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Abundance of antibiotic resistance in natural environmental flora in recent years has become a threat to public health. Various studies during the last decade have shown that the soil micro flora is a huge reservoir of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG). These environmental resistome is in continuous equilibrium with clinical pathogens with exchange of antibiotic resistance factors through horizontal gene transfer. Anthropogenic activity has significantly increased this telltale series of exchange. Therefore, urban environmental soils are perfect study materials to find environmental ARB and ARGs which may lead to a better understanding of evolution of antibiotic resistance. In this report we discuss about the isolation and characterization of a third generation cephalosporin resistant Gram positive isolate from urban environmental soil. Moreover, the ability of the bacteria to form biofilm in presence or absence of antibiotic has also been discussed.

Key words: Antibiotics, Soil Bacteria, Antibiotic resistance, *Bacillus cereus*, environmental bacteria

INTRODUCTION

Antibiotic resistance is an emerging public health threat. The COVID 19 pandemic has indicated how a microscopic or sub-microscopic organism can put the whole world at a stake. In this respect, antibiotic resistant bacteria can aptly be said as sleeping demons which in no time can endanger the world public health and economy as well.

Until recently, antibiotic resistance emergence and distribution is discussed in terms of clinical pathogens. But in recent times, several studies have (Aminov, 2009) proved the importance of environmental bacterial flora in the propagation of antibiotic resistance. Although several studies have shown the importance of soil microflora in the evolution, development and dissipation of antibiotic resistance, the knowledge was somewhat limited to naturally antibiotic producing soil dwelling *Streptomyces* sp. and isolated cave microbiome. Since most of the antibiotics used are produced by soil bacteria, soil is a potential source of antibiotic resistant bacteria and antibiotic resistance factors. The presence of antibiotics in soil is believed to throw an evolutionary selection pressure to the soil dwelling microorganisms towards the production ofantibiotic resistance genes. This belief is based on studies, which have shown some clinically important antibiotic resistance genes such as blaCTX-M, qnrA and blaNDM to originate in the environmental bacteria *Kluyvera sp*., *Shewanella* sp. and *Erythrobacter litoralis*, respectively (Oliver *et al*. 2001). So, soil serves as a reservoir which can be a probable source of antibiotic resistance genes (ARGs) for the clinical pathogens. On the other hand, continuous release of antibiotics to the environment from hospitals, livestock facilities, and sewage treatment plants poses the soil bacteria under antibiotic selection pressure making them more prone towards the development of antibiotic resistance. These evidences force us to try for better understanding ofthe ecology and evolution of antibiotic resistance of the soil micro flora. The current study discusses the isolation and characterization of bacterial isolate TIUJB6 which

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was found to be resistant to 3rd generation cephalosporins. It showed strong biofilm forming ability in presence of Ampicillin

MATERIALS AND METHODS

Isolation and screening of Ampicillin resistant bacteria

The soil sample was collected aseptically from North 24 Parganas district of West Bengal, India (22º48' 58.7" N, 88º 24' 59.7" E) to isolate Ampicillin resistant bacteria. Soil (1g) was suspended in 9.0 ml sterile distilled water, agitated for a minute and 0.1 ml suspension was spread over enrichment medium agar plates containing 0.5% yeast extract, 0.5% tryptone, 1% magnesium sulphate, 3% sodium chloride, 0.15% potassium chloride, 0.05% sodium nitrite, 0.05% potassium nitrate, 1.5% agar and 20 μg/ml of ampicillin and incubated for 48 h at 37°± 1°C. Bacterial strains that were resistant to10 μg/ml of ampicillin were selected for further studies and werestored at "80 °C (as 50% glycerol stocks) for future experiments.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by Kirby-Bauer disc diffusion test using Mueller-Hinton agar plates (HiMedia). (Uwizeyimana *et al*. 2020).Twentyfour antibiotics were tested including Penicillin G [PEN] (10 μg), Ampicillin [AMP] (20 μg) Ceftazidime [CAZ] (30 μg), Aztreonam [AZT] (30 μg), Piperacillin [PIP] (100μg), Imipenem [IMI] (10 μg), Cefpodoxime [CPD] (10 μg), Cefpodoxime/ Clavulanic Acid [CEF/CLAV] (10/5 μg), Cefotaxime [CTX] (30 μg), Ceftriaxone [CTR] (30 μg). Kanamycin [KAN] (30 μg), Amikacin [AK] (30 μg), Streptomycin [STR] (30 μg), Ciprofloxacin [CIP] (5 μg), Chloramphenicol [CHL] (30 μg), Tetracycline [TET] (30 μg), Vancomycin [VAN] (30 μg), Rifampicin [RIF] (5 μg), Co- Trimoxazole [COT] (25 μg), Amoxyclav [AMC] (30 μg), Gentamicin [GEN] (10 μg), Ofloxacin [OF] (5 μg), Cefuroxime [CXM] (30 μg) and Tobramycin [TOB] (10 μg).The plates were incubated for 24 hour at 37ºC. After the incubation period, the susceptibility of the bacterial isolates was determined as recommended by CLSI (Wikler, 2006). The multiple antibiotic resistance (MAR) index was determined for all isolates and is defined as a/b where 'a' represents the number of antibiotics the isolate was resistant to and 'b' represents all the antibiotics the isolate was tested against (Krumperman 1983). A MAR index value of<or 0.2 indicates a very low antibiotic resistance while a MAR index value of >0.2 indicates that the isolate had resistance to more than two antibiotics. *E. coli* Dh5a was used as quality control strain.

