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Recombination of Plasmid-Borne Drug Resistant *Paenibacillus* sp. Isolated From Crab (*Portunus sanguinolentus*)

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Abstract

Twenty five numbers of crabs (*P. sanguinolentus*) were collected from Parangipettai fish market. *Paenibacillus* sp. were isolated from the gut of crabs. Then the isolated colonies were characterized for phenotypic and biochemical properties for identification. Out of twenty five crabs, twenty one showed the presence of *E.coli* while only four crabs showed the presence of *Paenibacillus* sp. Among these isolates *Paenibacillus* sp. showed drug resistance to 11 antibiotics and sensitiveness to 2 antibiotics, whereas *E.coli* was found to be the sensitive to all the 13 antibiotics which were provided in the 2 different antibiotic discs. The drug resistant plasmid present in *Paenibacillus* sp. was transformed into drug sensitive *E.coli*. The *E.coli* transformants shows resistant to all the three drugs employed (Tetracycline, Penicillin and Chloramphenicol). Transformation efficiency between the *Paenibacillus* sp. and *E.coli* were not calculated because of the formation of mat instead of individual colony in all the agar plates of Eosin Methylene Blue, Muller Hinton Agar and Luria Bertani agar. The contamination of sea foods caused by resistant *Paenibacillus* sp. will be very difficult to treat. Therefore, adequate cooking of sea foods before consumption is strongly recommended.

Keywords: *Paenibacillus* sp; Recombination; *Portunus sanguinolentus*; Plasmid-borne drug resistant bacteria; Resistant bacteria

Introduction

Antibiotic resistance is now a linked global problem. Dispersion of successful clones of multidrug resistant (MDR) bacteria is common, often via the movement of people [1]. Antimicrobial resistance increases the morbidity, mortality and costs of treating infectious diseases. The threat from resistance (particularly multiple resistances in bacterial strains that have disseminated widely) has never been so great. The key factors driving this threat are increased antibiotic usage (in both human and animal medicine), greater movement of people and increased industrialization. Ineffective of the antibiotics against the bacterial infection is known as resistance [2]. Bacterial resistance is increasingly seen as a public health threat since few new antibiotics options are being introduced. The Infectious Diseases Society of America (IDSA) education campaign of "Bad Bugs, No Drugs" was begun to raise public awareness of this problem. There are many factors that could be responsible for the increase in antibiotics resistance in developing countries [3]. Therefore, the objective of the present study was undertaken to assess the multiple antibiotic resistance of bacteria isolated from the crab *P. sanguinolentus* and focuses on the activity of bacteria to transform R-plasmid to *E.coli* in natural conditions.

Materials and Methods

Collection and processing of samples

The crab samples were collected from fish market, Parangipettai (Lat.11˚29'N; Long.79˚46' E), Tamil Nadu. Twenty five crab samples were collected with meticulous care aseptically and transported in a sterile poly bag in ice to laboratory.

Isolation of gut microbiota

They were washed several times with sterile sea water to prevent contamination from shell surface and mantle fluid and subsequently the gut of the crabs were aseptically removed. The tissues adhering to the gut were carefully removed using a sterile forceps. The gut alone was homogenized with 9 ml of 50% sterile sea water. Serial dilutions were made from homogenate and from that 0.1 ml were spread into petriplates containing Zobell's marine agar for enumeration of *Paenibacillus* sp. The plates were incubated at 37ºC for 24 hours following which the isolated colonies were picked for further identification.

The isolated colonies were characterized for phenotypic and biochemical properties for identification. The details of characterization methodology are given below.

The isolates were grown on Zobell's marine agar plates to study their colony morphology. The culture plates were observed for size, form, margin, elevation and the data were recorded.

To the tube containing the liquid medium, the bacterial isolate was stab inoculated and the tube was incubated for 24-48 h. After incubation period, the tubes were observed for motility of the isolates. Presence of diffused growth is indicated as positive and absence as negative for motility tests.

Smear of bacterial culture was prepared by loopful of the culture in a clean slide and heat fixed using a spirit lamp. The smear was flooded with crystal violet and allowed to act for 1 min. It was rinsed with tap water and few drops of Gram's iodine were added to cover the smear and were allowed to react for 30 sec to one min. It was then rinsed with tap water and then decolourized with 95% ethanol. The smear was flooded with safranin, allowed to act for one min and rinsed with tap water. The stained culture smear was blot dried and examined under microscope.

