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### RESEARCH ARTICLE

#### EFFLUX AS AN ARISING CAUSE OF DRUG RESISTANCE IN E. COLI.

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#### Abstract

Multidrug resistant strains of bacteria which are characterized as serious public health problem may arise due to over expression of efflux pumps. Efflux pumps in bacteria can be detected by using specialized instruments. In the present study simple instrument free agar cartwheel method was used to detect efflux pumps in *E.coli* with some modification. Four isolates of *E.coli* were collected and processed. Biochemical analysis of all the strains was done using standard protocol. Further cartwheel assay was performed. MIC method was used to confirm the observations of agar cartwheel assay. In Cartwheel assay, a total of 4 strains of *E.coli* were analyzed for the presence of efflux pumps, active efflux pump was detected in 1 isolate while it was not observed in 3 strains. Bacterial isolate which contains efflux pump also showed high MIC value for the antibiotics used. But in the presence of an EPI, a considerable decrease in the MIC of antibiotics was observed among isolate containing efflux pump, while there was no decrease in MIC of antibiotics for strains without efflux pumps. Present study revealed that cartwheel assay to detect efflux pumps is a reliable, fast and sensitive technique, which may reduce time and efforts to detect efflux pumps.

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#### Introduction:-

*Escherichia coli* is a principal facultative organism present in the human gastrointestinal tract. Pathogenic strains of *E. coli* have the capability to cause different types of diseases in humans and animals because of the presence of definite colonisation factors, virulence factors and pathogenically associated genes. *E. coli* major cause of nosocomial infections in humans is a Gram-negative, flagellated, rod-shaped, Oxidase negative, motile, facultative anaerobe and classified under the family Enterobacteriaceae<sup>1</sup>. *E. coli* is genetically classified as the most versatile bacteria and is major source of many plasmid and phage mediated genes. It causes septicemia and diarrhoea in overall hosts including man, avian and animals such as cattle, piglets, kids, foals, lambs and buffaloes. The pathogenic strains of *E. coli* which are isolated from hospitalized patients were more resistant to amoxicillin, ciprofloxacin, cephalexin and Gentamycin compared to those from outpatients<sup>2</sup>. Some strains of *E. coli* develop resistance to third-generation cephalosporin's and monobactams. Most members of gram negative bacteria family are less susceptible to most classes of the antibiotics, mainly lipophilic and amphiphilic ones; this is due to the presence of outer membrane in gram negative bacteria. ). Constant increase in drug resistant strains of bacteria becomes a big threat to human health<sup>3</sup>. Multidrug efflux pumps unfavorably affect the clinical effectiveness of existing antibiotics and also affect the process of discovery of new antibiotics<sup>4</sup>. Active efflux is established as

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important factor of bacterial resistance in case of most classes of antibiotics. The mechanism of active efflux is mediated by efflux pumps<sup>5-7</sup>. Efflux pumps are known as membrane associated active transporters which help in expelling out toxic compounds, including antibiotics from the cell<sup>5-8</sup>.

