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Screening for Efflux Pump Mediated Resistance in *Enterobacterales* by Acridine Orange Agar Cartwheel Method

Anusha AP[1], Harish Kumar KS[2], Rani S Jayamohan[3], Deepthy BJ[4]

[1]Post-graduate Student, Dept. of Medical Microbiology, School of Medical Education, Centre for Professional and Advanced Studies, Kottayam, Kerala, India; [2]Professor and Head of the Department, Dept. of Medical Microbiology, School of Medical Education, Centre for Professional and Advanced Studies, Kottayam, Kerala, India; [3]Assistant Professor, Dept. of Medical Microbiology, School of Medical Education, Centre for Professional and Advanced Studies, Kottayam, Kerala, India; [4]Associate Professor, Department of Microbiology, Dr. Moopens Medical College, Meppady, Wayanad, Kerala, India.

ABSTRACT

Introduction: Aerobic gram negative bacilli such as *E. coli, Klebsiella pneumoniae* and *Enterobacter spp* are major causes of hospital-acquired infections. Efflux pump mediated resistance is one of the major mechanism contributing to antimicrobial resistance in *Enterobacterales*.

Aim/Objectives: The aim of our study was to detect efflux pump mediated resistance in *Enterobacterales* by Acridine orange agar method.

Materials and Methods: In this study, various *Enterobacterales* were isolated from clinical samples and identified using various biochemical tests. The screening for expression of efflux pump was done by Acridine orange agar cartwheel method.

Results: Among the *Enterobacterales*, *E. coli* showed highest prevalence of efflux pump activity followed by *Klebsiella pneumoniae* and *Enterobacter spp.*

Conclusion: In this study, it is implicated that Acridine orange can be used as an alternative to Ethidium bromide and screening for efflux pump can be done by Acridine orange agar method in a microbiology laboratory set up.

Key Words: *Enterobacterales*, Multidrug resistance, Extensively Drug Resistance, Efflux Pump Mediated Resistance, Acridine Orange Cartwheel Method, Antimicrobial Resistance Detection

INTRODUCTION

Antimicrobial resistance is a global health crisis which affects the well-being and development of a society. Undoubtedly, the selective pressure exerted by the excessive and indiscriminate use of antimicrobial agents such as antibiotics and sanitisers to kill or inhibit various bacterial pathogens has accelerated the emergence and spread of antimicrobial resistance among bacteria.[1,2] Bacteria have developed countermeasures to protect themselves against external stresses such as antimicrobials. In the presence of lethal stress, while the majority of bacterial cells perish, a subpopulation of bacterial cells known as persister cells can survive and resume growth after relief of the imposed lethal stress.[3,4]

The emergence of antimicrobial resistance among *Enterobacterales* have been increased recently in a dramatic pattern and has reached the pandemic scale. Among the *Enterobacterales*, *E. coli*, *Klebsiella pneumoniae* and *Enterobacter spp* are the important pathogens contribute to the antimicrobial resistance. Mechanisms of drug resistance fall into several broad categories, including drug inactivation/alteration, modification of drug binding sites/targets, changes in cell permeability resulting in reduced intracellular drug accumulation, and biofilm formation.[5,6,7]

Multidrug-resistant (MDR) phenotypes of clinical isolates have been shown to be related to overexpression of efflux pump systems.[8-13] Bacterial efflux pumps are proteins which are found in the plasma membrane of the bacterium and its function is to recognize toxic substances that penetrate the cell wall of the bacteria, and extrude them before they reach the intended target. These systems are found in Gram-

Corresponding Author:
Dr. Harish Kumar KS, Kaleeckal, Edappon, Ayaranikudy. P.O, Alappuzha, Kerala, India.
Contact No.: 9447265198; Email: drharishkumarks@gmail.com

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negative and Gram-positive bacteria; however, efflux mediated resistance in Gram-negative bacteria is more complex due to the molecular architecture of the cell envelope.[8,13] The screening of clinical isolates for overexpression of efflux pumps has been successfully conducted with the aid of the ethidium bromide (EB) agar method.[14] Although this method is user friendly, many countries rigorously control the use and disposal of EB since it is a toxic and mutagenic agent.[15,16,17] Acridine Orange (AO; *N,N,N',N'*-tetramethylacridine-3,6- diamine) is a cationic fluorescent chromophore used for vital staining of eukaryotic cells. It is cell permeable, and interacts with DNA and RNA by intercalation and electrostatic attractions, respectively. When bound to DNA, it is spectrally very similar to fluorescein, with an excitation maximum at 502 nm and an emission maximum at 525 nm (green). When it associates with RNA, the excitation maximum shifts to 460 nm (blue) and the emission maximum shifts to 650 nm (red). AO has been shown to be a substrate of bacterial efflux pumps. Because AO does not present with toxicity or mutagenicity when employed at concentrations used for the demonstration of efflux pumps or vital staining of eukaryotic cells, we selected AO as the substrate for the screening of bacteria that overexpress their efflux pumps compared to their wild-type counterparts. In this study, important nosocomial pathogens isolated from clinical samples and their efflux activity is monitored using Acridine orange agar method.