Microdilution method–determination of Ampicillin MIC for selected isolates

In 96 well plate, serial dilutions of ampicillin were prepared and standardized inocula of the isolates were added to obtain a final volume of 200 µL and ampicillin concentrations of 10-100 µg/ml. Medium (LB) without inoculum was negative control while positive control was a standardized inoculum. The plate was incubated at 37ºC for 24 h. Then absorbance was measured at 600 nm and MIC was defined as the lowest concentration at which no change in optical density from the negative control (only media) was observed.

Assay of biofilm formation and effect of Ampicillin on biofilm production

Biofilm assay was performed according to the protocol described by Bhattacharyya *et al*. (2019). The nutrient broth was prepared without ampicillin. It was then distributed 100 μl in each well of 96 well plate. Ampicillin was then added in increasing concentrations which were 25 μg/ml, 50 μg/ml, 100 μg/ml, 200 μg/ml, and 500 μg/ml. One well was without ampicillin. 5 μg overnight grown TIUJB6 was added to each well and incubated at 37°C for 48 hours. The plate was then washed using distilled water. Then 200 μl 1% crystal violet was added to each well and the plate was incubated at 37°C for 1 hour. The plate was again washed with distilled water and then it was kept in the incubator for drying. 90% ethanol was added to each well and incubated at 37°C for another hour. The O.D. of the wells were then measured at 590 nm.

Auto aggregation Test

Auto aggregation property was determined according to a previous study. (Sternišа*et al*. 2019). Overnight bacterial culture were centrifuged at 3000 rpm for 5 min, then washed twice and resuspended in Phosphate Buffer Saline (PBS) to get a final optical density of 1.00 at 600 nm. The absorbance of the upper phase of cell suspension was measured at 0 h, 4 h and 24 h at 600 nm. Auto aggregation was determined as the auto aggregation percentage (%AA) using the formula: %AA = [1 " (At/A0)] × 100

Hydrophobicity Test

Bacterial hydrophobicity was measured by measuring its adherence to xylene in a xylene-water system. (Kurin¡ci¡c*et al*. 2016). Bacteria were cultivated in nutrient broth, centrifuged for 5 min at 3000 rpm and washed twice in PBS buffer. 0.5 ml of 12.5% (v/v) p-xylene (HiMedia) was added to 3.5 ml of the cell suspension in PBS and vortexed for 2 min.The suspension was incubated for 20 min at room temperature. The absorbance was measured at 620 nm. The hydrophobicity was determined as a hydrophobicity percentage (%H) using the following formula: $\%H = [1 - (A/A_0)] \times 100$ where A_0 was the absorbance of the cell suspension (before phase separation) and A was the absorbance of an aqueous phase after 20 min incubation (after phase separation). These resulting percentages defined the degree of hydrophobicity [hydrophilic (<20%), moderately hydrophobic (20–50%) and hydrophobic (>50%)].