To the tube containing 3 ml of peptone water, the cultures were inoculated and incubated at room temperature for 48 h. After incubation period, drops of Kovac's reagent were added and noticed for cherry red ring formation. Peptone water without the isolate inoculation was used

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as control. The cherry red ring formation indicates positive reaction and recorded as (+) and the absence was recorded as (-).

To the MR-VP broth, the isolates were inoculated and incubated at room temperature for 24-48 h and after incubation few drops of methyl red indicator was added. MR-VP broth without the isolate inoculation was used as control. The colour change of broth culture to red colour was indicated as MR positive (+) and the absence indicated as negative reaction (-).

To the tube containing 3 ml of the MR-VP broth, cultures were inoculated and incubated at room temperature for 48 h. After incubation, 0.3 ml of α-napthol and KOH solutions were added and observed for pinkish red colour formation. MR-VP broth without the isolate inoculation was used as control. The pinkish red colour development indicates positive reaction and it was recorded as (+) and the absence was recorded as (-).

In the Simmons citrate agar slants, cultures were inoculated by means of a stab-and-streak technique. The tubes were then incubated at 28 ± 2°C for 24 to 48 h. Simmons citrate agar slant without the isolates inoculation was used as control. The colour change of the slant from green to deep Prussian blue indicates positive reaction and recorded as (+) and the absence of colour change was negative for citrate utilization and recorded as (-).

In the TSI slants, the isolated cultures were stabbed into the butt region, streaked on to the slant area and incubated at room temperature for 18 to 24 h. The slants were observed for the fermentation of sugars, production of gas and precipitation of hydrogen sulfide. TSI slant without the isolate inoculation was used as control. The formation of yellow colour on butt indicates the utilization of glucose, formation of yellow colour on slants indicates the utilization of lactose and sucrose and if slant was pink in colour it indicates that the organisms were unable to utilize lactose and sucrose. Black precipitation shows the production of H_2S and when gas is produced crack and break occurs in slant.

A typical carbohydrate fermentation medium was prepared with 5 g of carbon sources each of lactose, glucose and sucrose and mixed with the indicators phenol red, methyl red and bromocresol purple, respectively. 10 ml of each medium was taken in tubes with Durham's tubes placed inverted inside them. Bacterial isolate was inoculated into the tubes and kept for incubation for 48 h at room temperature. Tubes without bacterial inoculation were maintained as control. After incubation, the tubes were observed for colour change from red to yellow indicating the fermentation of carbon source. Production of gas can be found out by formation of air bubbles in the Durham's tube. Tubes that show positive result for carbohydrate fermentation and gas production was recorded as A/G and absence as no change.

Twenty four hours old fresh culture was taken in a clean glass slides and 3% H_2O_2 was added drop by drop. The culture was rubbed and the immediate production or absence of air bubbles was recorded. Production of air bubbles indicates the production of catalase.

Twenty four hours old fresh culture was picked and touched on a sterile oxidase disc. The production of violet colour within 30 sec indicates oxidase positive.

Isolated bacterial strains *Paenibacillus* sp. and *E.coli* from samples were tested for their antibiotics susceptibility tests by the antibiotic disc diffusion method [4]. The commercial antibiotics were used include Cephalothin, Clindamycin, Cotrimoxazole, Erythromycin, Gentamycin, Ofloxacin, Vancomycin, Amoxycillin, Cloxacillin, Tetracycline, Penicillin, Co-trimoxazole and Cephalexin. The petriplates containing 20 ml of Muller Hinton agar were seeded with 4 hours fresh culture of isolates. By making use of template drawn commercial antibiotics disc were dispensed on the solidified Muller Hinton agar. This was incubated at 37˚C for 24 hours in an incubator and was observed for the development of clearance/inhibition zones around the antibiotic disc.

The cells were grown overnight in Luria Bertani broth containing sodium chloride (LBS, NaCl, 2% w/v) and incubated at 37˚C in a shaker incubator (120 rpm) for 16–18 h. 1.5ml of culture were used for plasmid isolation by using Plasmid Isolation minispin kit (Chromous Biotech, Bangalore).

A bacterial colony was picked and streaked onto LB agar plate containing antibiotic and incubated for 24 hours. After incubation the culture (*Paenibacillus* sp.) were transferred into 5 ml of LB broth and incubated overnight at 37˚C in a shaker at 200 rpm. From that 1.5 ml of the culture was transferred into an eppendorf tube and centrifuged at 10,000 rpm for 1 min at 4˚C.