Antibiotic efflux was first reported in 1980, in case of *E. coli* bacteria as a mechanism for tetracycline resistance. Recently the role of efflux mechanism is reported in bacterial resistance in case of almost all major groups of antibiotics<sup>5,7,9-11</sup>. Bacterial resistance to most of the classes of antibiotics is provided by membrane transporter proteins which are known as drug efflux pumps<sup>12</sup>. Efflux pumps are present as single component system or multi-component systems. Increasing resistance of bacteria against most classes of antibiotics in recent days becomes a big problem<sup>13</sup>. Resistance in antibiotics develops very rapidly because of the changes in the expression of efflux pumps. So it is important to characterize new antibiotics, efflux pump inhibitors and the agents which are resistance modifying<sup>14</sup>. Resistance of bacterial isolates to most classes of antibiotics is widespread mostly in developing countries. Schedule monitoring of antibiotic resistance gives data for the antibiotic therapy and control of resistance, and evaluates the effectiveness of both of these<sup>15</sup>. In spite of the fact that antibiotics are originally emerged for the treatment of infectious disease caused by bacteria in humans, multidrug resistance to most classes of antibiotics becomes a worldwide problem. Efflux pumps are reported in both gram positive and gram negative bacteria and as well as in eukaryotic organisms. Some of the bacterial efflux pumps are selective for only one substrate while some of them are not. The non-selective group of efflux pumps are involved in shifting of wide range of compounds and different classes of antibiotics which confer a multiple drug resistance phenotype<sup>5,6-11</sup>. The AcrAB efflux system present in *E. coli* is multidrug efflux pump which is a major efflux system present in *E. coli* and responsible for intrinsic resistance of the bacteria against antibiotics, dyes and detergents<sup>16-17</sup>. The AcrAB efflux pump existing in *E. coli* belongs to resistance- nodulation- division (RND) family of efflux pumps which is a more usual in Gram – negative bacteria. AcrAB – TolC is specified as one of the best efflux pump in case of *E. coli*<sup>18-27</sup>. AcrAB efflux pump is the only single efflux pump of RND family in case of *E. coli* which plays an important role in antibiotic resistance against different antibiotics because of its prenominal expression. The Ethidium bromide (Et Br) agar cartwheel assay is a newly investigated simple, instrument free method used for the detection of efflux pump activity in bacteria<sup>28</sup>. The present study was aimed to detect efflux pumps in Gram negative bacteria *E.coli* with the help of EtBr agar Cartwheel assay.

## **Material and Methods:-**

### **Microbial strains:-**

The strains of *Escherichia coli* used in the present study were procured from Sabine Schuster and Winfried V. Kern, Center for Infectious Diseases and Travel Medicine, University Hospital, and Department of Medicine, Albert-Ludwig's-University, Freiburg, Germany and from MTCC and NCTC. The *E. coli* strains used were one knock out strain 1- DC14, wild type strain K-12 AG100 and two standard strains with NCTC number 12923 and MTCC number 1302.

### **Chemicals and reagents:-**

Nutrient broth, DMSO, Methanol, CCCP, Ethidium bromide, Tryptic soy agar were purchased from Hi-media Pvt. Ltd.

## **Methods:-**

### **Characterization of *Escherichia coli* strains:-**

Characterization of *Escherichia coli* was performed by gram staining and biochemical analysis.

### **Gram's staining:-**

Gram's staining was done following the methodology of Aneja<sup>29</sup>. The smear on a glass slide was covered with few drops of the primary stain (Crystal violet). After a minute of exposure to the staining solution, the slide was washed with water. The smear was treated with few drop of Gram's Iodine and allowed to stand for a minute. This results in formation of a dye-iodine complex in the cytoplasm. Gram's iodine serves as a mordant. The slide was again washed with water and then decolorized with absolute ethyl alcohol or acetone. After the smear was decolorized, it was washed with water without any delay. The smear was finally treated with few drops of counter stain (Safranin). The slide was washed in water; excess water was removed using a blotting paper, dried in air and heat fixed before observing under microscope.

**Biochemical Analysis:-**

Biochemical analysis was done according to Bergey's manuals of systematic bacteriology (1993). Following biochemical tests were performed for identification of *E. coli* isolates;

**Indole test:-**

Tryptone broth was prepared. The broth was dispensed into the tubes and sterilized. Culture of *E. coli* was inoculated into the tubes and one tube was left uninoculated as control. The tubes were inoculated at 37°C for 48 hours. After incubation 1 ml of KOVAC's reagent was added to all the tubes including control. The tubes were shaken gently and allowed to stand for 1-2 min.

**Positive test result:-** The indole reagent change colour to red.

**Negative test result:-** The indole reagent remains pale yellow.

**Methyl Red Test:-**

This test was performed by inoculating a colony of *E. coli* in 0.5 ml sterile glucose phosphate broth. After incubating overnight at 37°C for 24-48 hours. Few drops of methyl red reagent was added and the tubes were shaken gently.

**Positive test result:** Red colour change.

**Negative test result:** No colour change.

**Voges Proskauer Test:-**

Glucose phosphate broth was prepared and inoculated with *E. coli* culture. Inoculated tubes were incubated for 24-48 hours at 37°C. 1 mL of 40% KOH and 3 mL of 5% solution of the alpha naphthol was added in the inoculated test tubes. The medium was shaken at intervals for enhancement of the reaction by aeration.

