Journal of Tropical Resources and Sustainable Science

journal homepage: jtrss.org

Antibiotic and Heavy Metal Resistance of Bacteria Isolated from Diseased Mud Crab (*Scylla serrata*)

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Received 24 September 2014
Accepted 18 May 2015
Available online 1 December 2015

Keywords:
Scylla serrata, antibiotic, heavy metal, MAR index

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Abstract

A total of 320 bacteria isolated from marketable size and diseased mud crab (Scylla serrata) at a commercial farm. The isolated bacteria were Aeromonas spp. n = 70, Edwardsiella tarda n = 50, Vibrio alginolyticus n = 40, Vibrio parahaemolyticus n = 20, Salmonella spp. n = 70 and Klebsiella spp. n = 70. All the bacterial isolates were tested for antibiotic susceptibility against 16 types of antibiotics by using disk diffusion method. The antibiotics tested in this study were nalidixic acid (30 µg/disk), oxolinic acid (2 µg/disk), compound sulphonamides (300 µg/disk), doxycycline (30 µg/disk), tetracycline (30 µg/disk), novobiocin (30 µg/disk), chloramphenicol (30 µg/disk), kanamycin (30 µg/disk), sulphamethoxazole (25 µg/disk), flumequine (30 µg/disk), erythromycin (15 µg/disk), ampicillin (10 µg/disk), spiramycin (100 µg/disk), oxytetracycline (30 µg/disk), amoxycillin (25 µg/disk) and fosfomycin (50 µg/disk). Heavy metal resistance pattern of the present bacterial isolates was also characterized against mercury (Hg2+), chromium (Cr6+), copper (Cu2+), and Zinc (Zn2+) by using two fold agar dilution method. The percentage of antibiotic sensitivity of the present bacterial isolates was ranged from 12.5 % to 100 % in which most of the present bacteria isolates were not sensitive to ampicillin whereas all the bacteria isolates were sensitive to nalidixic acid, flumequine and oxytetracycline. Overall, the total of antibiotic sensitive case was reported as 72.7% whereas antibiotic resistance and intermediate sensitive case was recorded as 19.7% and 7.4 %, respectively. The Multiple Antibiotic Resistance (MAR) values were range of 0.03 to 0.29 in which Aeromonas spp (0.29) showed the highest value of MAR. This was followed by Salmonella spp. (0.21) and Klebsiella spp. (0.21), Edwardsiella tarda (0.20), Vibrio alginolyticus (0.09) and Vibrio parahaemolyticus (0.03). The MAR value indicated that the commercial S. serrata were not contaminated to the test antibiotics. Furthermore, low resistance activity of the present bacterial isolates to the tested heavy metals (Cr6+: 20.7 % to 30.8 %, Zn2+: 0 % to 40 %, Cu2+: 18.8 % to 25 % and Hg2+: 30 % to 33.3 %) was observed.

1. Introduction

Giant mud crab (Scylla serrata) is a popular mud crab in Asia. This edible mud crab has been identified as a potential aquaculture species [1] as its attributes of rapid growth, large size, accept a wide range of diets, high value market and hugh demand from market. In Malaysia, the production of mud crab through aquaculture has increased significantly in the last decade. However, bacterial disease became a major constraint in mud crab farming. Many studies were carried out to characterize antibiogram and heavy metal resistance pattern of bacteria isolated from diseased aquatic animals in Malaysia. For

ISSN Number: 2289-3946

instance, Lee et al. [2] characterized antibiogram and heavy metal resistance pattern of bacteria isolated from giant freshwater prawn, Macrobrachium rosenbergii. Other studies that revealed antibiogram and heavy metal resistance pattern of bacteria isolated from white leg shrimp [3], silver catfish, Pangasius sutchi and red hybrid tilapia, Tilapia sp. [4], American bullfrog, Rana catesbeina [5] and Asian seabass, Lates calcarifer, [6] were well documented in the literature.

Based on the literature survey, there are many researches on mud crab studies especially in larval culture technique [7-10] but very few or none have focused on antibiotic and heavy metal resistance of bacteria from diseased mud crab. There is no information available on antibiogram and heavy metal resistance profile of bacterial isolates from mud crab (S. serrata) in the literature. Thus, this study was carried out to characterize bacteria in S. serrata, may be useful for mud crab health management in captivity.

2. Materials and Methods

2.1. Bacterial Isolation and Identification

100 pieces of diseased mud crab (Scylla serrata) with body weight approximately 118 to 211 g were collected from a commercial mud crab farm located at Pasir Puteh, Kelantan, Malaysia. Fluid from external and internal part of mud crab was collected by using sterile cotton bud and spread plated on five types of agar plate medium, namely Thiosulphate Citrate Bile Salt (TCBS) (Merck, Germany), Mac Conkey, Cytophaga Agar (CA) (Merck, Germany), Xylose Lysine Deoxycholate (XLD) (Merck, Germany) and Glutamate Starch Pseudomonas (GSP) (Merck, Germany). All the inoculated media were incubated at room temperature of 30oC for 24 to 48 h in inverted position. The bacterial colonies that growth on the selective media was further selected for the identification test such as Gram staining, oxidase test and indole production test. The bacterial isolates were identified using conventional biochemical tests [5] and were confirmed with commercial identification kit (BBL, USA) [6].