MATERIALS AND METHODS

This study was conducted in Department of Medical Microbiology, Centre for Professional and Advanced Studies (CPAS), SME, Gandhinagar from January 2022 to December 2022. A total of 100 isolates of *Enterobacterales* including *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter spp*. were collected from various diagnostic microbiology laboratories in Kerala.

Identification of isolates and antimicrobial susceptibility testing of *Enterobacterales*

All the isolates were identified by routine biochemical testing and from the isolates obtained, members of the *Enterobcterales* were selected. Antibiotic susceptibility testing was done by disc diffusion method as recommended by Clinical Laboratory Standards Institute (CLSI) guidelines.[18] The following antibiotics were tested; Gentamicin (10 µg), Amikacin(10 µg), Imipenam (10 µg), Cefuroxime(30 µg), Cefoxitin(30 µg), Ciprofloxacin(5 µg), Aztreonam(30 µg), Ampicillin(10 µg), Amoxyclav (20/10 µg), Tetracycline(30 µg), Cefixime(5 µg), Ceftazidime(30 µg), Ceftazidime/ Clavulanic Acid, Cefotaxime(30 µg), Cefotaxime/Clavulanic Acid.

Based on the recommendations of the Centers for Disease Control and Prevention (CDC) and European Centre for Disease Prevention and Control (ECDC), isolates were termed as MDR which are non-susceptible to atleast one agent in three or more antimicrobial categories.[25] XDR is defined as non-susceptibility to atleast on agent in all but two or fewer antimicrobial categories (i.e., bacterial isolates remain susceptible to only one or two categories). PDR is defined as non-susceptibility to all agents in all antimicrobial categories (i.e., no agents tested as susceptible for that organism). ESBL production was detected phenotypically by disc combination method as recommended by guidelines of CLSI by using ceftazidime (30µg)–ceftazidime/clavulanic acid and cefotaxime (30µg)– cefotaxime/clavulanic acid. A difference of ≥ 5mm between cephalosporin discs and their respective cephalosporin/clavulanic acid disc is taken as ESBL producing strains.

Screening of Efflux pump mediated resistance in *Enterobacterales* by using Acridine Orange agar method

Screening of efflux pump was done on Mueller Hinton agar plates supplemented with various concentration of Acridine orange (0.01mg/L, 0.02mg/L, 0.03mg/L, 0.04mg/L, 0.05mg/L).2-3 colonies of bacteria were inoculated onto Mueller Hinton Broth.The inoculated tubes were incubated overnight at 37^0C. The bacterial isolate cultured on the Mueller Hinton broth then inoculated onto Mueller Hinton Agar plates supplemented with Acridine orange using a sterile swab by cartwheel streaking method.The inoculated plates were incubated overnight at 37^0C.

After overnight incubation fluorescence of the overlying bacterial streak was determined with a source of UV illumination by placing the plates inside the Biotech New Gel documentation system. The higher the concentration needed to produce fluorescence, the greater the ability of the bacterial efflux pump to extrude the dye.

STATISTICAL ANALYSIS

The study was approved by the institutional ethical committee at School of Medical Education(IEC/05/MICRO/SME-GNR/2021). The data was analysed using Microsoft excel 2019 and statistical package for the social sciences (SPSS-18).

RESULT

In the present study, a total of 100 isolates of *Enterobacterales* belonging to three genera were obtained from various clinical samples. The isolates obtained were *E. coli* (n=50), *Klebsiella pneumoniae* (n=25), *Enterobacter spp*. (n=25)

