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Research Article

EMERGENCE OF A MULTIDRUG RESISTANT *HALOMONAS HYDROTHERMALIS* STRAIN VITP09, PRODUCING A CLASS-A β-LACTAMASE, ISOLATED FROM KUMTA COAST

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ABSTRACT

A multiple antibiotic resistant halophilic bacterium (VITP09) was isolated from the Head-Bunder Lake (Kumta coast, Karnataka, India). The bacterium was found to be Gram negative, motile, moderately halophilic and showed considerable growth in 3 to 5% of sodium chloride and can tolerate upto 21% of sodium chloride. Its 16S rRNA gene sequence indicated 99% identity to *Halomonas hydrothermalis* strain SMP3M. Among the different antimicrobial agents used, the halophilic bacterium was found to be resistant to ampicillin (1.02 mg/ml for MIC_{90}), methicillin and vancomycin. β-lactamase assay in the presence of sulbactam confirmed the involvement of class-A β-lactamase in *Halomonas hydrothermalis* VITP09 resistance. Presence of sodium chloride (1 to 11%) in the growth medium does not affect the production of β-lactamase. Analysis of the enzymatic parameters revealed a K_M of 88.33 µM, which is comparable to the β-lactamase reported for the enzyme isolated from the clinical isolates. The presence of such β-lactamase mediated antibiotic resistance in halophilic organisms could be a potential environmental concern and the emergence of such resistant halophiles and the spread of antibiotic resistance among halophiles, is dangerous for organisms of aquatic, terrestrials and human health.

Keywords: β-lactam antibiotic, β-lactamase, Ampicillin, *Halomonas hydrothermalis,* Halotolerant, Multi-drug resistance.

INTRODUCTION

The emergence of multidrug resistant bacteria and the increasing ability of the bacteria to produce β-lactamases with different substrate profiles remains an important public health concern throughout the world[1-3]. The Infectious Diseases Society of America has recognized that the management of Gram-negative pathogens as one of the most problematic bacterial challenges in the infectious disease community today[4]. Resistance to β-lactam antibiotic in Gram negative organisms are mostly due to the secretion of β-lactamases[5], and a variety of extended-spectrum β-lactamase (ESBL) enzymes have been detected from these organisms[6]. It is surprising to note that most of the β-lactamase producing organisms are obtained from the community and not in hospitals[7]. It is increasingly evident that most of these microorganisms, pathogens and commensals, are replete with drug resistance genes[8] and the number of microorganisms with drug resistance is still on the raise. Environmental microbes that are either non-pathogenic or opportunistic pathogens have also been found to be more drug resistant in comparison to the bacteria typically associated with disease and therefore the role of these organisms as potential reservoirs of resistance genes is becoming a focus of research[9-12]. There is increasing evidence that resistance genes can be transferred between different bacterial Populations, including transfer from commensal bacteria to pathogenic bacteria[13,14] and *vice-versa*.

Halomonas are organisms that thrive in high salt environment and belong to the family *Halomonadaceae*[15]. *Halomonas venusta*, a moderately halophilic, non fermentative Gram-negative rod, was reported for the first time in *Halomonas* species as a human pathogen in a wound that originated from fish bite[16]. The consumption of raw or insufficiently cooked seafood may lead to their transmission of infection to humans[17]. Also *Halomonas phocaeensis*, highlighted the pathogenic potential of the genus *Halomonas*[18]. Recently, three novel *Halomonas* species have been isolated from the blood of two patients and from the dialysis machines in a renal care center[19]. Though many species of *Halomonas* has been identified and reported from many sources, their multiple antibiotic resistances and possible pathogenicity is not much investigated.

In the present study we report a new isolate of *Halomonas hydrothermalis* VITP09, from the Head-Bunder Lake of coastal Karnataka (India) that exhibited multidrug resistance. This property was found to be associated with class-A β-lactamase production. To our knowledge, this is the first report on the presence of class-A β-lactamase in *Halomonas* bacterium.

