Advanced Journal of Microbiology Research Vol. 2016
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research Paper

Antibiotic resistance microbes in tropical mangrove sediments in east coast peninsular, Malaysia

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Accepted 8 April, 2015

The study has been conducted at Tanjung Lumpur, mangrove swamp on January 2009 to isolate and identify the bacterial community in mangrove soil and their resistance against antibiotics. Identified bacteria were *Aeromonas hydrophila* group 1 and 2, *Escherichia coli* 1, *Chryseomonas luteola, Chromobacterium violaceum, Pseudomonas aeruginosa, Serratia rubudaea, Klebsiella pnuemoniae* and *Enterobacter cloacae*. The identified bacteria were introduced to fourteen different antibiotics to determine the bacterial susceptibility. All the isolates showed 100% resistant towards -lactam antibiotics (ampicillin, amoxicillin and penicillin), vancomycin, sulphafurazole, gentamicin, erythromycin, tetracycline, novobiocin, clindamycin and bacitracin indicates the presence of bacterial amidases and -lactamases in the bacteria which inhibit the action of - lactam antibiotics. Bacteria isolated from mangrove soil showed 66.7 and 77.8% resistance against chloramphenicol and streptomycin, respectively, suggesting that the lipid composition might play a key role in preventing the entrance or binding of antibiotic to the cell. All the isolates were susceptible to ciprofloxacin since it inhibits the enzyme topoisomerase II that cause the negative super coil in DNA and thus permits transcription or replication. All bacterial isolates showed Multi Antibiotic Resistance (MAR) index higher than 0.2 and proved high-risk sources of contamination of the environment. This study proved the presence of antibiotic resistant bacterial strains in mangrove soil that could be used for further studies.

Key words: Tanjung Lumpur, mangrove swamp, bacterial community, -lactam antibiotics, multi resistance antibiotic index.

INTRODUCTION

Antibiotic resistance is a concern for the management of disease in humans, animals and plants. Most of the known resistance determinants have been discovered in clinical and veterinary bacterial isolates, whereas other environmental reservoirs of antibiotic resistance are not well characterized (Nwosu, 2001; Seveno et al., 2002). Microorganisms produce diverse group of chemical called antibiotics that have microstatic or microcidal activity. Function of these microorganisms is to disrupt microbial metabolism by a variety of mechanisms. The discovery and clinical use of antibiotics just over 50 years ago coupled with improvements in immunization drastically reduced human suffering and deaths from infectious diseases (Nwosu, 2001). In recent years, there were diseases (Nwosu, 2001). In recent years, there were several researches that documented high level of antimicrobial resistance among bacteria isolated from food animal and in the environment (Jensen et al., 2001). Antibiotic utilization in medicine, veterinary medicine, agriculture and aquaculture has been steadily increasing (Mudryk, 2004) . However, knowledge about the amount of the antibiotic entering the environment after their use is very limited (Hirsch et al., 1999). The extent of the antibiotic use is indicative of the selection pressure exerted on bacteria (Schwartz et al., 2003).Emergence of bacteria resistant to antibiotic is common in areas where antibiotics are used, but occurrence of antibiotic-resistant bacteria is also increasing in aquatic environments (Schwartz et al., 2003). Earlier studies have shown that

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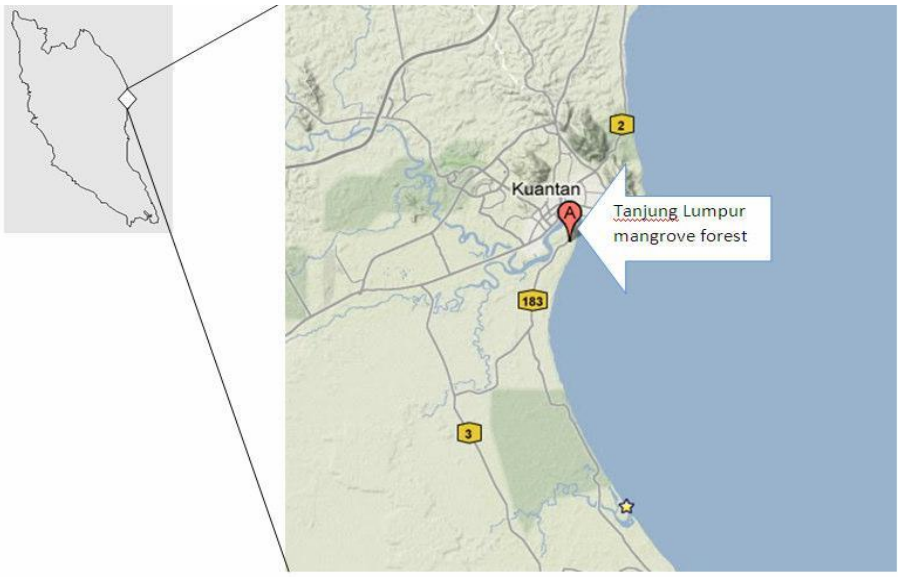