**Positive test result:** Colour change to pink.

**Negative test result:** No colour change.

**Citrate Utilization Test:-**

Citrate utilization test is used to determine the ability of bacteria to utilize sodium citrate as its carbon source. Simmons's citrate agar slant were prepared and streaked with 18-24 hours old culture of *E. coli*. The slants were incubated at 37°C for 24-48 hours.

**Positive result:** Growth in citrate medium or growth with colour change to blue in Simmons citrate tube.

**Negative result:** No growth in citrate medium.

**Urease Test:-**

Urease broth was prepared and inoculated with a loopful of *E. coli* culture. Then inoculated broth was incubated at 37°C for 24-48 hours.

**Positive test result:** Change in colour to red.

**Negative test result:** No colour change.

**Nitrate Reduction Test:-**

Nitrate broth was prepared and inoculated with *E. coli* culture. The inoculated broth was incubated at 37°C for 24-48 hours. After that 4-5 drops of mixture of solution A (8g of sulphanilic acid in 1000mL of 5N acetic acid) and solution B (5 of alpha naphthylamine in 1000mL of 5N acetic acid) were added.

**Positive test result:** Red colour after addition of sulphanilic acid and alpha-naphthylamine.

**Negative test result:** No colour change after addition of sulphanilic acid and alpha naphthylamine.

**Catalase test:-**

This test demonstrates the presence of catalase enzyme which catalyses the release of oxygen from hydrogen peroxide. This test was used to differentiate those bacteria which produces catalase enzyme from non-catalase producing bacteria. A small amount of colony of *E. coli* culture was transferred to surface of a clean, dry glass slide using an inoculating loop. A drop of hydrogen peroxide was placed on the slide.

**Positive test results:** Gas formation in the form of bubbles shows that the bacterium is catalase positive.

**Negative test result:** No gas formation shows negative result.

**Carbohydrate fermentation test:-**

Four test tubes were taken containing four different kinds of broth namely glucose, lactose, sucrose, Mannitol. Each tube consist of 1% sugar, 1% peptone water and 1% Andrade's reagent. Inverted Durham tube were kept in each tube. Tubes were autoclaved at 15 lb. (121°C) for 30 minutes. After cooling tubes were inoculated with loopful culture of *E.coli* and incubated at 37°C for 24 hours. The broth was examined for acid and gas formation.

**Positive test result:** Acid production was indicated by the colour change from red to yellow.

**Negative test result:** No colour change.

**Motility test:-**

Semi solid motility medium was prepared in a test tube and inoculated with *E.coli* culture with a straight wire by making a single stab down the center of the tube about 8- 10 mm deep into the medium and incubated at 37°C for 24hours. Examined after one day.

**Positive result:-**

Diffuse, hazy growths that spread throughout the medium rendering it slightly opaque.

**Negative result:-** Generally give growths that are confined to the stab-line, have sharply defined margins and the surrounding material completely transparent.

**Oxidase test:-**

Oxidase test is used to identify bacteria that produces oxidase enzyme of bacterial electron transport chain. A filter paper was soaked in substrate tetra methyl-p-phenylenediamine dihydrochloride. A loop full colony of *E.coli* culture was smeared onto the filter paper strip.