MATERIALS AND METHOD

Bacterial Isolation and Screening

Pure bacterial colonies, from the soil and water samples of Head-Bunder lake (Coastal Karnataka, India, 14.42°N and 74.4°E), were isolated[20] and cultured aerobically in Zobell marine broth at 37 °C and maintained as pure cultures in LB agar stabs and glycerol stocks. β-lactamase producing organisms were selected by β-lactamase iodometric assay and the potential strain (designated as VITP09 from series of isolates) with highest β-lactamase secretion was selected for further characterization. Unless otherwise stated all chemicals, antibiotics and medium were purchased from Hi Media (Mumbai). All experiments were performed in triplicate and the graphs were plotted using Sigma Plot V-10.

Microbial Characterization

Morphological and physiological characteristics of the isolate VITP09 were studied as per the standard protocols[21]. The strain was further identified by 16S rRNA amplification and nucleotide sequencing (Chromous Biotech Pvt. Ltd. Chennai, India). The nucleotide sequence was analyzed using CLUSTAL W and the phylogenetic relation was arrived using the neighbor joining method.

Antimicrobial Susceptibility

The antimicrobial susceptibility tests were performed by Kirby Bauer method[22] on Mueller- Hinton agar plates. The antimicrobial discs used were amikacin (30 µg), ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), gentamycin (10 µg), kanamycin (30 µg), linezolid (30 µg), nalidic acid (30 µg), methicillin (10 µg), rifampicin (5 µg), tetracycline (30 µg), vancomycin (10 µg) and ampicillin (10 µg) with sulbactam (10 µg). Growth on the agar medium without antibiotic disc was taken as the control.

Microbial Growth

The overnight grown culture (with an optical density of 1.0 at 550 nm) of VITP09 strain was used to inoculate the synthetic medium containing peptone (5.0 g/l) and yeast extract (1.0 g/l). The growth (at 37°C and agitation rate of 120 rpm) was monitored at regular time intervals by measuring the optical density of the culture at 550 nm. To study the effect of salt on the growth and production of β-

lactamase, sodium chloride (0 to 23% w/v) was included in the growth medium.

β-lactamase Assay

Assay for β-lactamase was performed as per the method by Sagent *et al*[23]. Accordingly, the crude extract obtained after centrifugation (20000 x g for 30 min) of cell lysate (in 0.1 M phosphate buffer, pH-7) was added to 5 µl (135 µM) of ampicillin with rapid mixing. After incubation at 30°C for 30 min, 125 µl of 1 µmol iodine reagent [prepared by adding 5 ml of iodine solution (0.16 M iodine and 1.2 M potassium iodide) to 95 ml of the acetate buffer pH 4.0] was added to stop the reaction. The absorbance was then measured at 490 nm using ELISA reader (Bio Rad, Model 680). One unit of enzyme activity was defined as the amount of enzyme which hydrolyses 1 µmol of substrate per minute under the specified conditions. The assay was also performed in the presence of β-lactamase inhibitor (sulbactam). To determine the steady state kinetic parameters, the enzyme was incubated with varying ampicillin concentrations (in the range of 50–300 µM).

Determination of Minimal Inhibitory Concentration (MIC)

The minimal inhibitory concentration (referred as MIC_{90}) of the antibiotic was determined using the micro dilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2009)[24]. Accordingly, 100 µl of the sterile medium was pipetted into the wells of a sterile microtitre plate. To this, 100 µl of antibiotic solution (stock 100 mg/ml) was added. The solution was serially diluted and inoculated with VITP09 strain. The plates were incubated at 37°C for 24 h, and the optical density was measured at 550 nm using an ELISA reader. MIC_{90} was obtained from the correlation between the antibiotic concentration and percentage inhibition.

RESULTS

Bacterial Strain Selection and Characterization

Halophilic bacterial strains isolated from the Head-Bunder Lake of coastal Karnataka were screened for antibiotic resistance against ampicillin. Bacterial strain VITP09 which contributed maximum β-lactamase activity (0.219 U/ml) was used for further investigation. This isolated strain was subjected to morphological characterization. The strain, VITP09, was Gram negative, rod shaped, non flagellate and motile. The 16S rRNA was partially sequenced (1441 bases) and the sequence analysis indicated that the organism belonged to the *Halomonas* cluster (Figure 1). The sequence exhibited 99% identity with *Halomonas hydrothermalis* strain SMP3M and therefore identified as *Halomonas hydrothermalis* VITP09. The 16S rRNA sequence has been submitted at the GenBank (Accession no. JN657266).

