

## Detection of efflux pump in multidrug resistant acinetobacter Species using Naphthyl-Methyl-Piperazine

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### Abstract

Acinetobacter, Gram negative bacterium, has proven to be recently important to research due to multidrug resistance. Multidrug resistance is directed against antibiotics like Aminoglycosides, Carbapenems, Cephalosporins, Quinolones etc. The presence of efflux pump, primarily the Resistance-Nodulation-Cell Division (RND) type, is an active transport mechanism which allows it to efficiently reduce antibiotic levels within the cell. 1-(1-naphthylmethyl)-piperazine (NMP) is an efflux pump inhibitor (EPI) which mechanically inhibits the efflux pump in bacteria resulting in increased concentration of antibiotic intracellularly, thereby killing the cell.

**Objectives:** To detect the efflux pumps phenotypically using NMP and to establish the cut-off zone diameter to detect efflux pump in Acinetobacter species.

**Materials and Methods:** Acinetobacter species were isolated from patient samples using conventional techniques of inoculation, identification and antibiotic susceptibility testing. The efflux pump was detected phenotypically by disc diffusion method on Mueller Hinton agar with NMP and chloramphenicol, tetracycline discs as substrates. The zone diameters were recorded & values compared using t-test. Tipping analysis was done to determine the mean zone difference to identify the presence of efflux pump.

**Results and Conclusion:** 61% of the Acinetobacter species were multidrug resistant of which 63% had efflux pump as detected by chloramphenicol substrate which is better than Tetracycline ( $p < 0.05$ ). A difference of 5 mm in the zone diameter can be used to detect efflux pump. The gene encoding for the efflux pump – AdeFGH was present in 72% of the isolates. NMP can be a potential adjuvant with common antibiotics to treat MDR Acinetobacter infections.

**Keywords:** Efflux pump, Acinetobacter, Naphthyl-Methyl-Piperazine.

### Introduction

Acinetobacter, a Gram negative bacterium of emerging complications, has proven to be recently important to research due to multidrug resistance. Its resultant infections have been troublesome especially within intensive care units where contact transmission is prevalent. Such contact transmission is facilitated by indwelling devices which cause bacteremia, ventilator associated pneumonia, meningitis, urinary tract infection, central line infections and wound infections. It is responsible for almost 45% of the ventilator associated pneumonias in tertiary care hospital across the world.<sup>1</sup> It is responsible for 48% of mortality due to pneumonia & sepsis along with another non fermenting bacilli – Pseudomonas.<sup>2</sup> Multidrug resistance of Acinetobacter is directed against antibiotics such as Aminoglycosides, Penicillins, Monobactams, Carbapenems, Cephalosporins, Quinolones and many others. In India, carbapenem resistance ranges from 9.1-100%<sup>3,4</sup> in *Acinetobacter* spp. isolated from various patient populations in different sample types, mostly consisting of respiratory specimens and wound swab samples. Mechanisms of antibiotic drug resistance have been studied in detail and include enzyme production, presence of efflux pumps, alteration of antibiotic binding sites and alteration of metabolic pathways.<sup>5</sup> In regards to Acinetobacter, the presence of efflux pump primarily the Resistance-Nodulation-Cell

Division (RND) type pump is governed by the expression of AdeFGH gene. Efflux pump inhibitors (EPIs) viz Phenylalanine-Arginine  $\beta$ -Naphthylamide (PA $\beta$ N), 1-(1-naphthylmethyl)-piperazine (NMP) and carbonyl cyanide-m-chlorophenylhydrazone (CCCP) are compounds which mechanically inhibits the efflux pump in bacteria resulting in increased concentration of antibiotic intracellularly, thereby killing the bacteria.<sup>6</sup> The efflux pump inhibitor can be used to study the presence or absence of the efflux pump in a particular isolate. 1-(1-naphthylmethyl)-piperazine (NMP) has been shown to be effective in case of inhibiting efflux pumps of Acinetobacter species.<sup>7</sup> Limited amounts of studies have taken place in India regarding the efflux pump as a mechanism for resistance and the EPIs as a possible adjuvant to antibiotics. This study aims to establish the burden of resistance due to efflux pump in Indian scenario. Most of the studies have used the broth dilution method to study the change in Minimum inhibitory concentration (MIC) of antibiotics.<sup>8</sup> The procedure is labor intensive and time consuming. Other disadvantage include greater rates of contamination and reader bias in interpretation of MIC. To overcome the disadvantages of broth dilution method, disc diffusion method was adopted and the zone diameter cut off was determined.

## Materials and Methods

This study was a prospective cross sectional study conducted at a tertiary care hospital. Inclusion criteria being Acinetobacter species isolated from all samples which are atleast resistant to 3 groups of antibiotics viz Cephalosporin, Fluoroquinolone, Aminoglycoside. The identification and susceptibility were done using conventional methods using MacConkey's agar, routine biochemical reactions and Kirby Bauer disk diffusion methods. Mueller Hinton agar with the Efflux pump inhibitor - Naphthyl Methyl Piperazine (100 µg/ml) was prepared. These plates were then blinded by labelling the plates with random numbers (generated using SAS software) for 2 groups. One group of numbers were for plain MHA plates and the other group for MHA with NMP. 2 plates (1 from each group) were packed together and used for conducting the experiment.

