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Plasmid curing of multidrug-resistant bacteria by using phenothiazine

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ABSTRACT: Antibiotic-resistant genes are found in baScterial plasmids, hence removing them aids in the lowering of antibiotic resistance. The method of removing plasmids from bacterial cells is known as plasmid curing. Phenothiazine has a plasmid-curing action and plasma membranes of prokaryotes and eukaryotes are the primary targets of phenothiazines. The prokaryotic plasma membrane is affected by efflux pumps, their energy sources, and energy-supplying enzymes like ATPase, as well as genes that govern and code for a bacterium's permeability. The response of multidrug-resistant and extensively drug-resistant tuberculosis to phenothiazines indicates a new treatment option for these horrible diseases, which are claiming an increasing number of lives throughout the world every year. In this study, it has been found that those bacteria, which were resistant to some antibiotics after plasmid curing with phenothaizine, become sensitive to some of the drugs. Not much antibiotic sensitivity was achieved in Salmonella choleraesuis. Other isolates, on the other hand, demonstrate, better antibiotic sensitivity after plasmid curing with phenothaizine.

KEYWORDS: Plasmid curing, Multidrug resistance, Phenothiazine, Antibiotic sensitivity, Efflux pump.

I. INTRODUCTION

Antimicrobial resistance (AMR) is a worldwide issue that makes treating bacterial illnesses challenging. Many components of contemporary medicine are becoming less effective as a result of it. This gives the bacteria the ability to proliferate in antibiotic concentrations that would ordinarily be hazardous to them. AMR genes (ARGs) are commonly found on chromosomal and extrachromosomal plasmids. Plasmids are self-replicating DNA elements with the number of environmental and genetic variables that confer resistance to a single drug or a group of medications in bacteria [1]. Plasmid elimination is one method of establishing the source of bacterial drug resistance [2]. They're routinely passed from bacteria to the bacterium, and some have even spread globally. Plasmid curative and anti-plasmid techniques could reduce ARG prevalence and make bacteria more susceptible to antibiotics, which are both essential to tackle AMR. Plasmid curing is the process of removing plasmid DNA from bacterial isolates to determine the link between plasmid DNA and multidrug resistance [3]. Chemicals (such as detergents and intercalating agents), ascorbic acid, and phenothiazine, among others, are used as curing agents [4].

Phenothiazines are a class of nitrogen and sulfur-containing heterocyclic chemicals that are used to treat schizophrenia, bipolar disorder, nausea and vomiting, and other psychotic conditions with delusional symptoms. Phenothiazines were developed and utilized in the United States as the first commercial antipsychotic therapy in the 1950s [5]. Because there are so many compounds evaluated for antiplasmid properties, QSAR studies can be done to see if there is a link between the antiplasmid action and the supramolecular chemistry of these plasmid curing drugs. Plasmid elimination in vitro is a method for isolating plasmid-free bacteria for biotechnology applications without the risk of mutations [6]. Heterocyclic chemicals called phenothiazines show antibacterial activity against a wide range of microorganisms [7,8]. Agents with similar actions, such as promethazine and acridine orange, have also been demonstrated to reverse antibiotic resistance in several bacterial species when used as controls [9,10].

II. MATERIALS AND METHODS

Isolation of pathogenic Gram-negative bacteria from urine samples

Urine samples were collected from Indira Gandhi Medical College (IGMC), Shimla (H.P.). Information about their sex and age was also collected. The isolation of clinical samples was

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carried out according to standard protocol [11]. The samples were serially diluted (up to 10^{-8} dilutions) in the sterile saline and 100µl of each dilution was spread on the agar plates containing MacConkey agar. The plates were incubated at 37^{O}C for 24-48 hours for the growth of morphologically distinct pure bacterial colonies. The isolates were identified by Biochemical kit- KB003-Hi25^{TM}

Antibiotic sensitivity test [12]

The Epsilometer test (E-Test) was used to test the in vitro susceptibility of the pathogenic bacterial isolates to various antibiotics like ceftazidime, ceftriaxone, cefepime, etc. The inoculum was prepared according to a standard method and 30µl of inoculum of test organisms was swabbed on Muller-Hinton agar plates. Various antibiotic strips were dispensed onto the surface of inoculated media and incubated at 37ºC for 24h. The zone of inhibition was measured using a strip scale.