Figure 1. Location of sampling area at Tanjung Lumpur Mangrove Forest.

resistance can be transferred between bacteria from diverse origins in the environment (Kruse and Sørum, 1994). Bacteria have adapted defenses against these antibiotics and continue to develop new resistances, even as we develop new antibiotics. There is no doubt that the use of antibiotics provides selective pressure that result in antibiotic resistant bacteria. Some resistant bacteria are found naturally in the environment as pathogens and nonpathogens and are released into the environment in several ways. (Gaston, 1998; Reinsenfeld et al., 2004) It was suggested that soil microorganisms harbour antibiotic resistance gene with considerably more genetic diversity.

Cultured microorganisms have been the source of almost all characterized antibiotic resistance genes; therefore, most previous studies have ignored the potential reservoir of antibiotic resistance genes in uncultured bacteria. The majority of bacteria are not readily cultured on standard laboratory media (Giovannoni et al., 1990; Ward et al., 1990; Amann et al., 1995; Suzuki et al., 1997; Hugenholtz et al., 1998), and the diversity of the uncultured majority is vast (Head et al., 1998; Torsvik et al., 1998; Whitman et al., 1998; Béjà et al., 2002). Despite recent progress in culturing methods culture-independent techniques are required to access the genetic diversity of most bacteria. Identifying antibiotic resistant bacteria will pave the way to understanding its genetic makeup and ultimately help in development of new antibiotics.

The development of effective antibacterial drugs has been accompanied by the emergence of drug-resistant organisms. This phenomenon of resistance imposes serious constraints on the option available for the medical treatment of many bacterial infections. Resistance in bacterial population can be spread in three ways: (1) by transfer of bacteria between people, (2) by transfer of resistance genes between bacteria (usually on plasmids) and (3) by transfer of resistance genes between genetic elements within bacteria, on transposons (Rang et al., 2007).

Mangrove soil has been appreciated for decades for the incredible diversity of bacteria that it harbours. Mangrove area at Tanjung Lumpur is dominated by *Avicennia alba* and *Sonneratia albican* at the front of the area. Abundance of *Rhizophora* spp was observed at the back of the swamp. This study was carried out to establish the occurrence of antibiotic resistance soil bacteria in mangrove ecosystem and to determine the susceptibility of the isolated soil bacteria towards different antibiotics.

MATERIALS AND METHODS

Samples collection

48 soil samples were collected from the study area (Figure 1): Tanjung Lumpur, mangrove swamp at low tides, where they were scooped into sterile Falcon tube prior to processing. Each station consisted of four transects, first transect was near the waterline, second was 5 m from the water line, third was 10 m from the waterline and lastly was nearby the mangrove plant's root. These transact sampling would help in understanding how the antibiotic resistant microbes are distributed near the water line and in nearby mangrove area. At each transect, soil samples were collected in triplicates and the psychological parameter such as temperature and pH were recorded.

Sample preparation and microbiological analysis

This study involved isolation and inoculation of the bacteria from mangrove soil samples followed by subsequent incubations and subcultures (Timoney et al., 1978; Mudryk, 2004). Nutrient agar and

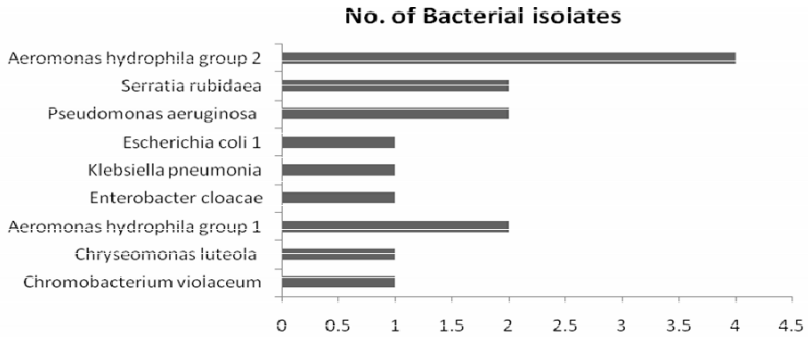

Figure 2. The total number of bacterial isolates identified from mangrove soil.

