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Pathogenic Bacteria Isolated from Diseased Giant Freshwater Prawn in Shrimp Culture Ponds

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***Abstract*—**Pathogenic bacterial flora was isolated from giant freshwater prawns, *Macrobrachium rosenbergii*. Infected shrimp samples were collected from BuaBan Aquafarm in Kalasin Province, Thailand, between June and September 2018. Bacterial species were isolated by serial dilution and plated on Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar medium. A total 89 colonies were isolated and identified using the API 20E biochemical tests. Results showed the presence of genera *Aeromonas, Citrobacter*, *Chromobacterium*, *Providencia*, *Pseudomonas*, *Stenotrophomonas* and *Vibrio*. Maximum number of species was recorded in *Pseudomonas* (50.57%) with minimum observed in *Chromobacterium* and *Providencia* (1.12%).

***Keywords*—**Biochemical test, giant freshwater prawn, isolation, salt tolerance, shrimp diseases.

I. Introduction

FRESHWATER prawn culture has undergone significant growth in tropical freshwater aquaculture research during the past decades. Aquaculture is an economically important venture with high commercial value. This intensification in aquaculture has generated research into the favorable environmental required conditions for pathogenic bacterial growth in culture systems [1]. Culture practices of freshwater prawn, particularly, the giant river prawn, *Macrobrachium rosenbergii* known as scampi are rapidly expanding in many countries. In Thailand, *M. rosenbergii* is one of the most important commercial crustaceans with high demand in both domestic and export markets. Environmental factors and microbiological parameters affect quality and health of shrimp. Survival and growth of *M. rosenbergii* are influenced by density of prawn populations, water volume/surface area, pH level of water, and food addition [2].

Low quality freshwater prawn production results from several factors, especially environmental parameters and microbes in the culture water. Diseases caused by bacteria, viruses, protozoa, fungi and helminthes are major problems encountered during *M. rosenbergii* culture. Microorganisms have been implicated in several adverse conditions, while viral infections are the leading cause of hepatopancreatic, and white tail diseases. Bacterial diseases, bacterial necrosis, larvae mid-cycle disease due to *Alcaligenes* spp., and *Enterobacter* spp., Spiroplasma disease due to a novel pathogen Spiroplasma MR-1008, and black spot, brown spot and shell disease are caused by *Vibrio* spp., *Pseudomonas* spp., and *Aeromonas* spp. Fungal agents as, *Fusarium solani*, *Debaryomyces hansenii* and *Metschnikowia bicuspidate* cause idiopathic muscle necrosis (IMN) in larvae [2].

Previous research in Brazilian prawn farms identified black-spot bacterial necrosis and gill obstruction. Brady and Lasso characterized *Aeromonas* spp., *Bacillus* spp., and *Pseudomonas* spp., which are the major causes of prawn haemolymph infection. Both *Aeromonas* and *Pseudomonas* have been isolated from the hepatopancreas (HP) of obviously healthy prawns. These bacteria can produce five extracellular products (ECPs) as protease, gelatinase, chitinase, lipase, and hemolysin. For this reason, *Aeromonas* spp. is considered to be a major threat to commercial aquacultural cultivation of *M. rosenbergii* as the causative agents of disease in Malaysia, Sri Lanka, Taiwan, Brazil, India, China, Japan and Thailand during the past few years [3]. Here, isolation and identification of pathogenic bacteria from infected prawns were investigated.

II. Materials and Methods

A. Prawn Sampling and Bacterial Isolation

Diseased shrimps were collected from BuaBan Aquafarm in Kalasin Province, immediately stored in ice and analyzsed in the laboratory within 3-4 h. Samples were washed thoroughly with sterile distilled water. The liver, HP and intestine were dissected with sterile scissors and homogenized in physiological saline under aseptic conditions. Appropriate dilutions of homogenized samples from the shrimp body parts were plated on TCBS agar (HiMedia) by spreading. Isolated bacteria from the body parts of shrimps were considered as shrimp disease isolates [4]. Pure colonies were subcultured on a Tryptic Soy Agar (TSA), HiMedia slant at 37 ^{o}C for 48 h and then stored at 4 ^{o}C until required for phenotypic characterization studies.

