Research Article
AMERICAN JOURNAL OF PHARMACY AND HEALTH RESEARCH
www.ajphr.com
2014, Volume 2, Issue 8
ISSN: 2321–3647(online)

Identification of Multidrug Resistant *Stenotrophomonas maltophilia* Strain from Vellar Estuary Sediment, Tamil Nadu

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ABSTRACT

Stenotrophomonas maltophilia has arisen as an important opportunistic pathogen, causing infections whose management is often difficult due to its intrinsic resistance to many antibiotics. Objective of the study was to identify accurately *S. maltophilia* isolates from environmental samples; the site selected being the Vellar estuary based on the extent of pollutants being discharged and the land waterways being polluted by aquaculture discharge, wastes and effluents being dumped by hospital as well as households. The surficial sediment sample from the site was tested for species isolation and identification. The isolate was identified as *S. maltophilia;* the identity was confirmed by conventional biochemical analyses and molecular techniques. The strain's antibiotic sensitivity/resistivity was checked by doing disk diffusion assay. The proposed approach enables isolation and identification of *S. maltophilia* from nonclinical environments. The drug resistant strains may associate with the indigenous microbial communities resulting in the rise of resistant varieties. The main risk for public health is that drug resistance genes are transferred from environmental bacteria to human pathogens. So awareness should be created among hospital staff and nearby communities.

Keywords: *Stenotrophomonas maltophilia; E*stuary; Multi Drug Resistant; Sediment; Resistance

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Received 16 August 2014, Accepted 28 August2014

Please cite this article as: Pingal K *et al.,* Identification of Multidrug Resistant *Stenotrophomonas maltophilia* Strain from Vellar Estuary Sediment, Tamil Nadu American Journal of Pharmacy & Health Research 2014.

INTRODUCTION

Antibiotics and their metabolites are discharged into the aquatic environments as a result of their indiscriminate use in medical, veterinary, agriculture, animal husbandry and aquaculture practices.[1]Coastal areas and adjoining water bodies receive inputs from the adjacent river runoff and back waters containing all types of terrestrial discharges. The discharges may contain medical and microbial contaminated wastes along with lots of nutrients which will adversely increase the existing microbial load. The drug resistant strains may associate with the indigenous microbial communities resulting in the rise of resistant varieties. So it becomes essential to study the prevalence of antibiotic resistant strains, which may subsequently enter and disrupt the food chain.[2] *Stenotrophomonas maltophilia* is an aerobic, non-fermentative, Gram-negative bacillus.[3] Originally classified as *Pseudomonas maltophilia*, *S. maltophilia* was also grouped in the genus *Xanthomonas* before finally becoming the type species of the genus *Stenotrophomonas* in 1993.[4,5]*S. maltophilia* is a ubiquitous free living microorganism found in aqueous environments, soil, and plants food, and hospital settings.[4]This bacterium is generally considered to be an opportunistic pathogen [4,6,7], causing wide spectrum of diseases, including bacteremia, urinary tract infections, respiratory tract infections, skin and soft tissue infections, endocarditis, meningitis and ocular infections.[6,8] *S.maltophilia* adheres avidly to medical implants and catheters, forming a biofilm that renders natural protection against host immune defenses and different antimicrobial agents [9,10,11,12]. The risk factors for infection by *S. maltophilia* include prolonged hospitalization requiring invasive procedures, previous exposure to broad-spectrum antibiotics, mechanical ventilation, and severe mucositis.[13, 14,15, 16,17]Although *S. maltophilia* causes mainly nosocomial infections [4,18], community-acquired infections may also occur.[19] It can be isolated from water but is found more often in soils and especially in the plant rhizosphere.[20] The bacterium has also been recovered from a variety of soil.[21,22] The objective of our present study was to isolate antibiotic resistant microorganisms, the site selected being the Vellar estuary, Parangipettai, Tamil Nadu, India, based on the extent of pollutants being discharged and the land waterways being polluted by aquaculture discharge, hospital waste and the effluents and waste being dumped by the households. Waste effluent from hospitals contains high numbers of drug resistant bacteria and antibiotic residues at concentration able to inhibit the growth of susceptible bacteria.[23]The main risk for public health is that drug resistance genes are transferred from environmental bacteria to human pathogens .There are several routes of entry of antimicrobial agents into the environment. Studies have shown that introduction by these

routes has altered the antibiotic susceptibility of the microbes in those environments.[24] So awareness should be created among hospital staff and nearby communities.