Antimicrobial susceptibility pattern of *Enterobacterales*

In the current study, out of 100 clinical isolates, 79% were sensitive and 21% were resistant to Gentamicin (as shown in Table 1). Amikacin resistance was seen in 20% while 68% were sensitive and 12% showed intermediate susceptibility. Imipenem resistance were 20% with 61% sensitivity and 19% intermediate susceptibility. *Enterobacterales* exhibited 49.33% resistance towards cefuroxime while 29.33% were sensitive and 21.33% classified as intermediate susceptibility. 53.33% sensitivity, 38.66% resistance and 8% intermediate susceptibility observed in cefoxitin. 53% were sensitive, 35% were resistant and 12% were intermediate towards ciprofloxacin. 61% of isolates were sensitive to aztreonam while 29% were resistant and the remaining 10% were of intermediate susceptibility. 64% Resistance observed in ampicillin while 34% were sensitive and 2% were intermediate. Sensitivity and resistance towards amoxiclav observed as 42.66% and intermediate susceptibility recorded as 14.66% intermediate. 66% were sensitive and 28% were resistant and 6% were intermediate towards tetracycline. Sensitivity towards cefixime recorded as 54% while 42% were resistant and 4% were intermediate respectively. 40% resistance observed in ceftazidime, 38% sensitivity and the remaining 27% classified as intermediate susceptibility. 47% resistance and 37% sensitivity and 16% intermediate susceptibility observed towards cefotaxime.

Prevalence of XDR, MDR strains in *Enterobacterales* - Among the clinical isolates tested, 36% (n=18) of *E. coli* and 24% (n=6) of *Klebsiella pneumoniae* and 8%(n=2) of *Enterobacter spp* were XDR and 10% (n=5) of *E. coli* and 16% (n=4) of *K pneumoniae* and 8% (n=2) of *Enterobacter spp.* were MDR.

Prevalence of ESBL production in *Enterobacterales* - In this study, among the clinical isolates, 60% (n=30) of *E. coli* were ESBL producing while 76% (n=19) and 12% (n=3) were ESBL producing in *Klebsiella pneumonia and Enterobacter spp.* respectively.

Screening of efflux pump by acridine orange agar method – *E. coli*

Among the 50 isolates of *E. coli* tested, all the strains showed no fluorescence at 0.01mg/L concentration of Acridine Orange. As the concentration of acridine orange increased exhibition of fluorescence also increased (as shown in figure 1). All the isolates of *E coli* tested are considered as Efflux positive, as they did notexhibit any fluorescence at the lowest acridine agar concentration as shown in figure 2.

Screening of efflux pump mediated resistance – *Klebsiella pneumoniae*

Among the *Klebsiella pneumoniae* isolates, 80% (n=20) showed no fluorescence at 0.01mg/L concentration of Acridine orange,(figure 3) therefore it can be concluded that 80% of *klebsiella pneumoniae* were Efflux positive (as shown in figure 4).

Screening of efflux pump by acridine orange agar method- *Enterobacter* spp.

Among the *Enterobacter spp* isolated 76% (n=19) showed no fluorescence at 0.01mg/L concentration of Acridine orange(figure 5). Therefore, they were considered as efflux positive (as shown in figure 6).

DISCUSSION

Antimicrobial resistance represents an enormous global health crisis and one of the most serious threats humans face today. Antimicrobial resistance mechanisms fall into four main categories: (1) limiting uptake of a drug; (2) modifying a drug target; (3) inactivating a drug; (4) active drug efflux. Gram negative bacteria make use of all four main mechanisms, whereas gram positive bacteria less commonly use limiting the uptake of a drug (don't have an LPS outer membrane), and don't have the capacity for certain types of drug efflux mechanisms.[19]

The identification of efflux mediated MDR clinical isolates is labour intensive and requires specialized instruments such as fluorometers, flow cytometers or radioactivity detectors.[20,21,22] Other methods currently used for evaluation is a combination of the drug with an efflux pump inhibitor, which evaluates reduction in MIC of a given antibiotic when an efflux pump inhibitor is present in the medium.[23] This can be quite expensive and, therefore, may not be feasible in all laboratories. We employed the Acridine orange agar cartwheel method, as it is a simple instrument free method that uses agar plates containing increasing concentrations of Acridine orange and can be easily adapted by a routine clinical microbiology laboratory. The Acridine orange cartwheel method has the advantage that multiple strains can be tested on the same agar plates.

In this study, the MDR and XDR strains isolated were 37% (n=37) out of which, *E. coli* found to have higher percentage of XDR strains which is 36% and *Klebsiella pneumoniae* found to have 24% XDR strains and *Enterobacter spp.* reveals 8%. It can be compared with study done by Ramakrishna MS et al. [24] in which,*E coli* reveals 21% XDR and *Klebsiella pneumoniae* found to have 33.66% XDR strains. In our study, the ESBL producing strains in total *Enterobacterales* is 52% (n=52), while it is 40% (by combination disk test of cephalosporin / clavulanic acid) in study done by Ramakrishna MS et al. and it is 64% in the study done by Harish Kumar K S et al.[25] in which, *Klebsiella pneumoniae* contributes 37.31% and *E. coli* contributes 35.42% while in our study, it is 76% and 60% respectively.