B. Phenotypic Characterization of Bacterial Isolates

Bacterial isolates selected from single colonies on TSA, were tested using Gram-staining, Indole, Methyl red, Voges Proskauer, Citrate, H_2S production in TSI agar, Lysine, fermentation of cellobiose, catalase and oxidase as described by [5], [6]. Growth patterns at 37 and 42 ^{o}C were also observed.

C. Growth on Salt Medium

Isolates were tested for salt tolerance using NaCl. Nutrient broth (HiMedia) was modified with addition of 0-10% NaCl.

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Isolates were inoculated in these modified broths at 37 °C for 48 h. Any degree of turbidity was considered for positive growth [6].

D. Biochemical Identification of Shrimp Bacteria

Biochemical characteristics of bacterial isolates from prawn were performed using API 20E strips (bioMerieux, France), according to the manufacturer's instructions. Identification of bacterial pathogens characteristics was compared with Bergey's Manual of Systematic Bacteriology [7].

III. Results and Discussion

A total of 89 pathogenic bacterial colonies were isolated from the infected *M. rosenbergii*.

A. Physical and Biochemical Study

Initially 89 suspected colonies were selected from the TCBS agar plate. Results of the biochemical tests presented in Table I show that 25 isolates were yellow, and 64 were green colored on TCBS agar. All isolates showed oxidase negative. 54 isolates were found with positive catalase, 43 isolates were found with positive MR, 28 isolates were found with positive VP and 45 isolates were found with positive LDC. All 89 isolates grew at 37 and 42 °C.

B. Growth of Isolates on Salt Media

Pathogenic bacteria can tolerate considerable amounts of salt. Here, we tested all 89 isolates for growth in nutrient broth containing NaCl 0-10%. All isolates grew in media without NaCl and showed salt tolerance up to 6% NaCl, while 87.64% and 48.32% of the isolates were found to grow at 8% and 10% concentration of NaCl respectively (Fig. 1). Therefore, all the isolates were considered as halotolerant. Increase of salt concentration causes a change in sensitivity toward antibiotics from susceptibility to phenotypic resistance [6].

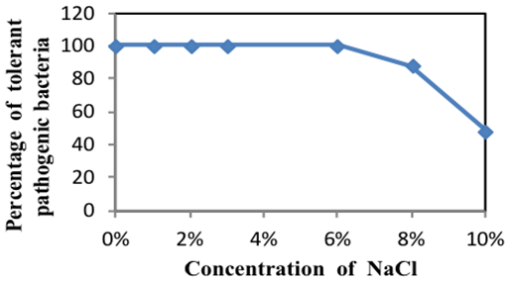


Fig. 1 Effect of salt concentration on isolated pathogenic bacteria

C. Occurrence and Distribution of Isolates

Among the pathogenic bacteria studied, *Pseudomonas aeruginosa*, *P. fluorescens* and *P. luteola* (50.57%) were the most prominent, followed by *Aeromonas hydrophila*, *A. salmonicida* (20.22%), *Citrobacter braakii*, *C. freundii*, *C. koseri*, *C. youngae* (15.73%) and *Stenotrophomonas maltophila* (8.99%) as shown in Fig. 2.

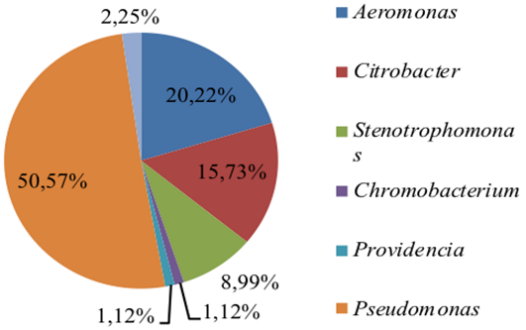


Fig. 2 Percentage occurrence of pathogenic bacteria in the samples

D. Species Diversity

During the study, 89 bacterial isolates belonging to the genera *Stenotrophomonas*, *Pseudomonas*, *Chromobacterium*, *Aeromonas*, *Citrobacter*, *Providencia* and *Vibrio* were examined for pathogenic bacteria in prawns. Species composition of the isolates is given in Figs. 3 and 4. In shrimp culture ecosystems, pathogenic bacteria play a negative role as they compete with shrimps for food and oxygen while causing stress and diseases [8]. Generally gram-negative bacteria were found to be the dominant forms in shrimp culture ponds [9].