MATERIALS AND METHODS

Sample collection:

Surficial sediment sample was collected from the shoreline of Vellar estuary(Lat 11º 29´ N Long 79º 46´ E), under sterile conditions and brought to laboratory immediately for further studies.

Primary screening:

One gram of soil sample was mixed in 9 ml of sterile 50% seawater and subsequently serially diluted up to 10^{-5} times. Next 100 µl dilution from 10^{-2} to 10^{-5} concentrations were swabbed on solid Luria Bertani (LB) media (HiMedia) made in 50% sea water with Ampicillin (100µg/ml) (HiMedia) mixed in it (to reduce the occurrence of sensitive microbes).

Incubation:

The culture plates were kept for incubation at 30±5^{o}C for 18-24 hours.

Screening of multi drug resistant strains:

Morphologically different and isolated Ampicillin (Amp) resistant colonies were selected for broth culture. The culture was spread evenly over Mueller Hinton media (HiMedia) with sterile swab. The occurrence of antibiotic resistant bacteria was determined by the disc diffusion method according to the Bauer-Kirby method[25] as per the Clinical Laboratory Standards Institute (CLSI), 2010 guidelines[26]. Within 15-30 min after inoculation, 11 disks impregnated with antibiotics (HiMedia) were applied to the surface of the inoculated plates. The plates were kept for incubation at 30±5^{0}C for 18-24 hours after which the zone of inhibition was measured accurately. The antibiotics are listed in Table 1.

Table1. Antibiotics used for susceptibility testing of *S.maltophilia isolate*

Sl.No.	Antibiotic	Symbol	Disc content
1.	Amikacin	AK	30mcg
2.	Amoxiclav	AMC	30mcg
3.	Co-trimoxazole	COT	1.25/23.75 mcg
4.	Cefotaxime	CTX	30mcg
5.	DoxicyclineHCl	DO	30mcg
6.	Levofloxacin	LE	5mcg
7.	Polymixin-B	PB	300units
8.	Vancomycin	VA	30mcg
9.	Imipenem	IPM	10mcg
10.	Meropenem	MRP	10mcg
11	Rifamipicin	RIF	5mcg

Morphological/ Biochemical characterization:

Morphological and biochemical characterization were done for determining the shape and gram character as well as to detect the presence/ absence of enzymes namely gelatinase, catalase, amylase, urease. Other tests were also performed like indole test, methyl red and Voges Proskauer test, H_2S production test respectively according to Gilardi, 1973 [27].

Molecular identification:

Genomic DNA was isolated using the Phenol-chloroform method[28]. 16S rRNA amplification was done[29] to confirm the strain identification[30] by employing the universal primers 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') & 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') using Mastercycler gradient (Eppendorf, Germany).Components for PCR master mix like PCR buffer (1X) (containing 10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5 mM $MgCl_2$ and 0.001% gelatin); Deoxynucleotides i.e. dATP, dCTP, dGTP, & dTTP (each 200µM); Taq pol (0.05 units/µl)and primers (10 picomole each) were purchased from Sigma-Aldrich(USA). Amplification of rRNA genes by the polymerase chain reaction [29] provides access to these genes for sequence analysis.

RESULTS AND DISCUSSION

The strain selected for this study was identified as *Stenotrophomonas maltophilia* after doing biochemical characterization and 16S rRNA sequence analysis. The specific strain was selected for further experiments as it was found to exhibit multi drug resistance against 10 antibiotics following CLSI guidelines[26].

Table 2.Resistivity/ Sensitivity of the *S. maltophilia* isolate

Sl.No.	Antibiotic	Resistivity/ Sensitivity
1.	Amikacin	R
2.	Amoxiclav	R
3.	Co-trimoxazole	R
4.	Cefotaxime	R
5.	DoxicyclineHCl	R
6.	Levofloxacin	R
7.	Polymixin-B	S
8.	Vancomycin	R
9.	Imipenem	R
10.	Meropenem	R
11	Rifamipicin	R

(S= Sensitive R= Resistant)