2.2. Antibiotic Susceptibility Test

Antibiotic susceptibility test were performed according to Kirby-Bauer disk diffusion method [2]. Bacterial isolates were randomly selected from the plates were cultured in Tryptic Soy Broth (TSB) for 18 to 24 h at room temperature, 30oC. The bacterial cells were then centrifuged at 14500 rpm for five minutes using minispin (Eppendorf, Germany). The concentration of the bacterial cells were adjusted to 106 colony forming unit (CFU) by using saline and monitored with Biophotometer (Eppendorf, Germany). The bacterial inoculums were swab on Trypticase Soy Agar (TSA) with zig zag pattern using sterile cotton swabs. Antibiotics that applied in the present study were Nalidixic acid (30µg), Oxolinic acid (2µg), Compound sulphonamides (300µg), Doxycycline (30µg), Tetracycline (30µg), Novobiocin (30µg), Chloramphenicol (30µg), Kanamycin (30µg), Sulphamethoxazole (25µg), Flumequine (30µg), Erythromycin (15µg), Ampicillin (10µg), Spiramycin (100µg), Oxytetracycline (30µg), Amoxycillin (25µg) and Fosfomycin (50µg) (Oxoid, England). Sterile forceps were use to place the antibiotic disc from antibiotic disc dispenser onto the agar surface. After incubation, the diameter (in mm) of inhibit zone that produced from each antibiotic disc was measured using ruler. Interpretation of the results namely as sensitive (S), intermediary sensitive (I) and resistance (R) according to the reference to the standard provided by the National Committee for Clinical Laboratory Standards [11] and was made in accordance to the standard measurement of inhibitory zones in millimeter (mm).

2.3. Multiple Antibiotic Resistance (MAR) Index Determination

Multiple Antibiotic Resistance (MAR) Index of all the present isolates against the 16 tested antibiotics were calculated based on the formula as follows [11]:

Multiple Antibiotic Resistance (MAR) Index = X / (Y x Z)

Where,

X = total of antibiotic resistance case;
Y = total of antibiotic used in the study;
Z = total of isolates

ISSN Number: 2289-3946

A MAR Index value of equal or less than 0.2 were defined as those antibiotics were seldom or never used for the animal in term of treatment whereas the MAR Index value higher than 0.2 is considered that animal have received high risk exposure to those antibiotics.

2.4. Heavy Metal Resistance Test

The heavy metal resistance test was conducted by using agar dilutions method with bacterial tolerance to four elements of heavy metal as follows: mercury (Hg^{2+}), chromium (Cr^{6+}), copper (Cu^{2+}) and Zinc (Zn^{2+}). Bacteria suspension was spread onto the Tryptic Soy Agar (TSA) medium which incorporated with five different concentrations of Kalium Dichromate ($K_2Cr_2O_7$), Zinc Sulphate ($ZnSO_4$), Copper II Sulphate ($CuSO_4$) and Mercury II Chloride ($HgCl_2$) (Merck, Germany). The concentration for Zn^{2+} and Cr^{6+} was ranging from 25 to 400 µg mL-1 while the concentration of Hg^{2+} and Cu^{2+} was ranging from 2.5 to 40 µg mL-1 and 150 to 2400 µg mL-1, respectively. The bacterial were considered resistance if they were able to grow at concentrations of 10 µg mL-1 Hg^{2+}, 100 µg mL-1 Cr^{6+} and 600 µg mL-1Cu^{2+} [11-12].

3. Results and Discussion

In the present study, a total of 320 bacterial isolates (Aeromonas spp. n = 70, Edwardsiella tarda n = 50, Vibrio alginolyticus n = 40, Vibrio parahaemolyticus n = 20, Salmonella spp. n = 70 and Klebsiella spp. n = 70) were successfully isolated from mud crab. All the present study bacterial isolates were found sensitive to nalidixic acid, flumequine and oxytetracycline (Table 1). More than 80 % of the bacterial isolates were found sensitive to oxolinic acid, compound sulphonamides, doxycycline, tetracycline, chloramphenicol and kanamycin. This study was also documented 75 % and 71.9 % of bacterial isolates were sensitive to erythromycin and fosfomycin, respectively. The percentage of sensitivity of present bacterial isolates to novobiocin, sulphamethoxazole, ampicillin, spiramycin and amoxycillin was ranged from 12.5 to 50 %. Overall, the total of sensitive case (72.7%) was reported higher than the resistance case (19.7%) and followed by intermediate sensitive case (7.4%).

Table 1: Percentage of sensitivity of present bacterial isolates against 16 types of antibiotics.