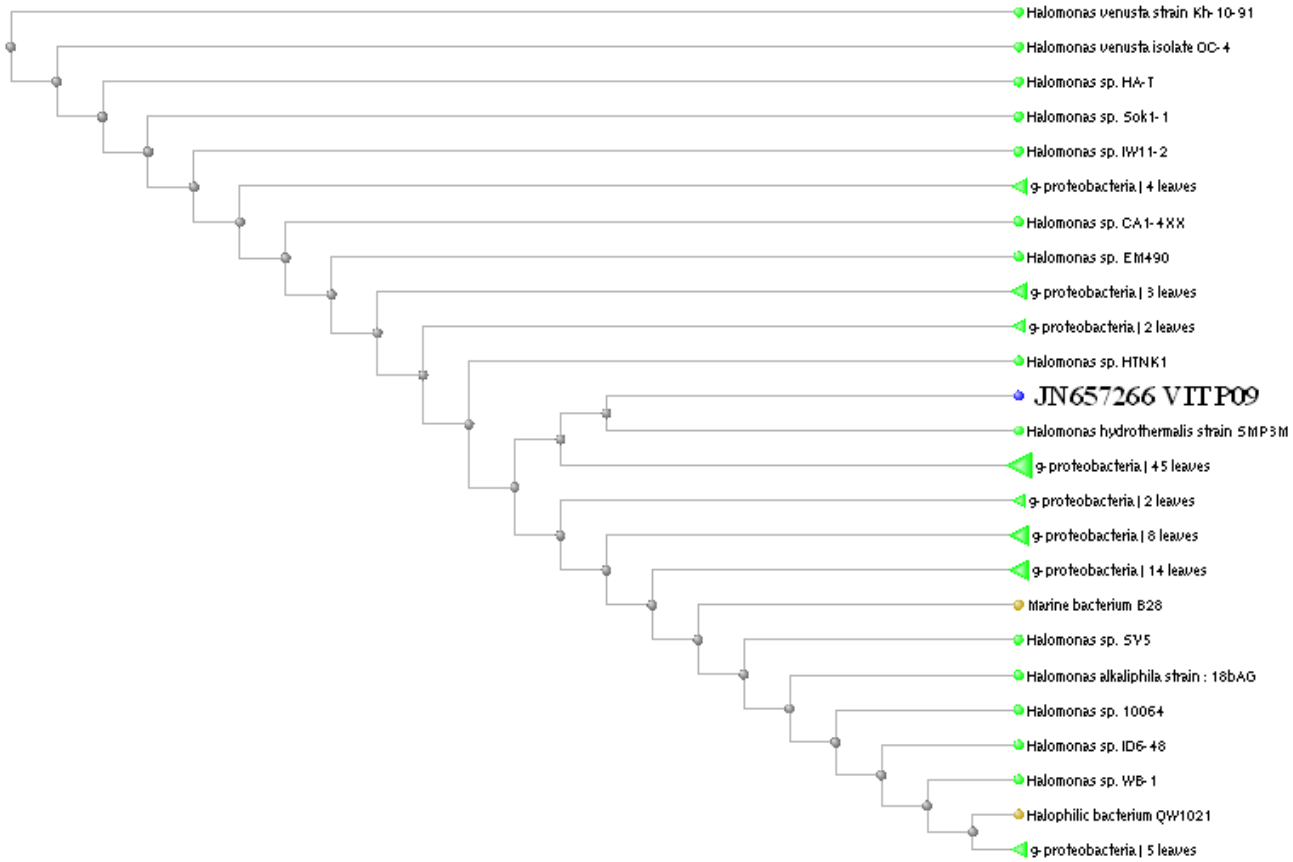

Fig. 1: Phylogenetic analysis of the isolated VITP09 strain. The tree was generated using the neighborhood joining method. The isolated strain belongs to the *Halomonas* cluster and shows 99% sequence similarity to *Halomonas hydrothermails*.