The supernatant was removed and pellet was collected. The pellet was then suspended in 250 µl of DNA suspension buffer (in cold condition). The bacterial cells were completely suspended by vortexing until no clumps remain. 250 µl of DNA extraction buffer was added and gently mixed by inverting 3-4 times. Immediately 350 µl of Plasmid Binder was added and mixed immediately by gently inverting the vials 3-4 times. The vials were then centrifuged at 13,000 rpm for 10 min at room temperature. The supernatant containing plasmids were decanted into an empty spin column along with the collection tube (600 µl at a time). The column was centrifuged at 13,000 rpm for 1 min at room temperature. The contents of collection to tubes were discarded and the spin column was placed back in the same collection tube. 500 µl of clean-up buffer was added to the column and spinned at 13,000 rpm for 1 min at room temperature. The contents of collection to tubes were discarded and the spin column was placed back in the same collection tube. 500 µl of 1X wash buffer was added to the column and spinned at 13,000 for 1 min at room temperature. The contents of collection to tubes were discarded and the spin column was placed back in the same collection tube. The empty column along with collection tube was centrifuged at 13,000 rpm for 3 min at room temperature. The spin column was then replaced in a 1.5 ml fresh eppendorf tube. 50 µl of elution buffer was added to the centre of the membrane of spin column. The vial along with spin column was kept at room temperature for 2 min and then centrifuged at 15,000 rpm for 1 min at room temperature. The spin column is then removed from the vial and the isolated plasmid was collected in the eppendorf tube. The isolated plasmid was then subjected to electrophoresis.

Electrophoresis was performed using 0.8% agarose gel system (Bangalore Genei, India) in Tris acetate buffer. Gels were stained with ethidium bromide. The resolved bands were visualized on a UV-transilluminator at a wavelength of 360 nm.

5 µl of Plasmid DNA was added to 100 µl of competent cells prepared (*E.coli*). The vial was gently tapered and kept on ice for 20 min. The vial was incubated in the water bath at 42˚C for 2 min such that the competent cells are immersed. After completion of 2 min the vials were quickly removed and chilled the vial on ice for 20 min. 1 ml of LB broth was aseptically transferred to the vial and incubated for 1 hour at 37˚C to allow bacteria to recover and express the antibiotic resistance. Then it was plated onto the LB plate containing antibiotic (Tetracycline), X–Gal and IPTG. The results were noted on the basis of colour colonies developed on the plates.

Results

Out of twenty five crabs (*P. sanguinolentus*) that were collected

from Parangipettai fish market twenty one showed the presence of *E.coli* while only four crab showed the presence of *Paenibacillus* sp. Table 1 when cultured on Zobell's marine agar and were identified upto the genus level by biochemical tests and selective medium (Table 2).

Spread plate technique was performed to isolate the colonies. The isolates were fast growing and were mucoid in nature. However, a little variation was observed in their morphology of the colonies. The colonies were picked and pure cultured and maintained in slants. The results of various biochemical tests have been tabulated in table 3.

Paenibacillus sp., shows resistance to the eleven antibiotics and it was sensitive to two antibiotics. Whereas *E.coli* shows complete sensitive to all the thirteen antibiotics presents in the two different antibiotics disc (Table 4).

Plasmid was extracted from the multiple drug resistance *Paenibacillus* sp. and run on agarose gel electrophoresis along with the 2000 bp ladder and the bands were observed in the gel documentation system. The molecular weight of the isolated plasmid DNA was found to be 1300 bp.

Media	*E. coli*	*Paenibacillus sp*
EMB	Metallic Sheen coloured colonies	-
Tryptic Soy agar	-	Yellow coloured colony

Table 1: Identification of Bacteria from different selective media.

S:No	*E. coli*	*Paenibacillus sp*
1	*E. coli*	21
2	*Paenibacillus sp*	4

Table 2: Incidence of bacteria isolated from crab.

S: No	Biochemical test	*Paenibacillus sp*	*E. coli*
1	Gram staining	+	-
2	Shape	Rod	Rod
3	Motility	+	+
4	Indole test	-	+
5	Methyl Red test	-	+
6	Voges Prauskauer test	-	-
7	Citrate utilization test	+	+
8	Triple sugar iron agar test	A	A
9	Catalase	+	+
10	Oxidase	-	-

Table 3: Identification Features of Bacteria from gut of crab.