Presence of pathogenic bacteria in typical organs of giant freshwater prawns showed maximum percentage in the HP. Matyar [10] reported that six species found with gram-negative bacterial genera at relatively high frequencies were *Stenotrophomonas maltophila* (19.6%), *Acinetobacter lwoffii* (14.4%), *Proteus vulgaris* (9.3%), *P. penneri* (8.3%), *Burkholderia cepacia* (8.3%) and *Pseudomonas aeruginosa* (7.2%). This may be due to seasonal variation of water and environmental conditions. However, opportunistic species may be expected to vary from one geographical area to another and from one hatchery to another within a country as well as between different countries [11]. Abdolnabi et al. [3] revealed that a wide variety of fish and shellfish including giant freshwater prawns were susceptible to *A.hydrophila* which is also believed to be a pathogen of emerging importance for humans through consumption of contaminated fish and shellfish. Prawn intestines collected from culture ponds contained *Escherichia coli*, *Pseudomonas* spp., *Enterobacter* spp., *Vibrio* spp., *Aeromonas* spp. and *Staphylococcus aureus* [12]. Members of the genus Pseudomonas are a ubiquitous group of gram-negative, rod-shaped, motile bacteria showing metabolic versatility. They can survive in environment which are hostile to many other bacteria, as one of the most diverse bacterial genera, containing over 60 validly described species *P.aeruginosa* was identified as harmful to *M.rosenbergii* found to be 30% from farm cultured prawns [12]. Ramalingam and Ramarani [13] infected giant freshwater prawns with *P.aeruginosa* MTCC1688 to determine the histopathological effects in vivo. Their results revealed characteristic degenerative changes in both body muscle and HP.

TABLE I
BIOCHEMICAL CHARACTERIZATION OF BACTERIAL ISOLATES FROM INFECTED SHRIMP *MACROBRACHIUM ROSENBERGII*

No. of isolate	Gram staining	Color on TCBS	Cell shape	Indole	Methyl Red	Voges-proskauer	Citrate	Urease	Catalase	Oxidase	H_2S production	Growth at 37°C	Growth at 42°C	Identification of bacteria
KSGV01	-	G	Rod	-	-	-	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV02	-	Y	Rod	-	+	-	-	-	-	-	-	+	+	*Vibrio fluvialis*
KSGV03	-	Y	Rod	+	+	-	-	-	-	-	-	+	+	*Aeromonas hydrophila*
KSGV04	-	Y	Rod	-	+	-	-	-	-	-	-	+	+	*Vibrio fluvialis*
KSGV05	-	G	Rod	-	+	-	+	-	+	-	-	+	+	*Citrobacter youngae*
KSGV06	-	G	Rod	-	+	-	+	-	-	-	-	+	+	*Pseudomonas fluorescens*
KSGV07	-	Y	Rod	-	+	+	+	-	-	-	-	+	+	*Stenotrophomonas maltophila*
KSGV08	-	G	Rod	-	+	-	+	-	+	-	-	+	+	*Citrobacter youngae*
KSGV09	-	Y	Rod	+	-	+	+	-	+	-	+	+	+	*Aeromonas hydrophila*
KSGV10	-	G	Rod	-	+	-	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV11	-	G	Rod	-	+	-	+	-	+	-	+	+	+	*Citrobacter youngae*
KSGV12	-	G	Rod	-	+	-	+	-	+	-	+	+	+	*Citrobacter youngae*
KSGV13	-	Y	Rod	+	+	+	-	-	-	-	-	+	+	*Aeromonas hydrophila*
KSGV14	-	G	Rod	-	+	+	+	-	-	-	-	+	+	*Pseudomonas aeruginosa*
KSGV15	-	Y	Rod	+	+	+	+	-	+	-	-	+	+	*Chromobacterium violaceum*
KSGV16	-	G	Rod	-	+	+	+	-	+	-	+	+	+	*Citrobacter youngae*
KSGV17	-	G	Rod	-	+	-	-	-	+	-	-	+	+	*Citrobacter freundii*
KSGV18	-	G	Rod	-	+	-	+	-	+	-	-	+	+	*Pseudomonas aeruginosa*
KSGV19	-	G	Rod	-	-	-	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV20	-	Y	Rod	+	-	+	-	-	+	-	-	+	+	*Aeromonas hydrophila*
KSGV21	-	G	Rod	-	-	+	+	-	-	-	-	+	+	*Pseudomonas aeruginosa*
KSGV22	-	G	Rod	-	-	-	+	-	-	-	+	+	+	*Citrobacter freundii*
KSGV23	-	G	Rod	-	-	-	+	-	-	-	-	+	+	*Pseudomonas aeruginosa*
KSGV24	-	Y	Rod	-	+	+	+	-	+	-	-	+	+	*Stenotrophomonas maltophila*
KSGV25	-	Y	Rod	-	-	-	+	-	-	-	+	+	+	*Stenotrophomonas maltophila*
KSGV26	-	Y	Rod	+	-	+	-	-	-	-	+	+	+	*Aeromonas hydrophila*
KSGV27	-	G	Rod	-	-	-	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV28	-	Y	Rod	-	-	-	+	-	+	-	-	+	+	*Stenotrophomonas maltophila*
KSGV29	-	G	Rod	+	+	-	+	-	+	-	-	+	+	*Providencia rettgeri*
KSGV30	-	Y	Rod	+	+	+	-	-	-	-	-	+	+	*Aeromonas hydrophila*
KSGV31	-	G	Rod	-	+	-	-	-	+	-	-	+	+	*Citrobacter freundii*
KSGV32	-	G	Rod	+	+	-	-	-	+	-	-	+	+	*Citrobacter braakii*
KSGV33	-	G	Rod	+	+	-	+	-	+	-	-	+	+	*Citrobacter koseri*
KSGV34	-	G	Rod	+	+	-	+	-	+	-	-	+	+	*Citrobacter koseri*
KSGV35	-	Y	Rod	-	+	-	-	-	-	-	-	+	+	*Aeromonas salmonicida*
KSGV36	-	G	Rod	-	-	-	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV37	-	G	Rod	-	-	-	+	-	-	-	+	+	+	*Pseudomonas luteola*
KSGV38	-	G	Rod	-	-	-	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV39	-	G	Rod	-	-	+	+	-	-	-	+	+	+	*Pseudomonas aeruginosa*
KSGV40	-	G	Rod	-	-	+	+	-	+	-	-	+	+	*Citrobacter freundii*
KSGV41	-	G	Rod	-	-	-	+	-	-	-	+	+	+	*Pseudomonas aeruginosa*