nutrient broth media were used to culture soil bacteria from mangrove soil samples and obtained pure culture were cryo-preserved for further biochemical analysis. 25 g of soil samples were weighted and transferred into sterile Falcon tube. 25 ml of sterile distilled water were added into each Falcon tube and vortexed for homogenization. This mixture was spinned in a large centrifuge for 3 min at 9000 rpm (G force = 7711). A fixed amount of 200 l of supernatant was pipetted and transferred into freshly prepared nutrient agar plate. Then the mixture transferred was spread on individual plates for each sample. The plates were incubated at 37°C for 24 h.

After incubation, each different colonial type was picked using sterile toothpick and then place on different sections of the agar plate and incubated at 37°C for 24 h. Then the secondary plates were checked for purity of colony. Upon examination, the colonies were preserved in 15% glycerol solution and stored at -20°C to maintain the viability of pure colonies. (Giraffa et al., 2004).

All the 15 bacterial isolates were identified using API 20 identification system. Gram staining was also performed for all the isolates. Bacterial samples were tested against antibiotics using disc -diffusion method. The turbidity of the bacteria was compared with 0.5 Mc Farland Standard using spectrophotometer at 625 nm. The amount of the bacteria was equivalent to 150 x 106 colony/unit.

200 l of cultured bacteria was spread using hockey stick on agar plate and left to dry before placing the antibiotic disc on the surface of the plate. All the samples were treated with fourteen different antibiotics to test their susceptibility towards the antibiotics. The antibiotics used were namely Novobiocin (NV, 30 g), Tetracycline (TE, 30 g), Erythromycin (E, 15 g), Chloramphenicol (C, 30 g), Streptomycin (S, 10 g), Bacitracin (B, 10 units), Ciprofloxacin (CIP, 5 g), Vancomycin (VA, 5 g), Gentamicin (CN, 10 g), Penicillin G (P, 10 units), Ampicillin (AMP, 10 g), Amoxycillin (AML, 25 g), Sulphafurazole (SF, 300 g) and Clindamycin (DA, 2 g) which are available from Oxoid in disc form. Each sample plates were divided into six sections and their antibiotic resistance characteristics were observed. The results were documented by measuring the inhibition zone of the colonies that formed after 24 h of incubation at 37°C on the treated plates to determine Multi Antibiotic Resistance (MAR index = a/b where a = number of resistant antibiotics, b = total number of antibiotics exposed) and it was compared with standards obtained from British Society for Antimicrobial Chemotherapy (BSAC).

RESULTS

There were four isolates identified as *Aeromonas hydrophila* group 2 followed by two isolates of *Aeromonas hydrophila* group 1, *Serratia rubidaea and Pseudomonas aeruginosa. Escherichia coli* 1, *Klebsiella pneumonia, Enterobacter cloacae, Chryseomonas luteola and Chromobacterium violaceum* represented with single isolates were identified from the mangrove soil samples (Figure 2).

The physicochemical parameters such as temperature and pH of the sampling area were determined using soil temperature profile sensor ST01 and ph meter. There were no much fluctuation in pH and temperature of the sampling locations. Slightly acidic pH were observed in all the area (6.2 – 6.4) and the temperature ranged between 22 – 25°C. This might be due to the location of the sampling area which is closer to the mangrove soil and near the water line (Figure 3).

The distribution of gram positive and gram negative bacteria were identified from different transacts of the fourstations. Compare to other stations, station 1 showed higher number of gram negative bacterial isolates at the third transact (n = 13) followed by station 1 fouth transact and station 3 fouth transact (n = 5). Se-cond and fourth transacts of station 2 showed 4 isolates of gram negative bacteria and it was comparitively more than station 4 gram negative isolates. More focus was shown to gram negative bacterial isolates since they are pathogenic to human (Figures 4 and 5).