Plasmid isolation [13]

The cultures were grown on MacConkey agar medium for 24hours at 37ºC and centrifuged at 6000 rpm for 10 min. pellets were resuspended in lysis solution and then centrifuge it at 8000 rpm for 10min. The supernatant was transferred immediately to a fresh vial and ethanol was added precipitate the DNA. The vials were mixed inverting and incubated at room temperature for 10-15 min. The contents were spined at 10,000 rpm for 20 min and the supernatant was discarded. The vial was inverted on blotting paper to drain out the leftover supernatant. The pellet was resuspended in 20µl of 1X TE (added along the sides); mixed by tapping the vial so that DNA goes into solution. 5µl of RNase was added to vials and incubated at 37ºC for 20 min. The 1% agarose gel was prepared and 2µl of gel loading buffer was added to each of the samples. The 2µl of extracted DNA along with 3µl of control DNA sample was loaded on 1% agarose gel and electrophoresis was conducted at 100 volts for 2h. The gel was visualized under a UV transilluminator.

Plasmid curing [14]

LB broth was inoculated with a single colony of bacterial isolate and varying concentration of curing agent (phenothiazine) was added to each flask containing media. The culture was incubated for 24h at 37ºC with vigorous shaking.

Antibiotic sensitivity test after plasmid curing with phenothaizine [12]

The antibiotic sensitivity test was done to check the sensitivity of pathogens against the antibiotic after plasmid curing.

Plasmid isolation after plasmid curing with phenothaizine [13]

The plasmid isolation was done to check the effect of phenothaizine on plasmids after the antibiotic sensitivity test.

III. RESULTS AND DISCUSSION

Isolation of pathogenic Gram-negative bacteria

Total 64 urine samples were collected from IGMC, Shimla (H.P.). Out of these 21 isolates were Gram-negative bacteria and out of these seven bacteria were found the most resistant against a number of antibiotics (Table 1).

Table 1. Antibiotic sensitivity test of pathogenic Gram-negative bacteria

Antibiotic used	Salmonella Choleraesuis	Enterobacter sakazakii	Enterobacter gergoviae	Morganella morganii	E.coli	Enterobacter cloacae	Klebsiella pneumonia
Minimum inhibitory concentration in (µg/ml)							
Ampicillin	0	0	0	1.5	0	0	0
Aztreonam	0	0	0	0	12	0	0
Penicillin	0	0	0	0	0	0	0
Ceftriaxone	0	0	0	0	0	0	0
Norfloxacin	0	0	0	0	1.5	0	0
Amikacin	0	0	6	4	0	3	0
Amoxicillin	0	0	0	1	0	0	0
Cefepime	0	0	6	12	0	0	0
Streptomycin	64	24	3	4	32	24	0
Trimethoprim	0	0	0	0	0	2	0
Levofloxacin	0	0	0	0	0	0	0
Ciprofloxacin	0	0	0	0	0	0.50	0
Gentamicin	0	0	0	16	0	0	0
Erythromycin	128	0	0	128	0	64	0
Ceftazidime	0	0	0	0	0	0	0

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These most resistant bacteria are identified as Salmonella Choleraesuis, Enterobacter sakazakii, Enterobacter gergoviae, Morganella morganii, E.coli, Enterobacter cloacae, Klebsiella pneumonia. These seven isolates were the most resistant against penicillin, ceftriaxone, levofloxacin, ceftazidime,but they were sensitive against streptomycin.

The E-test was used to identify the minimum inhibitory concentration for amikacin, ceftazidime, ceftriaxone, ciprofloxacin, gentamicin, imipenem, and piperacillin [15]. Ipenem-4 mg/L, ceftazidime-8 mg/L, pipercillin-16 mg/L, ciprofloxacin-1 mg/L, gentamicin-4 mg/L were the breakpoints for susceptible and resistant groups. Among Gram-positive isolates, coagulase-negative staphylococci were the most common cause of bacteremia. Gram-positive isolates had a moderate level of antimicrobial resistance (60–80%), but Gram-negative bacteria had a high level of resistance (>80%) to ampicillin and amoxicillin [16].

Plasmid profiling of multidrug-resistance bacteria

Plasmid-mediated analysis of different multidrug-resistant bacterial isolates from different urine samples were observed by agarose gel electrophoresis which showed plasmid bands of different combinations. Different bacterial isolates show different sizes of plasmids on agarose gel (Fig. 1).