S: No	Biochemical test	*Paenibacillus sp*	*E. coli*
1	Cephalothin	R	S
2	Clindamycin	R	S
3	Cotrimoxazole	R	S
4	Erythromycin	R	S
5	Gentamycin	S	S
6	Ofloxacin	S	S
7	Penicillin	R	S
8	Vancomycin	R	S
9	Tetracyclin	R	S
10	Chloramphenicol	R	S
11	Amoxycillin	R	S
12	Cloxacillin	R	S
13	Cephalexin	R	S

R- Resistant; S- Sensitive

Table 4: Antibacterial activity of selected isolates.

The plasmids were successfully transformed to *E.coli* were found on EMB plates with antibiotic, forming a mat of colonies upon the media. Since colonies were forming like a mat, three times it was analysed to count the individual colonies but all the three times it was difficult to count the individual colonies due to the formation of mat and hence the recombination percentage was not calculated. Transformation was also analysed on Luria Bertani agar plate with the antibiotic tetracycline and the formation of blue-white colonies were observed indicating the absence and presence of both the transformed and untransformed colonies.

Discussion

Various factors contribute to antimicrobial resistance, some of which are the use of antimicrobials in animal feeds in low doses, availability of antimicrobials over-the-counter in many countries, misuse by health professionals, poor patient compliance, antimicrobial application in agriculture, aquaria and family pets and eating raw or undercooked foods. The most notable causes are improper antimicrobial use and inappropriate empiric therapy selection.

Yokota et al. [5] in Japan discovered a phenomenon of transfer of bacterial resistance from clinical isolates of Shigella flexneri and E. coli to sensitive bacteria. It was the first discovery of R factors.

According to Akinbowale et al. [6] although there are no products registered for use in aquaculture, antimicrobial resistance is present in isolates from aquaculture and marine environments. Terrestrial bacteria entering into the seawater with antibiotic-resistant plasmids may also be responsible for the prevalence of resistance genes in the marine environment6. In the present study it was found that resistant plasmid of *Paenibacillus* sp. was transformed into the drug sensitive *E.coli*. According to Peter and Annie [7] and Baya et al. [8] exposure to heavy metals and other toxicants can confer resistance to some antibiotics as well. Transfer of antibiotic resistant bacteria to humans via the food chain is a significant health concern. Tendencia and Leobert [9] studied and established that multiple drug resistance (MDR) and intermediate sensitivity test reaction of the drug-resistant bacterial strains affect future of crab as well as human with diseases which are associated with antimicrobial use thus, the use of antibiotics in aquaculture even at low concentration should be avoided.

Tetracycline resistance is due to three different mechanisms, which are all plasmid-mediated in the present result also showed the tetracycline resistance when the resistance plasmid was transformed into sensitive *E.coli* , Skerman et al. [10] suggested that plasmids carried by fish pathogens may potentially serve as a reservoir for resistance to other antimicrobials. Eleoner et al. [11] reported that the incidence of resistance to chloramphenicol was the least observed among the individual antibiotics in their study. But in the present study *Paenibacillus* sp. was found to be resistant towards the antibiotic chloramphenicol.

No change in the number of isolates was apparent when susceptibility was tested against to the antibiotics prior to and after plasmid curing experiments, which indicates that resistance to these antibiotics is found to be chromosomal. Skerman et al. [12] discovered the role of bacterial plasmids in the production of enterotoxins The present study pointed out that when antimicrobial agents are used more often in an environment, the higher will be the occurrence of resistant microorganisms in that site. This is because exposure to antimicrobials may also result to cross-resistance between other aquaculture antibacterials [13] assessed and investigated the efficacy of

different organic acids in decreasing the heat resistance of *Paenibacillus polymyxa* spores and the relationship between concentration of the undissociated form of different organic acids and decrease in heat resistance. The heat resistance of *P. polymyxa* spores was tested in distilled water at 85, 90 and 95˚C, at pH 4 and in the presence of 50, 100 and 200 m mol l^{-1} of the undissociated form of lactic, citric or acetic acid and sodium citrate or acetate. The undissociated form of organic acids was responsible for increasing the heat sensitivity of spores.

Upon the introduction of a new antibacterial into the market, the development of resistance is simply a matter of time. Hence, the knowledge of specific resistance pathways, including information regarding molecular mechanisms in atomic detail, can provide crucial insights into the development of novel drugs. Molecular knowledge and behavioral changes must progress hand-in-hand if serious progress is to be made in the war against the development of antibiotic resistance [13].

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