Motility Test

The swimming motility was determined according to (O'May and Tufenkji, 2011; Von Rosenvinge *et al*. 2013). 1 µL of overnight bacterial culture was placed in medium with 0.3% (w/v) agar (Himedia). The motility zones were measured (in mm) after 24 h incubation at 37 ºC. The motility defined as " negative if zone diameter is 0 mm; + positive if diamter1–23.3 mm; ++ moderately positive if diameter 23.4–46.5 mm; +++ medium positive if diameter 46.6–69.9 mm); ++++ strong positive if diameter e"70 mm.

RESULTS AND DISCUSSION

Isolation of Ampicillin resistant bacteria

Inoculation of the sample on the Ampicillin containing medium resulted in the isolation of 47 colonies on the agar plate. The colonies were checked for colony morphology and appearance. 20 unique colonies were selected for further experiments.

Antimicrobial susceptibility testing

All of the 20 isolates were resistant to Penicillin and Ampicillin. 14 of them were resistant to combination of β–lactam and β–lactamase inhibitor clavulanic acid as well as to aztreonam (TIUJB1-11, TIUJB14, 15, 16, 17 and TIUJB19). Resistance to DNA gyrase inhibitors, polypeptide antibiotic vancomycin or protein synthesis inhibitors was absent except TIUJB6. TIUJB6 was found to be resistant to Tetracycline as well. Therefore, TIUJB6 was selected for the further experiments.The antibiotic resistance profile of TIUJB6 has been demonstrated in Fig.1. The MAR index of TIUJB6 was found to 0.45 which is indicative of moderate antimicrobial resistance.

Table .1 : Biochemical characters related to biofilm formation of TIUJB6

Biochemical characters	Activity
Hydrophobicity	Hydrophobic
Auto aggregation	Moderate
Motility	+++

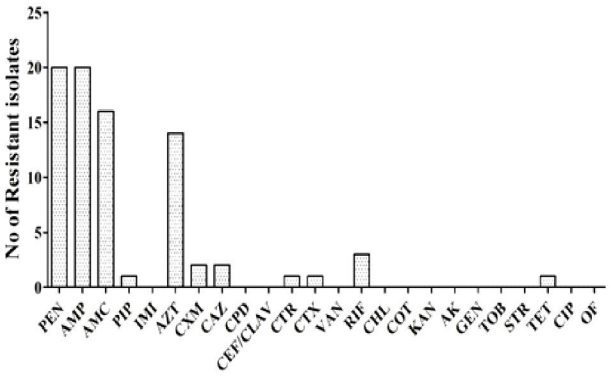

Fig.1 : Antibiotic resistance profiling of bacteria isolated from the soil sample

Ampicillin MICfor TIUJB6

The minimum inhibitory concentration of ampicillin was determined. TIUJB6 was resilient even up to 100 µg/ml of ampicillin as observed from the broth micro dilution method (Fig. 2A).

Effect of Ampicillin on biofilm formation

In this study, we observed the dynamics of biofilm formation by TIUJB6 in increasing concentration of ampicillin (Fig.2B). In lower concentration of ampicillin the TIUJB6 bacteria showed higher biofilm formation while increasing the antibiotic to much higher concentration led to the inhibition of biofilm formation, following Gaussian distribution.

Auto aggregation Test, Hydrophobicity Test and Motility

The bacterial hydrophobicity, auto aggregation and motility (Table 1) and their importance in ampicillin-

resistant isolate was investigated. Several studies showed the importance of biofilm formation in antibiotic resistance. It is a well established fact the hydrophobic properties of bacteria can be an important factor in biofilm formation. Therefore, hydrophobicity of TIUJB6 was studied and it was found to be hydrophobic cell surface properties. (Table 1). Auto aggregation is another property of bacteria which is important for biofilm formation. TIUJB6 showed increasing auto aggregation from 5 h to 24 h and found to be moderately auto aggregative. Motility is another decisive factor in bacterial biofilm formation. TIUJB6 showed strong motility on 0.3% agar plates (Table1).

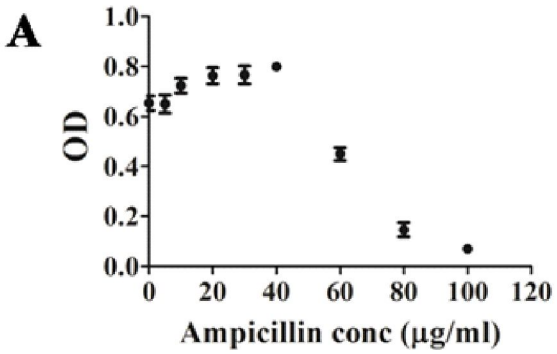

Fig. 2A: Growth dynamics of TIUJB6 in presence of increasing concentration of Ampicillin (ìg/ml) by measuring optical density at 600 nm.