Antimicrobial Sensitivity Test

In order to investigate the spectral range of antibiotic susceptibility of *Halomonas hydrothermalis* VITP09, the bacterial growth was monitored in the presence and absence of different antibiotics. Amikacin, chloramphenicol, ciprofloxacin, gentamycin, kanamycin, linezolid, nalidic acid, rifampicin, tetracycline and ampicillin was used in the study. With the exception of ampicillin, methicillin and vancomycin, the bacterial growth was inhibited by all other antibiotics. As ampicillin is a β-lactam antibiotic, it is therefore possible that the organism might produce hydrolytic enzyme to counter the effects of the ampicillin. In order to confirm this, the β-lactamase activity of the cell free culture supernant was performed in the presence and absence of β-lactamase inhibitor, sulbactam. Figure 2 depicts that the enzyme activity was reduced by 94% in the presence of sulbactam (10 µmol). This suggests that the ampicillin resistance is due to the production of class-A β-lactamase by *Halomonas hydrothermalis* VITP09.

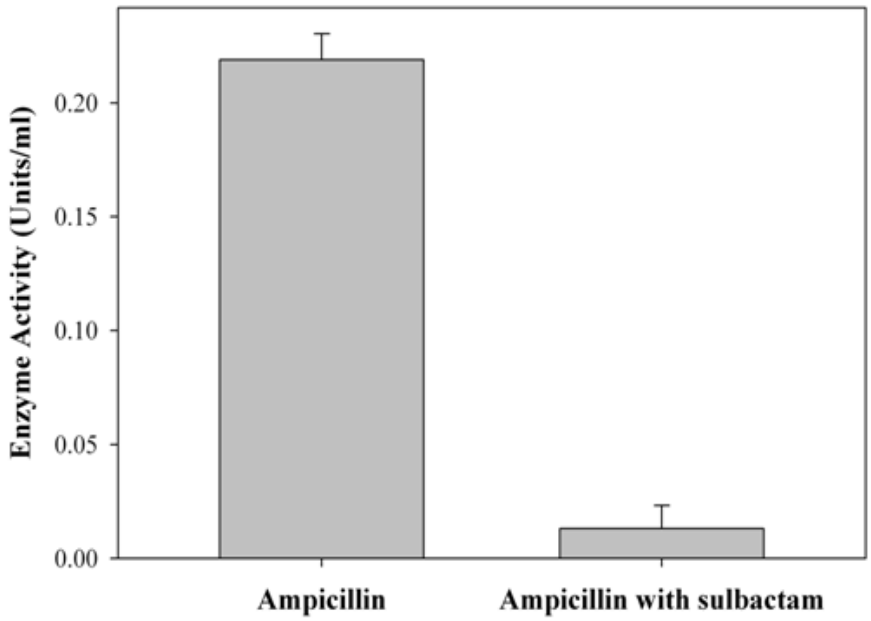

Fig. 2: β-lactamase activity of the *Halomonas hydrothermails* VITP09 in the presence and absence of β-lactamase inhibitor, sulbactam (10 µmole). The enzyme activity is reduced to 94% in the presence of sulbactam.

MIC of Ampicillin

In order to arrive at the minimum inhibitory concentration of the antibiotic, the β-lactamase activity was studied as a function of ampicillin concentration (Figure 3). The inhibitor concentration (MIC_{90}), defined as the concentration required for 90% inhibition, was found to be 1.02 mg/ml (2746 µM). Correspondingly, the MIC_{50} was found to be 0.69 mg/ml (1858 µM).

Effect of Salt on Cell Growth and β-Lactamase Production

As *Halomonas hydrothermalis* VITP09 belongs to the group of organisms in the halophilic cluster, we investigated the effect of salt on growth of this bacterial strain as well as on β-lactamase production. The organism exhibited considerable growth (Figure 4a) in the presence of different concentrations of sodium chloride and could tolerate upto a 21% (w/v) salt concentration, with a maximal growth after 36 h. Figure 4b indicates the number of units of β-lactamase produced as a function of both bacterial growth and concentration of sodium chloride. It was inferred that β-lactamase production was maximum in the presence of 5% (w/v) salt. It could also be observed that there is one- to- one correlation between the enzyme activity and the amount of biomass produced (as inferred from the cell growth). This indicates that the production of the enzyme is growth assisted.