Antibiotic (µg/disk)	Resistance (%)	Intermediary sensitive (%)	Sensitive (%)
Nalidixic acid (30)	0.0	0.0	100.0
Oxolinic acid (2)	6.3	3.1	90.6
Compound sulphonamides (300)	15.6	0.0	84.4
Doxycycline (30)	3.1	3.1	93.8
Tetracycline (30)	3.1	0.0	96.9
Novobiocin (30)	50.0	12.5	37.5
Chloramphenicol (30)	12.5	0.0	87.5
Kanamycin (30)	0.0	3.1	96.9
Sulphamethoxazole (25)	62.5	21.9	15.6
Flumequine (30)	0.0	0.0	100.0
Erythromycin (15)	6.3	18.8	75.0
Ampicillin (10)	68.8	18.8	12.5
Spiramycin (100)	21.9	28.1	50.0
Oxytetracycline (30)	0.0	0.0	100.0
Amoxycillin (25)	40.6	21.9	37.5
Fosfomycin (50)	28.1	0.0	71.9
Overall	72.7	19.7	7.4

The Multiple Antibiotic Resistance (MAR) values were range of 0.03 to 0.29 in which Aeromonas spp. (0.29) showed the highest value. This was followed by Salmonella spp. and Klebsiella spp. (0.21), Edwardsiella tarda (0.20), Vibrio alginolyticus (0.09) and Vibrio parahaemolyticus (0.03). The MAR value indicated that the mud crabs in the present study (Scylla serrata) were not highly exposed to the tested antibiotics.

Heavy metal test results showed the total percentage of bacteria resistant to Cr^{6+} was ranged from 20.7 % to 30.8 %. The total resistance percentage of bacteria to Zn^{2+} was ranged from 0 % to 40 % where all the bacterial isolates of V. alginolyticus and Klebsiella spp. were sensitive to Zn^{2+} whereas 40 % of E. tarda were resistant to Zn^{2+}. On the other hand, Vibrio parahaemolyticus and Salmonella spp. were recorded the lowest resistance case to Zn^{2+} activity (25 %). The percentage of the present study bacterial isolates

ISSN Number: 2289-3946

resistant to Cu2+ and Hg2+was ranged from 18.8 % to 25 % and 30 % to 33.3 %, respectively.

The information concerning the bacterial flora of crustaceans, particularly mud crab, appears to be limited in the literature. Therefore, this study was conducted to characterize the bacteria colonized in mud crab in terms of bacterial species, antibiotic sensitivity and heavy metal resistance pattern. All the isolated bacterial species in the present study are opportunistic bacteria species in which will only attack the host whenever the host becomes weak. These bacteria species were reported can cause serious problem in aquaculture especially in marine and brackish water farming like mud crab culture as described by Karunasagar et al. [13]; Abraham et al. [14]; Sahul Hameed and Balasubramanian [15]. Therefore, it is very important to monitor the existence of these opportunistic bacteria in the mud crab culture. In the present study, nalidixic acid, flumequine and oxytetracycline were found effective in controlling all the bacterial isolates in the present study. Therefore, we suggest that these antibiotics can be used as antimicrobial agent in commercial mud crab farm except for oxytetracycline due to this antibiotic is already banned by Malaysian government to be used in Malaysia aquaculture. As a result, the mud crab farmers may use nalidixic acid and flumequine as antimicrobial agent in their farm. However, flumequine may remain in the sediment and the surroundings after application and will effect directly on the external bacteria population within the aquatic ecosystem [16]. Hence, we suggest that mud crab farmers can use nalidixic acid instead of using flumequine continually.

More than 50 % of the present bacterial isolates were found resistant to novobiocin, sulphamethoxazole, ampicillin, spiramycin and amoxicillin. The incidence of bacteria resistant to these antibiotics may be due to the increasing input of antimicrobial agents into mud crab culture system or probably as a consequence of the disposal of untreated sewage, industrial wastes and agricultural activities. These human activities may also contribute to the incidence of heavy metal resistance pattern of the bacterial isolates in the present study. The MAR index indicated that the sampling site is still free of antibiotic residues contamination in which the farmed mud crabs were not highly exposed to the tested antibiotics. In terms of heavy metal resistance pattern of bacterial isolates in the present study revealed that no more than 40 % of the present bacterial isolates were resistant to the tested heavy metals. This clearly showed that the water source of the sampling sites is suitable to be used for aquaculture activity. Heavy metal resistance cases were observed among the present bacterial isolates could be the result of heavy metal contamination with fertilizer which contains heavy metal residues in which can contribute to heavy metal resistance cases among the bacteria in the present study. In additional, alternatives to antibiotics should be explored for aquaculture industry development to ensure its sustainability and food safety. Besides that, further studies should be carried out in the near future to reveal the incidence of antibiotics and heavy metal resistance among opportunistic bacteria concerning the possible risks to mud crab and public health.

4. Conclusions

In the present study, low MAR value and incidence of heavy metal resistance case indicating that the sampled mud crab were not highly exposed to the tested antibiotics and heavy metals. However, further study need to be carried out before we can come to a conclusion.

Acknowledgement

This project was funded by Universiti Malaysia Kelantan short term projects (R/SGJP/A03.00/00387A/001/2009/000018) and Fundamental Research Grant Scheme (FRGS) vot no: R/FRGS/A0.700/00387A/005/2013/00107.

ISSN Number: 2289-3946

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ISSN Number: 2289-3946