**Positive test result:** Dark blue-purple colour change within 10-30sec.

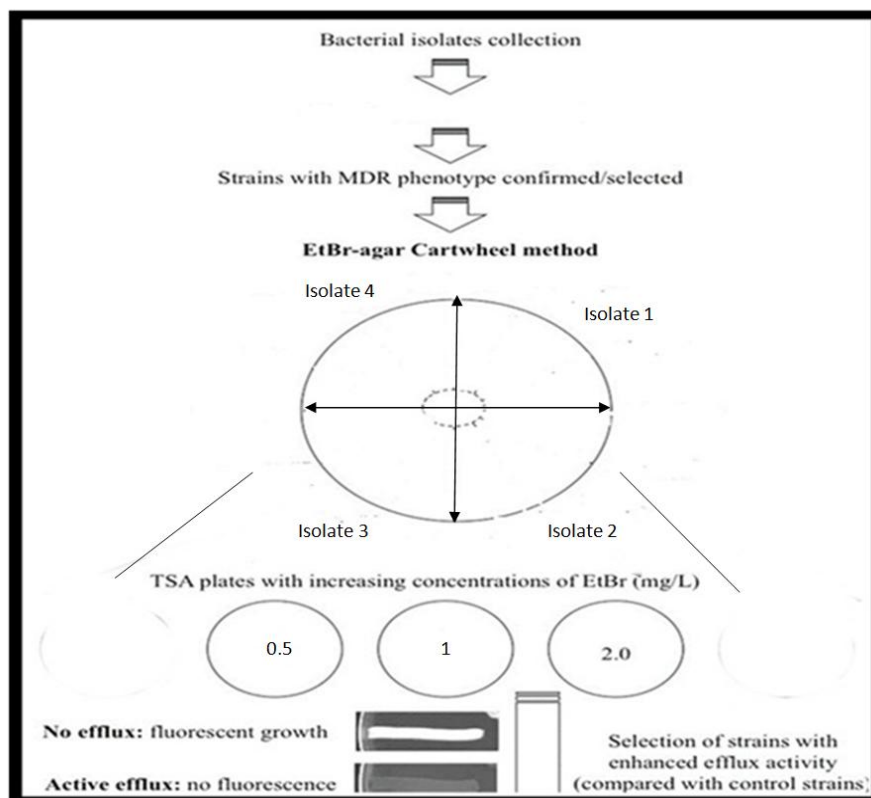
**Negative test result:** No colour change or colour change after more than 30 sec.

**Agar cartwheel assay for the detection of efflux pumps:-**

The Etbr- agar cartwheel assay method was used to assess the presence of efflux activity in *E.coli* strains following the methodology of with slight modifications<sup>28-30</sup>. All the strains of *E.coli* were grown in nutrient broth and incubated at 37°C for 24 hours. The TSA plates are then divided into four sectors, forming a cartwheel pattern. *E. coli* culture was inoculated by swabbing on tryptic soya agar plates starting from the center of the plate to the margin, these plates containing different concentrations (0.5µg/ml, 1µg/ml and 2.0µg/ml) of Etbr. Plates were incubated at 37°C for 16 hours and then observed under UV light (Gel Doc- it2 310 imager).The minimum concentration of Etbr that produces fluorescence of the bacterial mass was recorded. (fig.1)

**MIC Determination:-**

The MIC was determined in all strains of *E.coli* used for Etbr agar cartwheel assay to confirm the presence of efflux pumps. Pure cultures of *E.coli* were diluted in nutrient broth to a concentration between  $1 \times 10^5$  and  $1 \times 10^6$  cfu/mL. A stock dilution of the antibiotics i.e., Ciprofloxacin, Tetracycline and Erythromycin were made at approximately 100x the level of the expected MIC for *E.coli*. The concentrations used for each of the antibiotics were (0.003–128) µg/mL and Further 1:1 dilutions of antibiotics were made with nutrient broth in 96 well microtiter plates. Then the bacterial culture was inoculated from lower concentration to higher concentration of antibiotic in equal amount (30µl). Positive and negative control was also included. Then microtiter plates were incubated at 37°C for 24 hrs. And then plates were observed in ELISA plate reader (BioTek) at 490 nm. MIC was recorded as the lowest concentration where no growth was visually observed. Then MIC of antibiotics was again observed in the presence of an EPI (CCCP).



**Fig.1:-**Flowchart followed to test bacterial strains using the EtBr-agar Cartwheel method.

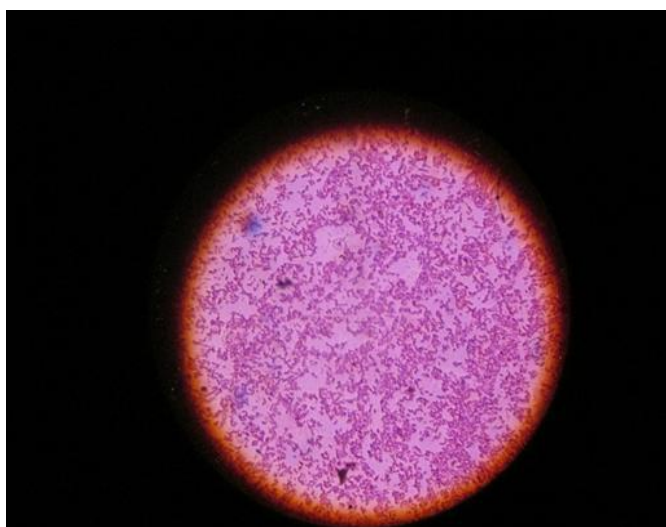
## Results:-

### Characterization of *E. coli* strains:-

Characterization of *E. coli* was performed by Gram's staining and biochemical testing.