E. coli 1 and *C. violaceum* were isolated from soil at station 1. Station 2 was dominated with *A. hydrophila* group 2 followed by *K. pneumonia. A. hydrophila* group 1 was observed in station 2 and station 4 soil indicates higher level of faecal contamination in these stations. *P. aeruginosa* and *E. cloacae were isolated from* station 3. Besides *A. hydrophila* group 1, two other bacterial isolates (*C. luteola* and *S. rubidaea)* were isolated from soil at station 4.

All the identified bacteria showed 100% of bacterial resistance towards -lactam antibiotics (ampicillin, amoxicillin and penicillin), vancomycin, sulphafurazole, gentamicin, erythromycin, tetracycline, novobiocin, clin-damycin and bacitracin. Bacterial resistance of 66.7 and 77.8% were observed against chloramphenicol and streptomycin. Isolated bacteria were susceptible to cipro-floxacin. All the isolates from mangrove soil that showed MAR Index value of >0.2 indicates high risk source of

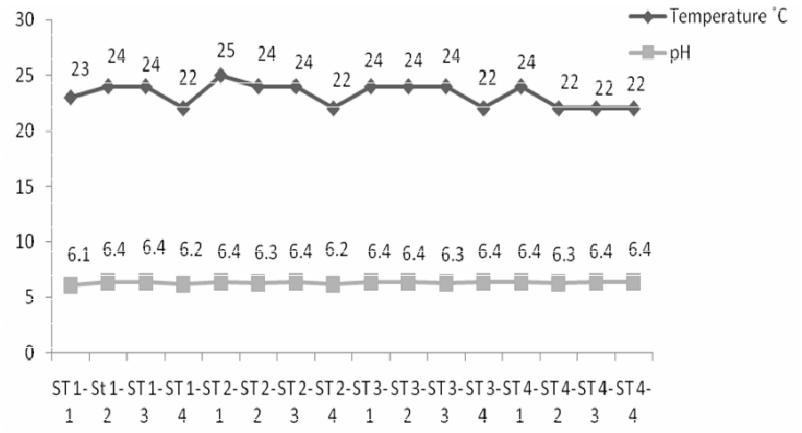

Figure 3. Soil pH and temperature in different transacts from four sampling stations.

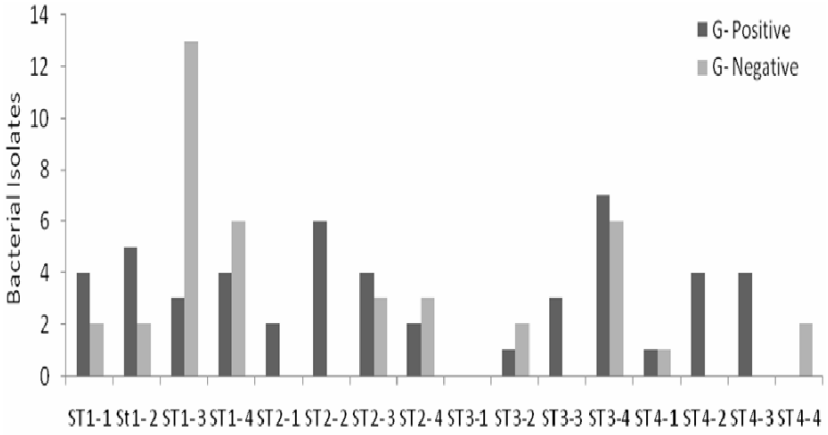

Figure 4. The distribution of gram positive and gram negative bacteria in different transacts from four sampling stations.

