 152 strains. The detailed explanation of antibiotic resistance are presented in Table 1.

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

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

Antibiotics	Percentage resistance% (Number of bacteria resistant)
Ni	98.25 (56)
Li	96.49 (55)
Ce	94.74 (54)
Ch	89.47 (51)
Tr	85.96 (49)
No	84.21 (48)

Er	57.89 (33)
Te	45.61 (26)
Ci	42.11 (24)
Of	33.33 (19)
Am	31.58 (18)
Ak	24.56 (14)
Li + Ni	94.74 (54)
Ce + Ni	92.98 (53)
Ce + Li & Ch + Ni*	89.47 (51)
Ch + Li	87.71 (50)
Ce + Ch & Li + No & Ni + No*	84.21 (48)
Ce + No & Ch + No*	82.45 (47)
Ce + Tr & Ni + Tr*	33.33 (19)
Ch + Tr & Li + Tr & No + Tr*	31.58 (18)
Ce + Ch + Li & Ce + Ch + Li + Ni*	82.45 (47)
Ce + Ch + Li + Ni + No	80.70 (46)
Ce + Ch + Li + Ni + No + Tr	31.58 (18)

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

159 3.3 Effect of EPIs on Antibiotic MICs

160 MICs of antibiotics determined in absence of efflux inhibitors were compared with those determined in
161 presence of efflux pump inhibitors. Two fold or more reduction in MIC levels was considered as an
162 indication of presence of efflux activity in antibiotic resistant bacterial strains [22]. It was observed that the
163 MIC levels of ciprofloxacin were lowered in 16 of 57 bacteria in the presence of piperine (Table 2). The
164 results indicate that 28.07% of bacterial strains attained antibiotic resistance due to active efflux pump of
165 ciprofloxacin. A total 16 bacterial strains declined the ciprofloxacin MIC to two fold out of which 11 were
166 Gram-positive and 5 were Gram-negative.

167 Similarly, MIC levels of ofloxacin were declined in 11 of 57 strains in the presence of plumbagin (Table 2).
 168 In the presence of plumbagin the MIC values for ofloxacin were found to decrease upto 4 fold in 1 and
 169 upto 2 folds in 10 strains. Out of 11 bacterial strains, 10 were Gram-positive and 1 was Gram-negative.
 170 MTCC bacterial strains have high prevalence (75%) of efflux pump of ciprofloxacin as compared to
 171 isolated bacterial strains, similar results were observed with efflux pump of ofloxacin but with a lower
 172 percentage (45.45%). Overall, the efflux pump overexpressed phenotype in fluoroquinolones
 173 (ciprofloxacin and ofloxacin) was observed in a significantly greater number (62.5%) in MTCC bacterial
 174 strains than isolated bacterial strains (37.5%).
 175 The magnitude of MIC didn't vary much and the range of MIC in case of ofloxacin-plumbagin combination
 176 was 5 to 2.5 µg/ml (two fold) except in one from 2.5 to 0.625 µg/ml (four fold) while in case of
 177 ciprofloxacin-piperine combination MIC was declined to half (Table 3).
 178 Table 2 Decline in MIC among 57 bacterial strains in presence of efflux inhibitors.

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

179
 180 Table 3 List of bacterial strains possessing decline in MIC of ciprofloxacin and ofloxacin in presence of
 181 piperine and plumbagin respectively.

Sr. No.	Bacterial strains	Ci+Piperine Decline in Ci MIC(µg/ml)		Of+Plumbagin Decline in Of MIC(µg/ml)	
1	<i>Bacillus cereus</i> (MTCC 430)	5	2.5	NE	NE
2	<i>Bacillus polymyxa</i> (NCDC 68)	NE	NE	5	2.5
3	<i>Bacillus pumilus</i> (MTCC 7411)	NE	NE	NE	NE
4	<i>Bacillus stearothermophilus</i>	2.5	1.25	NE	NE

	(MTCC 8505)				
5	<i>Bacillus subtilis</i> (MTCC 8509)	NE	NE	5	2.5
6	<i>Bacillus subtilis</i> (MTCC 121)	5	2.5	5	2.5
7	<i>Lactobacillus brevis</i> (NCDC 371)	NE	NE	NE	NE
8	<i>Lactobacillus plantarum</i> (NCDC 20)	NE	NE	NE	NE
9	<i>Staphylococcus aureus</i> (MTCC 3160)	2.5	1.25	NE	NE
10	<i>Staphylococcus aureus</i> (MTCC 109)	5	2.5	NE	NE
11	<i>Staphylococcus epidermidis</i> (MTCC 3086)	5	2.5	NE	NE
12	<i>Staphylococcus epidermidis</i> (MTCC 435)	2.5	1.25	NE	NE
13	<i>Staphylococcus hominis</i> (MTCC 4435)	NE	NE	2.5	1.25
14	<i>Escherichia coli</i> (MTCC 1885)	2.5	1.25	5	2.5
15	<i>Klebsiella pneumoniae</i> (MTCC 4030)	1.25	0.625	NE	NE
16	<i>Pediococcus acidilactici</i> (NCDC 252)	NE	NE	NE	NE
17	<i>Proteus vulgaris</i> (MTCC 426)	0.312	0.156	NE	NE
18	<i>Pseudomonas aeruginosa</i> (MTCC 424)	1.25	0.625	NE	NE
19	<i>Pseudomonas aeruginosa</i> (MTCC 7453)	1.25	0.625	NE	NE
20	SA-1	NE	NE	NE	NE
21	SA-2	NE	NE	5	2.5
22	SA-3	NE	NE	NE	NE
23	SA-4	NE	NE	NE	NE
24	SA-5	NE	NE	NE	NE
25	SA-6	NE	NE	NE	NE
26	SA-7	5	2.5	NE	NE
27	SA-8	NE	NE	5	2.5
28	SA-9	NE	NE	NE	NE