### Gram's staining:-

The results of gram's staining are summarized in figure 2. Gram- negative bacilli of *E. coli* were seen under the microscope. Rod shaped pinkish colony of *E. coli* were appeared.



**Figure 2:-** Gram-negative rod shaped bacilli of *E. coli*

**Biochemical Analysis:-**

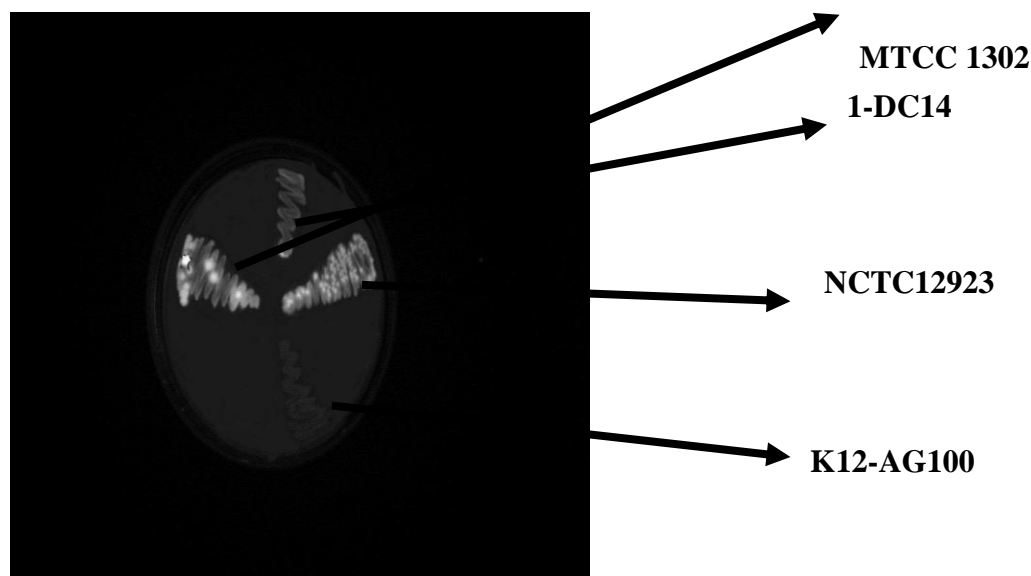
The results described in table 1 shows that all the strains of *E. coli* were positive for, Catalase test, Indole test, Methyl red test, Carbohydrate fermentation, Nitrate reduction and Motility test. The strains of *E. coli* showed negative results for, Oxidase test, urease test, Sucrose fermentation and Maltose fermentation.

**Table 1:-** Biochemical analysis of *E. coli* strains

Biochemical tests	<i>E. coli</i> strains			
	1-DC14	K12-AG100	MTCC	NCTC
Catalase test	Positive	Positive	Positive	Positive
Oxidase test	Negative	Negative	Negative	Negative
Indole test	Positive	Positive	Positive	Positive
Methyl red test	Positive	Positive	Positive	Positive
Voges Proskauer Test	Negative	Negative	Negative	Negative
Citrate utilization test	Negative	Negative	Negative	Negative
Urease test	Negative	Negative	Negative	Negative
Nitrate reduction	Positive	Positive	Positive	Positive
Glucose fermentation	Positive	Positive	Positive	Positive
Lactose fermentation	Positive	Positive	Positive	Positive
Maltose fermentation	Negative	Negative	Negative	Negative
Sucrose fermentation	Negative	Negative	Negative	Negative
Motility test	Positive	Positive	Positive	Positive

**Etbr Agar Cartwheel Assay:-**

Etbr agar Cartwheel assay was performed for the detection of efflux pumps. The result of Etbr assay is summarized in figure 3. Agar cartwheel assay was performed for all 4 strains of *E. coli*, out of these four strains efflux pump was detected only in K12-AG100 strain and the other three strains (knockout strain 1-DC14, MTCC strain 1302 and NCTC strain 12923) have shown florescence hence confirms the absence of efflux pump.