In this study, it is found that among the clinical isolates tested, 100% of the *E. coli* strains and 80% of the *Klebsiella pneumoniae* strains and 76% of *Enterobacter spp* showed no fluorescence at the lowest concentration of Acridine Orange, hence found to be efflux positive. In similar studies done by Ana Martins et al.[26] it is found that the strain *E. coli* AG100 began to fluoresce at 5 mg/l and at 10 mg/l fluorescence while the strains with overexpression of efflux pumps, namely, *E. coli* AG100$_{TET}$, did not fluoresce at high concentrations of AO. In our study, *E. coli* exhibited 100% and *Klebsiella pneumoniae* exhibited 80% efflux activity, which in study done by Shervin Gooyande et al.[27] was 80% and 52% respectively. The variability in number may be due to higher sample size and efflux action were only considered for MDR strains. *Enterobacter spp* exhibited efflux activity in 76% of isolates. We were not able to compare as we couldn't obtain any other research article on the same to best of our knowledge.

The results of this study demonstrates that Acridine orange can be used as an alternative to Ethidium bromide for the screening of multidrug resistant strains which overexpress their efflux pumps. Since the replacement of EB with AO eliminates the risks associated with the use of EB, the AO agar method bypasses the restrictions that have limited the use of EB in many countries. The basic concept behind the AO agar method is identical to the one using EB agar, and allows rapid and relatively easy screening of large numbers of strains whose multidrug resistance is due to overexpression of an efflux pump system. Our study has limitations as the sample size (n=100) is small. The reported data above require genotypic confirmation and further studies have been planned to investigate these isolates.

CONCLUSION

The activity of these efflux mechanisms needs to be recognized early as drug resistance rapidly develops in hospitalized patients undergoing treatment and corrective measures benefit the adjustment of therapeutic strategies that minimize the selection of resistant variants. Screening for efflux pump mediated resistance using Acridine orange can be used for *Enterobacterales*. In the present study, *E. coli* isolate exhibited higher prevalence of efflux pump positive strains than *Klebsiella pneumoniae* and *Enterobacter spp.* Acridine orange agar method affords the benefits of simplicity, consumption of inexpensive reagents, the use of inexpensive accessories normally found in a clinical laboratory, and the lack of need for expensive instrumentation. Therefore, Acridine orange can be used for the screening of efflux pump in a clinical microbiology laboratory.

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Author's Contribution: Anusha. A. P, Dr. Harish Kumar K. S, Rani S Jayamohan, Dr. Deepthy B. J contributed equally to the present study**.**

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Table 1: Antimicrobial susceptibility pattern of *Enterobacterales*

	Sensitive(%)	Resistant (%)	Intermediate (%)
Gentamicin	79	21	0
Amikacin	68	20	12
Imipenem	61	20	19
Cefuroxime	29.33	49.33	21.33
Cefoxitin	53.33	38.66	8
Ciprofloxacin	53	35	12
Aztreonem	61	29	10
Ampicillin	34	64	2
Amoxyclav	42.66	42.66	14.66
Tetracycline	66	28	6
Cefixime	54	42	4
Ceftazidime	38	40	27
Cefotaxime	37	47	16

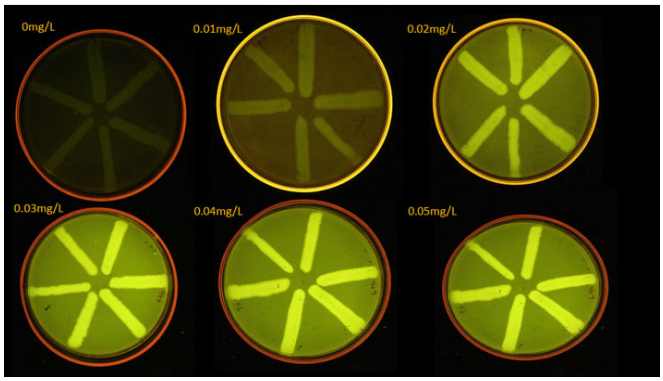

Figure 1: *E. coli* isolates exhibiting increasing fluorescence with increase in concentration of Acridine orange as viewed under 254 nm ultra violet light (Biotech Gel documentation system).