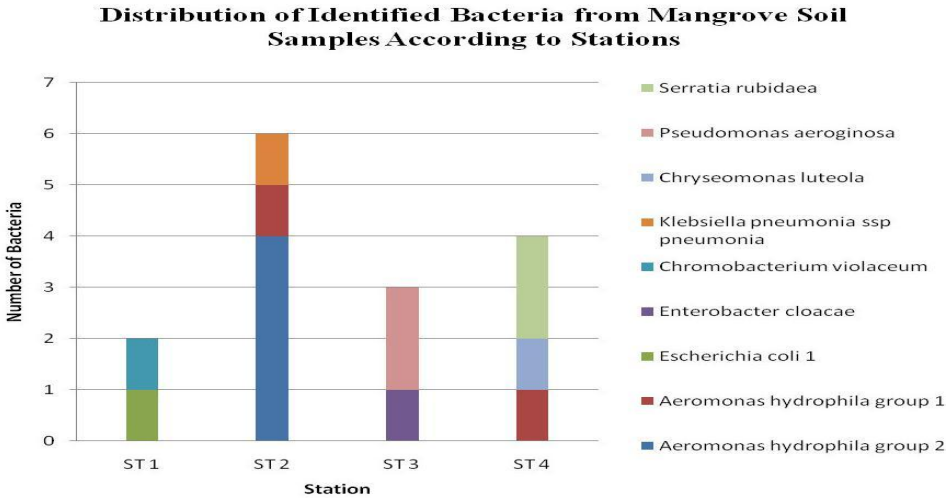

Figure 5. Description of inconsistent distribution of identified bacteria from mangrove soil samples from different sampling stations.

contamination in the water body.

DISCUSSION

The development of effective antibacterial drugs has been accompanied by the emergence of drug- resistant organisms. Resistance in bacterial population can be spread in three ways: (1) by transfer of bacteria between people, (2) by transfer of resistance genes between bacteria (usually on plasmids) and (3) by transfer of resistance genes between genetic elements within bacteria, on transposons (Rang et al., 2007). Antibiotic resistance test was done to all of 15 selected samples of gram-negative organisms. All the isolated bacteria showed 100% resistant towards -lactam antibiotics (ampicillin, amoxicillin and penicillin) indicates the presence of bacterial amidases and -lactamases enzymes in the bacteria that inhibit the action of -lactam anti-biotics. *A. hydrophila* group one and two were resistant to antibiotic bacitracin and this might be due to its inefficiency in penetrating into the cell wall of gram negative *Aeromonas sp.* Earlier studies revealed *that A. hydrophila* was susceptible to polymyxin B and gentamicin which possibly affect the protein synthesis of gram positive and gram negative bacteria particularly at aerobic condition (Subashkumar et al., 2006, Ranga et al., 2007) but present study showed its resistance towards gentamicin which indicates the possible insertion of gentamicin resistant gene into the genome of *A. hydrophila.* Bacteria isolated from mangrove soil showed 66.7 and 77.8% resistance against chloramphenicol and streptomycin, respectively. Differences in total lipid or fatty acid composition, or both, have been associated with an increased antibiotic resistance in various bacteria, and it has been suggested that the lipid composition may be of importance in preventing the entrance or binding of the antibiotic to the cell (Bishop and Bermingham, 1973). All the bacteria isolated from mangrove soil were susceptible to ciprofloxacin due to its broad spectrum nature. Ciprofloxacin act by inhibiting the topoisomerase II that cause negative supercoil in DNA and thus permits transcription or replication. Its broad-spectrum nature helps against both gram-positive and gram-negative organisms especially *Enterobacteriaceae*. Due to indiscriminate use of antibiotics the microorganisms might have developed resistance towards several antibiotics. Asha et al., 2005 reported that the presence of antimicrobial agents at low concentration through leaching or continued usage may lead to the development of drug- resistant strains and MAR in bacteria, which ultimately result in transfer of resistance to pathogenic bacteria and reduced efficacy of antibiotic treatment for human and animal diseases. Observed MAR index higher than 0.2 identifies organisms that originate from high-risk sources of contamination (Freeman et al., 1989). The sole source of pollutants in Tanjung Lumpur mangrove swamp is due to the management of adjacent port which leads to the discharge of of effluent directly into the mangrove soil.

Conclusion

Antibiotic resistance is considered to be a major problem because many disease causing bacteria are becoming more resistant to the commonly used antibiotics. New medications are slowly being developed suited to these resistant bacteria. Unfortunately, the problem of antibiotic resistance has been made to worse when antibiotics are not used and prescribed appropriately or used when they are not needed. The overuse and misuse of antibiotics can create the conditions for the development of antibiotic resistant bacteria. This study gives new finding which could be referenced to the State Government of Pahang about the existing of microbial pollution status in Tanjung Lumpur, mangrove swamp and able to prove the presence of antibiotic resistance bacterial strains that can be used as the basis for future studies.

ACKNOWLEDGEMENT

The authors wish to express their gratitude to Faculty of Science, Institute of Oceanography of Maritime Studies Laboratory teams, for their invaluable assistance throughout the sampling period.

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