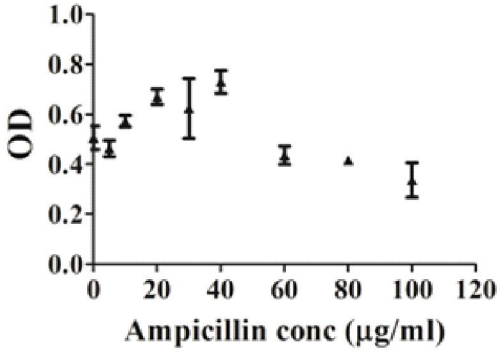

Fig. 2B: Effect of different concentrations of ampicillin (ìg/mL) biofilm formation (determined with crystal violet assay measured at 584 nm) of TIUJB6.

In this study, the urban soil was collected from North 24 Parganas district of West Bengal, India. Samples were collected from the surface layer (0–10 cm) to enumerate total bacteria resistant to ampicillin in that soil samples.A total of 47 ampicillin resistant isolates were obtained from the soil sample. Among them 20 isolates appearing unique in colony morphology were studied further (TIUJB1-TIUJB20). The strain TIUJB6 was found to be most resistant to different class of antibiotics. It was mainly resistant to β-lactams while resistance to aminoglycosides or polypeptide antibiotic vancomycin or DNA-gyrase inhibitors was not observed. The isolate was also resistant when β-lactams was combined with a beta-lactamase inhibitor (clavulanic acid).In addition to Penicillin G, ampicillin and amoxicillin/ Clavulanic acid resistance the strain was resistant to Aztreonam. Aztreonam resistance is important in this respect that Aztreonam resistant strains sometimes exhibited increased resistance to structurally unrelated antibiotics. The strain was also resistant to cephalosporins. It showed resistance to 3rd generation cephalosporins like ceftazidime, ceftriaxone and cefotaxime. But it was found to be susceptible to another 3rd generation cephalosporin, Cefpodoxime and to the carbapenems. It was also resistant to rifampicin antibiotic. Cephalosporin resistance is escalating these days with most of the Enterobacteriaceae became showing resistance towards even 3rd generation cephalosporins. Our study demonstrated the presence of environmental resistance to 3rd generation cephalosporins which is indicative of enhanced anthropogenic activity which might be influencing the dissemination of antibiotic resistance factors from clinical settings to environment. Bacteria employ several mechanisms to survive in extreme conditions. TIUJB6 was found to tolerate ampicillin concentration upto 100 µg/ml as observed by the broth dilution experiment.One of the ways to survive under stress is the formation of biofilm (Burmolle *et al*. 2010; Janez *et al*. 2021). Clinical isolates have shown greater survival against a high antibiotic selection pressure in the biofilm form (Tolker- Nielson, 2015). But similar studies with environmental isolates are not that prominent. In the present study, we observed that the dynamics of biofilm formationby TIUJB6 in the presence of antibiotic (ampicillin). It was observed that lower concentration of ampicillin promoted biofilm formation while higher concentration inhibited the biofilm formation.Several studies strongly suggest that low concentrations of antibiotics in the environment act as positive modulator of quorum sensing in bacteria which is necessary to trigger biofilm formation. (Balcazaret*et al.*2015). In higher concentration, the antibiotic might actually inhibit

or kill the planktonic bacteria leaving very less bacteria to form biofilm. This might explain the observation of inhibition of biofilm formation at higher antibiotic concentration. Overall, biofilm formation countered the effect of ampicillin on the growth of TIUJB6 and resisted the antibiotic's effect. Aggregation is a pre requisite of biofilm formation. TIUJB6 showed moderate auto aggregating property which comply with its strong biofilm forming ability. Bacterial motility is also a decisive factor in biofilm formation.TIUJB6 has high motility which most possibly involved in biofilm formation. Moreover TIUJB6 was hydrophobic in nature, a factor which might play a crucial role in its biofilm formation ability. Therefore, taken together the isolated bacteria TIUJB6 demonstrates most plausible case of horizontal gene transfer by which resistance determining factors get entry into soil from clinical settings either by waste material contamination or uncontrolled use of antibiotics in soil.

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