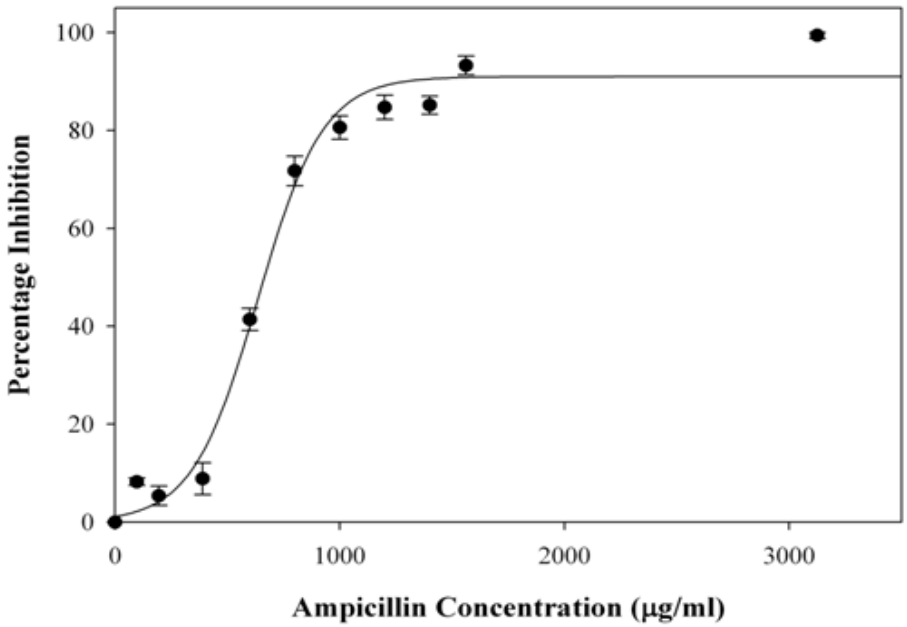

Fig. 3: Minimum Inhibitory Concentration of ampicillin. The inhibitor was inferred from the optical density values (corresponding to the growth of the organisms) at 550 nm. The MIC_{50} and MIC_{90} for ampicillin were found to be 0.69 mg/ml and 1.02 mg/ml respectively.