29	SA-10	NE	NE	Resistant	10
30	SA-11	NE	NE	NE	NE
31	SA-12	NE	NE	NE	NE
32	SA-13	1.25	0.625	NE	NE
33	SA-14	NE	NE	NE	NE
34	SA-15	NE	NE	NE	NE
35	SA-16	2.5	1.25	5	2.5
36	SA-17	NE	NE	NE	NE
37	SA-18	NE	NE	NE	NE
38	SA-19	NE	NE	NE	NE
39	SA-20	NE	NE	2.5	0.625
40	SA-21	NE	NE	5	2.5
41	E-1	NE	NE	NE	NE
42	E-2	NE	NE	NE	NE
43	E-3	NE	NE	NE	NE
44	E-4	NE	NE	NE	NE
45	E-5	5	2.5	NE	NE
46	E-6	NE	NE	NE	NE
47	E-7	NE	NE	NE	NE
48	E-8	NE	NE	NE	NE
49	E-9	NE	NE	NE	NE
50	E-10	NE	NE	NE	NE
51	E-11	NE	NE	NE	NE
52	E-12	NE	NE	NE	NE
53	E-13	NE	NE	NE	NE
54	E-14	NE	NE	NE	NE
55	E-15	NE	NE	NE	NE
56	E-16	NE	NE	NE	NE
57	E-17	NE	NE	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 subgrouped 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).

188 The III subgroup *i.e* Ce+Ni showed maximum antibiotic resistance prevalence (92.98%) and highest
 189 prevalence of the EPO phenotype in ciprofloxacin (75%). Similarly, the subgroup which demonstrated the
 190 highest prevalence of the EPO phenotype in ofloxacin was II subgroup (100 %). Overall comparison
 191 showed that (Ce + Ni) resistant strains subgroup showed maximum antibiotic resistance prevalence and
 192 EPO prevalence. The ciprofloxacin Efflux pump overexpression (EPO) strains possessed resistance
 193 against antibiotics like cefixime and nitrofurantoin (75%), while ofloxacin EPO, possessed resistance
 194 against cefixime and lincomycin in all eleven strains (Table 4).

195 Table 4 Comparison of Antibiotic resistant (AbR) prevalence and Efflux pump overexpression (EPO)
 196 prevalence within the subgroups of specific antibiotics.

Sr. No	Sub groups	Antibiotic Resistant Subgroups	AbR prevalence %	EPO prevalence %	
				ciprofloxacin+Piperine	ofloxacin+Piperine
1.	I	Ce + Ch	84.21 (48/57)	56.25 (9/16)	90.90 (10/11)
2.	II	Ce + Li	89.47 (51/57)	68.75 (11/16)	100 (11/11)
3.	III	Ce + Ni	92.98 (53/57)	75.0 (12/16)	90.90 (10/11)
4.	IV	Ce + Ch + Li	82.45 (47/57)	50.0 (8/16)	81.81 (9/11)
5.	V	Ce + Li + Ni	85.96 (49/57)	56.25 (9/16)	90.90 (10/11)

6.	VI	Ch + Li + Ni	87.71 (50/57)	56.25 (9/16)	81.81 (9/11)
7.	VII	Ce + Ch + Li + Ni	82.45 (47/57)	43.75 (7/16)	81.81 (9/11)

4. DISCUSSION

Antibiotic-resistance in microorganism acquired by 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 (94.74%) class i.e. cefixime (94.74%). Resistance against two or more of antibiotics (Li+Ni) which was observed in this study was higher than other earlier investigations conducted by Addis [25] 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, not recently exposed to antibiotic doses, so likelihood having less antibiotic resistance prevalence. Antibiotics are routinely used along with growth promoters in poultry and cattle feed [26]. The high prevalence of resistance seen after individual antibiotic use highlight the increasing threat posed by antibiotic resistance and suggest further evidence for the need for commitment to ensuring antibiotics are used as rational a manner as possible [3].

Efflux mechanisms have become broadly recognized as major components of resistance to many classes of antibiotics. Many of efflux pumps are of clinical relevance because they can render a bacterial infection untreatable by the agent(s) of choice [18].

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. Although among all studied strains (except one) the MIC decline potential was only upto 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 [27].

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 type proton motive force and ATP dependent extrusion system involved in fluoroquinolones resistance [28].

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 [24]. 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 [29].

Singh and co-workers, [28] 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 [23] 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, bacterial strains isolated 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 isolates or MTCC.

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

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 bacterial strain in which the MIC level was declined due to respective efflux pump inhibitors presence shall be carried for further studies.

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