In a prior study, the number of plasmids that were analyzed ranged from one to five, with sizes ranging from 2.9 to 66 kb [17]. According to another study, the number of plasmids ranged from 1 to 7. The plasmid number from E. coli isolates ranged from 1 to 5, with sizes ranging from 0.5 to 40 kb [18]. The average copy number of uropathogenic E. coli isolated from children was 5.5 (range from 1 to 10) with plasmid sizes ranging from 1 to 33 kb, according to a plasmid analysis study [19]. Previous research revealed that some isolates only had one plasmid, ranging in size from 5 to 9 kb [20].

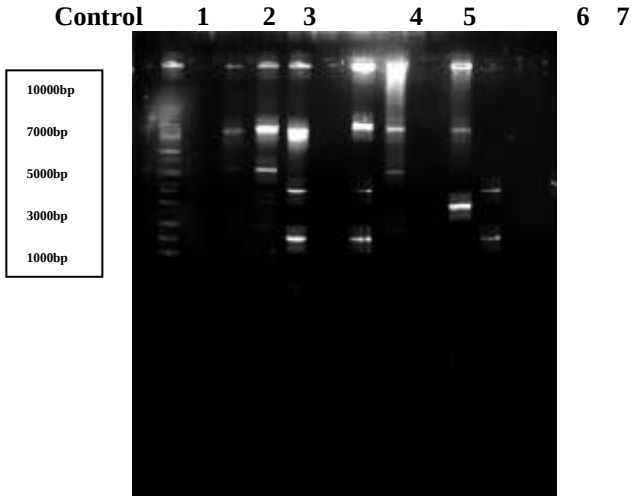

Fig. 1. Plasmid profiling of pathogenic Gram-negative bacteria (Lane 1- control, lane 2- Salmonella Choleraesuis , lane 3- Enterobacter sakazakii, lane 4- Enterobacter gergoviae, lane 5- Morganella morganii, lane 6- E.coli, lane 7- Enterobacter cloacae, lane 8- Klebsiella pneumoniae)

In this study, it was found that bacteria were resistant to some antibiotics but after plasmid curing with phenothaizine, they become sensitive to some drugs but not all. Salmonella Choleraesuis does not show a big difference in sensitivity against antibiotics. However other isolates show sensitivity against the antibiotics after the plasmid curing with phenothaizine (Table 2).

Table 2 Antibiotic sensitivity test after curing with phenothaizine

Antibiotic used	Salmonella Choleraesuis	Enterobacter sakazakii	Enterobacter gergoviae	Morganella mor	E.coli	Enterobacter cloacae	Klebsiella pneumonia

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Minimum inhibitory concentration in µg/ml							
Ampicillin	0 (resistive)	0 (resistive)	0.75 (sensitive)	0 (resistive)	0 (resistive)	0 (resistive)	0 (resistive)
Aztreonam	0 (resistive)	0.016 (resistive)	8 (sensitive)	0 (resistive)	16 (sensitive)	0 (resistive)	0 (resistive)
Penicillin	0 (resistive)	0 (resistive)	0 (resistive)	0 (resistive)	0 (resistive)	0 (resistive)	0 (resistive)
Ceftriaxone	0 (resistive)	0.19 (resistive)	0 (resistive)	0 (resistive)	0 (resistive)	0 (resistive)	0 (resistive)
Norfloxacin	0 (resistive)	0 (resistive)	0 (resistive)	8 (sensitive)	12 (sensitive)	0.016 (sensitive)	1.5 (sensitive)
Amikacin	0 (resistive)	2 (resistive)	6 (sensitive)	24 (sensitive)	1.5 (sensitive)	0 (resistive)	3 (sensitive)
Amoxicillin	0 (resistive)	0 (resistive)	0 (resistive)	0 (resistive)	0 (resistive)	0 (resistive)	0 (resistive)
Cefepime	0 (resistive)	0 (resistive)	16 (sensitive)	0 (resistive)	2 (sensitive)	0 (resistive)	0 (resistive)
Streptomycin	32 (sensitive)	12 (sensitive)	8 (sensitive)	32 (sensitive)	4 (sensitive)	4 (sensitive)	0 (resistive)
Trimethoprim	0 (resistive)	0 (resistive)	0 (resistive)	0 (resistive)	0 (resistive)	0 (resistive)	0 (resistive)
Levofloxacin	0 (resistive)	0 (resistive)	0 (resistive)	32 (sensitive)	0 (resistive)	0 (resistive)	0.38 (sensitive)
Ciprofloxacin	0 (resistive)	0 (resistive)	0 (resistive)	0 (resistive)	0 (resistive)	0 (resistive)	0 (resistive)
Gentamicin	0 (resistive)	0.25 (sensitive)	1 (sensitive)	2 (sensitive)	0.25 (sen	0 (resistive)	0 (resistive)