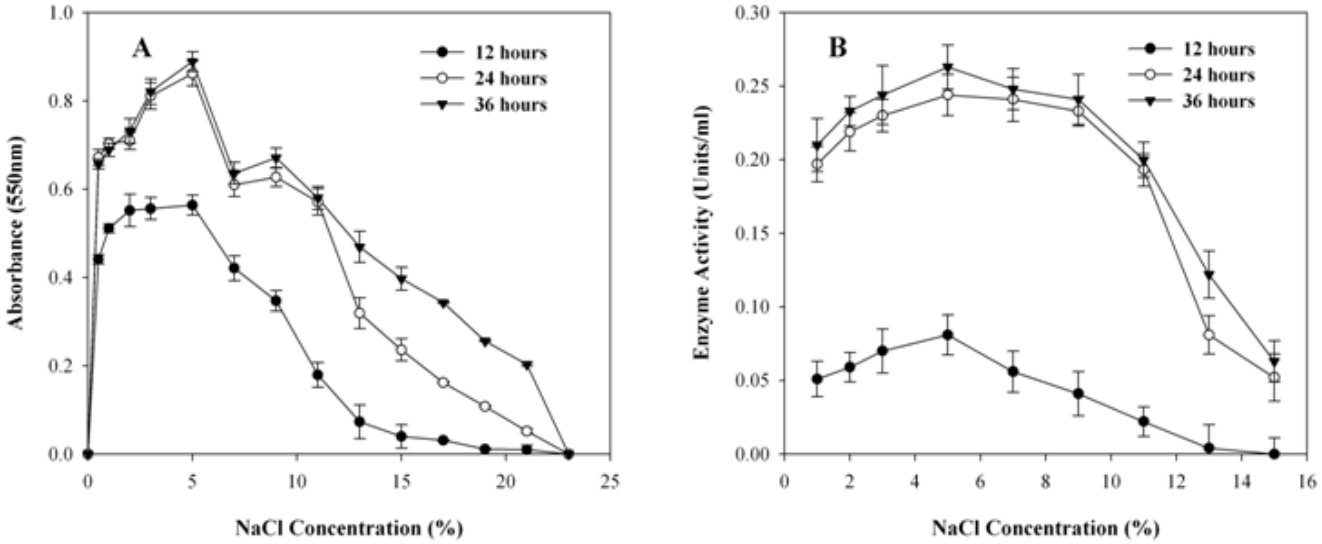

Fig. 4: Effect of NaCl on the (A) organism growth and (B) β lactamase production as observed at different growth periods of 12 h, 24 h, and 36 h. Both organism growth and β-lactamase production is high at 24 h and 36 h. Organism growth was optimal in the NaCl concentration range of 1- 8 % (w/v) and β-lactamase production was substantial upto 10 % (w/v) of NaCl concentration.

β-Lactamase Kinetics

In order to arrive at a few quantitative measurable values that could reflect the activity of the enzyme, the enzyme activity was measured as a function of ampicillin concentration (Figure 5). The data could be fit with the Michealis-Menten model for enzyme kinetics. Saturation was observed at the substrate concentration of 300 µM. The dissociation constant of the enzyme-substrate complex, as inferred from the Lineweaver- Burk plot was 88.33 µM and the initial rate of hydrolysis was found to be 0.48 sec^{-1}.

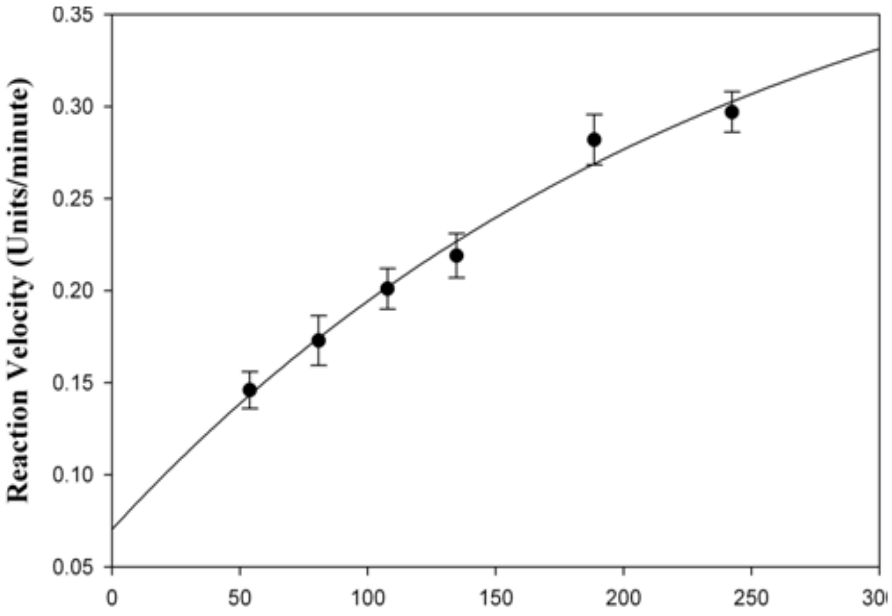

Fig. 5: Relation between ampicillin (substrate) concentration and reaction velocity. The data could fit with the Michealis- Mentons model of enzyme kinetics with a confidance level of 95%. Under the experimental conditions used, the dissociation constant of the enzyme-substrate complex was found to be 88.33 µM.

DISCUSSION

Microorganisms which require salt for growth are referred to either halophiles or halophilic organisms[25]. Samples collected from coastal areas, especially salterns, harbor such halotolerant, if not halophilic microbes. These are being exploited for the production of metabolites, biodegradation and also used as unique marine biocatalysts[26-28]. Recently, we have been exploiting the potential application of the bacterial strains from the saltern, which harbor halotolerant organisms. The isolated strains are found capable of producing extracellular hydrolases with novel properties[20,29,30]. In continuation of our efforts in this direction, we explored the possibility of the presence of antibiotic resistant bacterial strains in the sample.