One set of plates as packed previously was used for each isolate of Acinetobacter species. A suspension of these isolates were prepared using peptone water and matched to 0.5 McFarland standard. Then they were inoculated as a lawn culture on the agar plates and Chloramphenicol & Tetracycline disks (Efflux Pump substrates) were placed. The zone of inhibition around the disc was measured on both the petri plates and recorded on excel sheet.

The AdeFGH gene coding for efflux pump was detected by conventional Polymerase chain reaction technique. The following primers (forward & reverse) were used.<sup>9</sup> The amplified products were detected by gel electrophoresis method.

**P1: GGATTATGGCGACAGAAGGA - 104 bp**  
**P2: AATACTGCCCAATACCAG**

**Table 1: Acinetobacter species positive for efflux pump**

Antibiotic Disc	No. of isolates showing positive	Percentage
Chloramphenicol	169	63.2
Tetracycline	122	45.6

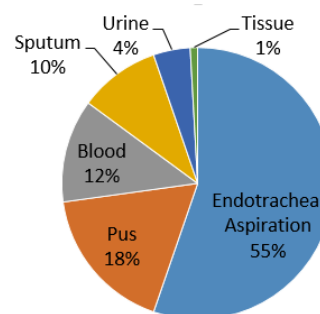


Legend – Left picture is plain MHA showing resistance (no zone of inhibition) & right picture showing MHA with PABN showing sensitive (large zone of inhibition)

**Fig. 2: Mueller Hinton agar plate with Efflux pump substrate disc**

## Results and Discussion

A total of 267 isolates of Acinetobacter were collected from different clinical specimen submitted to the microbiology laboratory. Endotracheal aspiration was the commonest (55%) clinical specimen which yielded Acinetobacter. This frequency of isolation is similar to other studies where an average frequency of isolation was 48%.<sup>10</sup> This indicates that the Acinetobacter colonizes the endotracheal tube commonly and causes pneumonia in artificially ventilated patients. Isolation from endotracheal aspiration is one of the criteria to diagnose ventilator associated pneumonia (VAP) according to the IDSA guidelines.



**Fig. 1: Clinical Samples that yielded Acinetobacter**

These 267 isolates were tested further for presence of efflux pump using Chloramphenicol & Tetracycline discs. This was done by placing the discs onto a lawn culture of the isolate on MHA plates with and without the NMP. The difference in zone diameter was calculated. Positive for efflux pump was arbitrarily defined as  $\geq 5$  mm difference between the zone diameter as observed around discs with and without the NMP.

More than half of the isolates showed presence of efflux pumps when tested with Chloramphenicol. The combination of efflux pump inhibitor - NMP and its significant effect on Chloramphenicol has been shown in the study conducted by Stefannie et al.<sup>10</sup> Hence Chloramphenicol is a better marker ( $p < 0.005$ ) to detect the efflux pump than Tetracycline as seen in this study.

The above results of detecting efflux pumps with Chloramphenicol disc was with the assumption of 5 mm difference in zone diameter being taken as significant. To check whether this is actually true in detecting the presence of efflux pumps and not as a confounding factor, the analysis of the results obtained were re-run using different cut off values viz 3 mm, 4 mm, 5 mm, 6 mm & 7 mm. T test was applied to determine the p value to establish the significance difference between the zone diameters. This was done only Chloramphenicol disc because the results obtained using this disc were better than Tetracycline disc. The following table shows the p values obtained for each these cut off zone diameters.

**Table 2: p value for various cut off value for zone diameter difference**

Difference in zone diameter	p value	Interpretation
3 mm	0.742	Not significant
4 mm	0.146	Not significant
5 mm	0.004	Significant
6 mm	0.926	Not significant
7 mm	0.995	Not significant

It can be observed that when the cut off of 5 mm is considered only then there is a significant difference between the groups which show presence of efflux pump and those which do not. This indicates that the assumption of 5 mm as cut off was indeed a correct value to detect the presence of efflux pumps. The literature available on the detection of efflux pumps by EPIs have used broth dilution methods to demonstrate 4 fold reduction in MIC of antibiotics. The broth dilution method is cumbersome and labor intensive. The biggest disadvantage being subjective interpretation of the turbidity after incubation. Since there are no studies which used disc diffusion method, it was difficult to decide on the cut off value to consider positivity for efflux pump. This additional analysis using different cut off values was done to overcome this deficiency and establish a cut off value for disc diffusion procedure.

The efflux pump gene - AdeFGH was detected by conventional PCR technique which showed 72% of the isolates had this gene. Since efflux pump was detected phenotypically in 63.2% of the isolates it can be concluded that there are many factors involved in expression of the gene.<sup>6</sup> The expression of these efflux pumps depends on a two-component regulatory system. Level of the expression of the pump, regulated by such

a system, is dependent on the presence of the stimulus (substrate) in environment of the cell, which activating the pump for removing substrate outside the cell. Antibiotics can serve as inducers and regulate the expression of some efflux pumps at the level of gene transcription or mRNA translation, by interacting with regulator systems.

## Conclusion

Acinetobacter, commonly isolated from endotracheal secretions from artificially ventilated patients exhibits efflux pump as the major mechanism of drug resistance. It can be detected by Naphthyl Methyl piperazine as the inhibitor. Chloramphenicol is a better substrate as compared to Tetracycline in detecting efflux pumps. Increase in zone size of 5 mm in presence of EPI indicates the presence of efflux pump.

EPIs like NMP can be a potential adjuvant drug that can be used clinically after modification to reduce its toxicity. Around 60% of the infections can be cured with a combination of conventionally used antibiotics along with EPI.

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