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					siti ve)		
Erythromycin	0 (resistive)	64 (sensitive)	0 (resistive)	32 (sensitive)	6 (sensitive)	2 (sensitive)	64 (sensitive)
Ceftazidime	12 (sensitive)	0.38 (sensitive)	0.19 (sensitive)	0.128 (sensitive)	12 (sensitive)	0 (resistive)	0 (resistive)

The response of multidrug-resistant and extensively drug-resistant tuberculosis to phenothiazines suggests a new treatment option for these dreadful diseases, which are claiming an increasing number of lives every year throughout the world. Many phenothiazines have been proven to have a synergistic effect with a variety of antibiotics, resulting in reduced antibiotic doses being given to patients with certain bacterial illnesses. Trimeprazine and trimethoprim have a synergistic effect. Penicillin and chlorpromazine have been reported to be synergistic with flupenthixol [21].

Plasmid isolation after plasmid curing with phenothaizine

The curing effect of phenothaizine on bacterial plasmids can be easily seen in Fig.2. Most of the plasmid bands were lost after the curing with phenothaizine.

Antibiotic-resistant genes found in bacteria with plasmids can result in catastrophic treatment failure [22,23] Antimicrobial Activity of phenothiazines manifested as a result of the selection of the resistant plasmid-containing strain [24,25]. Compound that can neutralize the potential impacts of plasmid antibiotic-resistant genes in a specific bacterial infection are clinically essential in light of these findings. Phenothiazines are known to aid in the removal of plasmids from infected bacteria to this level [26,27].

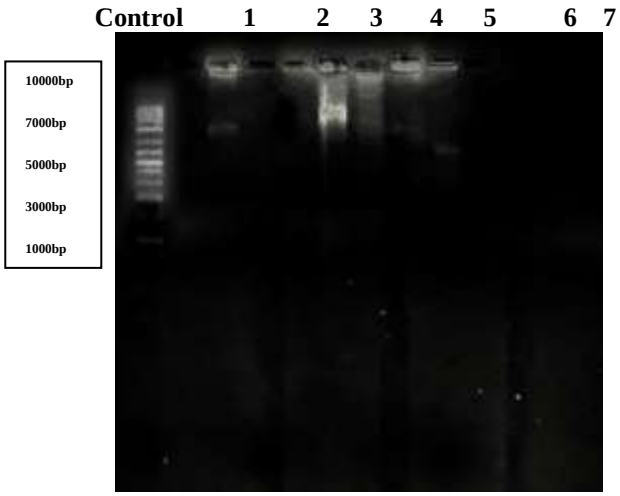

Fig. 3. Plasmid profiling of pathogenic Gram-negative bacteria after plasmid curing with phenothaizine (Lane 1- control, lane 2- Salmonella Choleraesuis , lane 3- Enterobacter sakazakii, lane 4- Enterobacter gergoviae, lane 5- Morganella morganii, lane 6- E.coli, lane 7- Enterobacter cloacae, lane 8- Klebsiella pneumoniae).

IV. CONCLUSION

In addition to the antibacterial properties, phenothiazines include plasmid curing properties. As in this study after the antibiotic sensitivity test, it was found that Salmonella Choleraesuis, Enterobacter sakazakii, Enterobacter gergoviae,

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and Klebsiella pneumoniae were revealed to be the most resistant bacteria. After antibiotic sensitivity testing penicillin, ceftriaxone, levofloxacin, and ceftazidime resistance was highest among the seven isolates, while they were sensitive to streptomycin. The sensitivity of Salmonella Choleraesuis to antibiotics after plasmid curing with phenothaizine is not significantly different however, some isolates demonstrated better antibiotic sensitivity.

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Conflict of interest

We declare that we have no conflict of interest.

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