The isolated bacterial strain, *Halomonas hydrothermalis* VITP09, exhibited optimal growth in the presence of 3 to 5% of sodium chloride. In general, organisms of *Halomonas* genera are found in a wide variety of habitats that encompass a broad range of salinity, temperature, hydrostatic pressure, organic carbon concentrations and pH conditions[31]. The strain reported in this study, *Halomonas hydrothermalis* VITP09, belongs to group II of *Halomonadaceae*

family. Available literature reveals that ampicillin-resistant isolates were predominantly Gram-negative organisms, and the major genera of these bacteria were *Acinetobacter*, *Alcaligenes, Citrobacter, Enterobacter, Pseudomonas, Serratia, Klebsiella* and *Proteus*[32], but none of these are from a saline environment. Very few antibiotic resistant organisms have been reported, till date, for strains belonging to *Halomonas genera.* Susceptibility of organisms belonging to this genus to various antibiotics is generally found varying[33-35]. *Halomonas hydrothermalis* strain VITP09 is resistant to ampicillin, methicillin and vancomycin but susceptible to ampicillin in presence of sulbactam. Methicillin and vancomycin resistant organisms are of great clinical concern and therefore detailed analysis of such antibiotic resistant strains is essential. In methicillin resistant *Staphylococcus aureus,* resistant to β-lactams is mediated by *mecA*, which encodes for pencillin binding proteins (PBP2a) and *blaZ* gene encoding for β-lactamase[36].

In the present study, resistance to ampicillin has been shown to be due to the secretion of class-A β-lactamase. Ampicillin is a β-lactam antibiotic, which act as a competitive inhibitor of the enzyme transpeptidase and activity is inhibited by β-lactamase production[37]. In general, class-A enzymes are susceptible to the commercially available β-lactamase inhibitors[38] (clavulanate, tazobactam, and sulbactam). Cefpodoxime is highly stable in the presence of β-lactamase enzymes. As a result, many organisms, which produce β-lactamase and are therefore resistant to penicillin and some cephalosporins, but susceptible to cefpodoxime[39]. The MIC for β-lactam antibiotic (penicillin) for *S. pneumoniae* was 0.04 µg/ml in 1940 and it increased to 0.12-1 µg/ml in 1980 and it is no longer used in the last decade due to significance increase in resistance[40], indicating the evolution of the organisms with adaptive resistance. In the present study, the MIC_{90} of ampicillin was found to be 1.02 mg/ml (2746 µM), which is almost comparable with the isolate *K. pneumonia* collected from swine in southwestern China, with MIC_{90} 1.024 mg/ml for ampicillin[41]. To our knowledge the MIC of ampicillin for other clinical isolates reported till date are much lesser than the value reported in this study. It is interesting to note that *Halomonas phocaeensis,* recovered from the blood cultures of six neonates in a neonatal intensive care facility (Tunis, Tunisia), was found to be susceptible to amoxicillin with an MIC_{90} of 0.67 µg/ml[18]. In addition, the dissociation constant of the enzyme-substrate complex was found to be 88.33 µM, indicating greater substrate affinity, which is the same order of magnitude found with metallo β-lactamase VIM-2 and Mb11b[42].

Overall the study indicates that microorganisms in coastal areas could also exhibit antibiotic resistance of great magnitude. This probably underlines the possibility of the development/ transfer of antibiotic resistance to other organisms in the coastal flora and definitely signs danger for organisms of aquatic, terrestrials and human health. Transferable antibiotic resistance in bacteria was first recognized in Japan[43]. There are also reports indicating the transfer of antibiotic resistances among multiple antibiotic resistant enterotoxigenic *E. coli* isolated from drinking water collected from different parts of Lucknow city[44]. The ability of the mobile elements themselves to replicate, transfer, and recombine, helps the movement of genes among bacteria. In reality antibiotic resistance genes are readily transferred horizontally, even to and from distantly related bacteria[45]. However more detailed investigation is required to prove the prevalence of such horizontal gene transfer mechanisms in halophilic organisms. Such investigations will not only reveal the biodiversity of these antibiotic resistant, β-lactamase producing, non-pathogenic strains (from non-clinical isolates), but also will pave way to unravel the transfer mechanisms of antibiotic-resistance-associated genes and its role as potential reservoir.

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