**Figure 3:-** Ethidium bromide fluorescence detected in three strains of *E. coli*. The resistant strain showing the absence of Etbr fluorescence confirming the presence of Efflux pump.

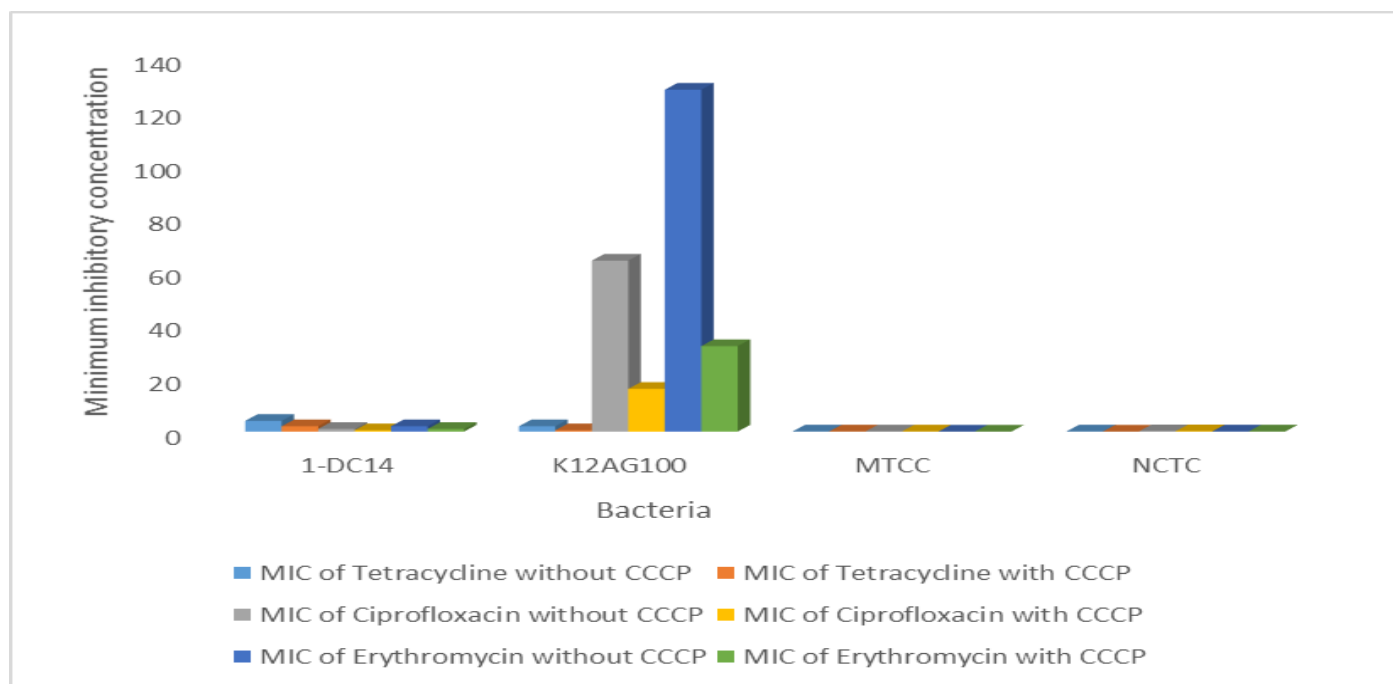
**Minimum Inhibitory Concentration:-**

The observations of Etbr agar cartwheel assay was further confirmed by the determination of MIC values for selected antibiotics, known to be efflux pump substrates viz., Tetracycline, Erythromycin and ciprofloxacin. The Wild type strain K12-AG100 containing AcrAB- TolC efflux pump has shown very high MIC values ranging from

0.5 µg/mL to 128 µg/mL for all antibiotics known to be efflux pump substrates. Further the assay was performed with an Efflux pump inhibitor EPI a significant decrease was observed in MIC of antibiotics for the resistant strain and these values were ranging from 0.5 µg/mL to 32 µg/mL. While three strains (1-DC14 Knockout strain, 1 MTCC strain and 1 NCTC strain) without efflux pump have shown very low MIC value of antibiotics which had remain constant even in the presence of an EPI (CCCP), (Table 2 and Fig 4). The observations of MIC assay were found similar to those of EtBr agar cartwheel assay.