Table 2 shows the antibiotic sensitivity of the test strain where letter 'S' indicates sensitivity and letter 'R' indicates resistivity of the strain towards specific antibiotics. Sensitivity testing

requires nonstandard culture techniques (incubation at 30°C).[31, 32] Sensitivity testing was done at 30±5°C as testing at the wrong temperature results in isolates being incorrectly reported as being susceptible when they are in fact resistant.[33, 34] The management of *S. maltophilia* infections is often problematic because this pathogen is frequently inherently resistant to multiple antibiotics, including β-lactams, aminoglycosides, carbapenems and quinolones.[35, 36] Co-trimoxazole appears to be an effective treatment for *S. maltophilia* infections, with susceptibility rates over 90% in most settings.[37,38] However, strains resistant to this agent have also been reported.[36,39] The test isolate was also resistant to co-trimoxazole. Although the mechanism of resistance to co-trimoxazole is not well understood, it is considered to be mediated by mobile genetic elements, either plasmids or class I integrons.[40,41,42] It is notable that ciprofloxacin and ceftazidime or ceftriaxone were often administered as monotherapy, with clinical success rates of 100% for ciprofloxacin and 66.7% for ceftazidime or ceftriaxone. However, *S. maltophilia* has gained resistance mechanisms for both classes of these antibiotics. Ceftazidime may be inhibited by the production of β -lactamases, as well as by efflux pumps.[43] The experimental isolate was also found resistant to Cefotaxime. Resistance to quinolones can also be quickly developed by mutations in outer-membrane proteins or via target-site mutation in DNA gyrase.[43] The test isolate was also resistant to Levofloxacin. *S. maltophilia* is naturally resistant to imipenem because it produces imipenemase.[44] *S. maltophilia* is also resistant to most β -lactam antibiotics because it overproduces broad-spectrum[45] β -lactamase. The selected isolate was resistant to imipenem. The results of morphological/ biochemical characterization experiments are shown in Table3.

The isolate was found to be Gram-negative and motile. It also has an ability to grow up over wide range of substrates such as glucose, fructose, galactose. This result indicates towards the aerobic metabolic pathway as these substrates could be easily assimilated and directly enters Tri Carboxylic Acid Cycle.[46]16S rRNA sequence analysis was performed by MCLAB (California, USA). N-BLAST search of the 16S rRNA sequence of isolated strain revealed that the isolate bears maximum similarity (99%) and identified as *Stenotrophomonas maltophilia strain PASVE***.** The sequence obtained was submitted to NCBI GenBank(Accession No. KM387320).The 16S rRNA gene was used for the strain identification[29] as it is highly conserved between different species of bacteria and archaea.[47] The use of 16S rRNA was pioneered by Carl Woese[48]; because of their high degree of conservation, rRNA sequences are useful for establishing phylogenetic relationships among organisms.[49]

Table 3.Morphological/ Biochemical characterization of *S. maltophilia*

Sl.No.	Test	Reaction
1.	Gram's staining	Gram negative, rod
3.	Growth at 18°C-37°C;	P
4.	No growth at 4°C or 41°C	P
5.	Motility (18°C-37°C)	P
6.	Catalase utilization	P
7.	Methyl red	N
8.	Voges-Prokauer	N
9.	Hydrogen sulphide	N
10.	Gelatin hydrolysis	P
11	Starch hydrolysis	N
12.	Urea hydrolysis	N
13.	Glucose as carbon source for growth	P
14.	Fructose as carbon source for growth	P
15.	Galactose as carbon source for growth	P
16.	Glucose fermentation	N
17.	Maltose fermentation	P
18.	Indole production	N
19.	Lysine decarboxylase	P
20.	Phenylalanine deaminase	N

(N = Negative; P= Positive)

CONCLUSION

The emergence of new multiple drug resistant (MDR) microorganisms found in nonclinical environments, the increasing reports of community-acquired infections and the spread of these pathogens in the clinical setting have all under-scored the need to monitor these organisms. *Stenotrophomonas maltophilia* is an environmental global emerging Gram-negative MDR strain that is most commonly associated with respiratory infections in humans. It can cause various serious infections in humans. The acquisition of genes from environmental bacteria by *S.maltophilia* emphasizes the importance of monitoring the antibiotic resistance of *S. maltophilia* clinical isolates. Such monitoring can provide insight into the environmental source of antibiotic resistance genes, validate how these genes are being spread among clinical isolates, and suggest prevention strategies to reduce the level of antibiotic resistance.

ACKNOWLEDGEMENT

This work was supported by grants received from Rajiv Gandhi National Fellowship Scheme, under University Grants Commission, New Delhi.

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