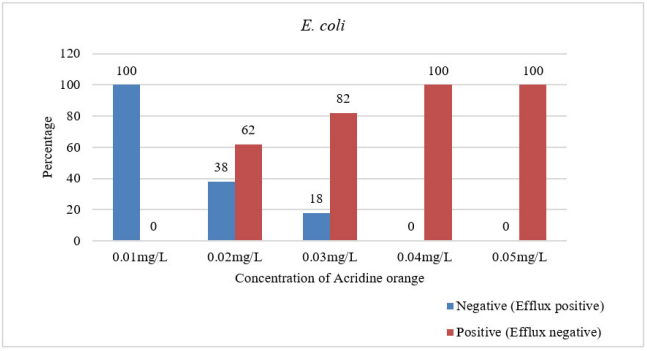

Figure 2: Results of efflux activity exhibited by *E. coli.*

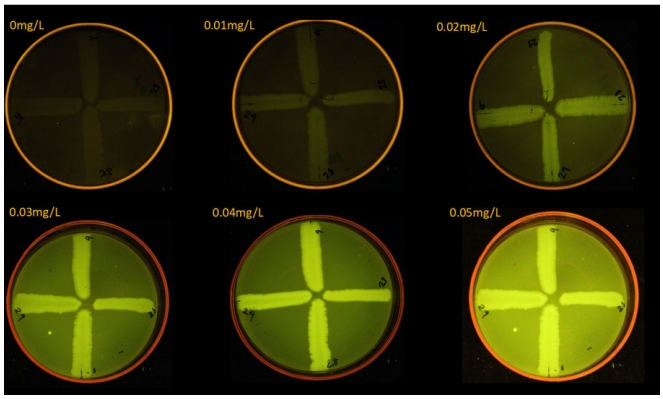

Figure 3: *Klebsiella pneumoniae* isolates exhibiting increasing fluorescence with increase in concentration of Acridine orange as viewed under 254 nm ultra violet light (Biotech Gel documentation system).

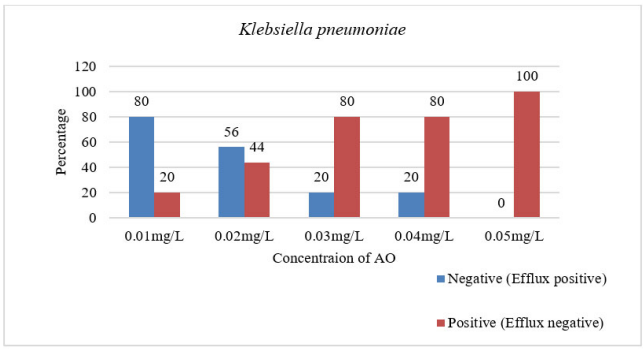

Figure 4: Results of efflux activity exhibited by *Klebsiella pneumoniae.*

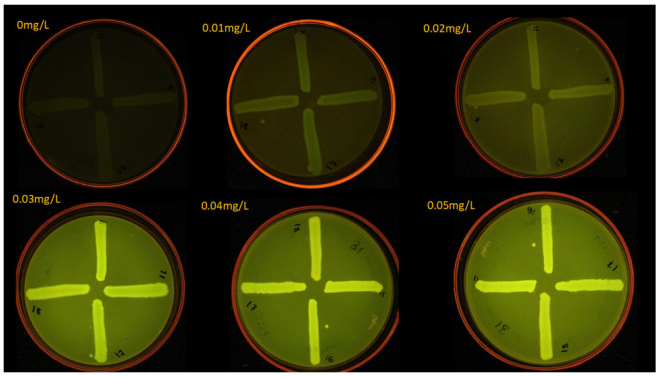

Figure 5: Enterobacter spp. isolates exhibiting increasing fluorescence with increase in concentration of Acridine orange as viewed under 254 nm ultra violet light (Biotech Gel documentation system).

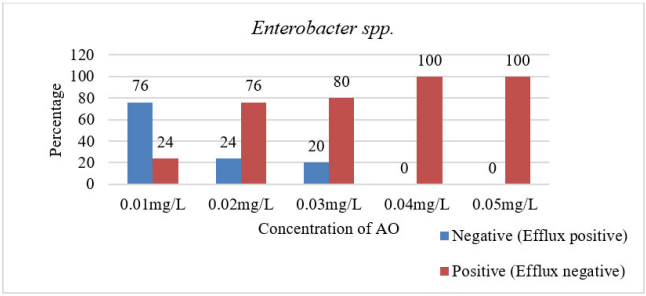

Figure 6: Results of efflux activity exhibited by *Enterobacter spp*.