**Table 2:-** MIC determination in *E.coli* strains for ciprofloxacin, Tetracycline and Erythromycin (µg/mL) to confirm the presence of active efflux pump

Sr.No	Name of strain	MIC for Tetracycline		MIC for Ciprofloxacin		MIC for Erythromycin	
		Without CCCP	With CCCP	Without CCCP	With CCCP	Without CCCP	With CCCP
1	1-DC14	4	2	1	0.5	2	2
2	K12-AG100	2.0	0.5	64	16	128	32
3	MTCC	0.06	0.03	0.05	0.05	0.003	0.003
4	NCTC	0.003	0.003	0.125	0.125	0.05	0.05



**Fig. 4:-** MIC of Ciprofloxacin, Tetracycline and Erythromycin (µg/mL) with or without CCCP for *E.coli* strains.

### Discussion:-

*Escherichia coli* has recognized as an emerging opportunistic pathogen of clinical relevance. The intrinsic resistance of *E.coli* has been attributed due to the outer membrane barrier. From many years, role of efflux mechanisms in bacterial resistance to most classes of antibiotics<sup>5,7,9,11</sup>. Ethidium bromide is a substrate for all efflux pumps and is not allowed to accumulate inside the bacterial cell<sup>32</sup>. Ethidium bromide used as a marker to detect efflux pump in resistant bacteria which have efflux pump mediated drug resistance mechanism. Those bacteria have efflux pump does not show EtBr fluorescence because they do not accumulate the EtBr and effluxed out. In the present study, EtBr Agar Cartwheel assay was performed to detect AcrAB-TolC efflux pump in *E.coli* isolates. In the present study, we observed the EtBr fluorescence among three strains 1-DC14 Knockout strain, MTCC-1302 and NCTC-12923 these strains get accumulated EtBr and shows EtBr fluorescence. While K12-AG100 wild type strain has not shown the EtBr fluorescence hence it confirms the presence of efflux pump.

Martins *et al.*, (2013) reported that Gram negative bacteria *Acinetobacter* has shown significant increase in the fluorescence after the incubation at 4°C while less fluorescence was observed at 37°C. The observations of the present study suggested that the drug efflux mechanism is an energy mediated mechanism and direct perpetuate to the temperature when the temperature decreased the energy of membrane decreased and the other factor may be responsible for less efflux is low metabolic rate at low temperature, which is not able to produce sufficient energy, hence the available energy is not sufficient to efflux out the accumulated compounds. Therefore, more fluorescence occurs at low temperature. In addition to this, the isolates having potentially less active efflux systems showed higher fluorescence and isolates having more active efflux systems showed less fluorescence<sup>32</sup>. In the present study, the MIC value was determined for the selected antibiotics, known as efflux pump substrates viz., Tetracycline, Erythromycin and Ciprofloxacin. K12-AG100 strain with known AcrAB-TolC efflux pump have shown very high MIC values ranging from 0.06 µg/mL to 128 µg/mL confirm the presence of AcrAB-TolC efflux pump. While the other three strains have shown very low MIC value. Further the assay was performed with an Efflux pump inhibitor (EPI) CCCP. A significant decrease was observed in MIC of antibiotics for the resistant strain and these values were ranging from 0.003 µg/mL to 64µg/mL. While the MIC of control sensitive strains (1 MTCC- 1302 and 1NCTC-12923) without efflux pump was not change and remain constant even in the presence of an EPI (CCCP). Similarly, Abdi- Ali *et al.*, (2006) has reported significant decrease in MIC of resistant antibiotics used in combination with EPI (CCCP). The observations of the study again suggested that the drug resistance in MDR isolates is mediated by an active efflux pump which effluxed out the accumulated drug.

### Conclusion:-

EtBr agar cartwheel assay is a simple trustworthy method and can be used for the detection of efflux pumps in bacteria and on the basis of present study it can be concluded that efflux pump over expression is an leading cause of drug resistance in *E.coli* therefore proper measures should be used to solve this problem.

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