No. of isolate	Gram staining	Color on TCBS	Cell shape	Indole	Methyl Red	Voges-proskauer	Citrate	Urease	Catalase	Oxidase	H_2S production	Growth at 37°C	Growth at 42°C	Identification of bacteria
KSGV42	-	G	Rod	-	-	-	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV43	-	Y	Rod	-	-	-	-	-	-	-	+	+	+	*Stenotrophomonas maltophila*
KSGV44	-	G	Rod	-	+	-	+	-	+	-	-	+	+	*Pseudomonas aeruginosa*
KSGV45	-	G	Rod	-	+	-	+	-	+	-	-	+	+	*Pseudomonas aeruginosa*
KSGV46	-	G	Rod	-	-	+	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV47	-	G	Rod	-	-	+	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV48	-	G	Rod	-	-	+	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV49	-	Y	Rod	+	+	+	-	-	+	-	-	+	+	*Aeromonas hydrophila*
KSGV50	-	Y	Rod	+	+	-	-	-	-	-	-	+	+	*Aeromonas hydrophila*
KSGV51	-	G	Rod	-	-	+	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV52	-	G	Rod	-	-	+	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV53	-	G	Rod	-	-	-	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV54	-	G	Rod	-	+	-	+	-	-	-	-	+	+	*Pseudomonas aeruginosa*
KSGV55	-	G	Rod	-	+	-	+	-	-	-	-	+	+	*Citrobacter freundii*
KSGV56	-	G	Rod	-	-	+	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV57	-	G	Rod	-	-	+	+	-	-	-	+	+	+	*Pseudomonas aeruginosa*
KSGV58	-	G	Rod	-	-	+	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV59	-	G	Rod	-	-	+	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV60	-	G	Rod	-	-	-	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV61	-	G	Rod	-	+	-	+	-	+	-	-	+	+	*Pseudomonas aeruginosa*
KSGV62	-	G	Rod	-	-	-	+	-	-	-	+	+	+	*Pseudomonas aeruginosa*
KSGV63	-	G	Rod	-	-	-	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV64	-	Y	Rod	+	+	-	-	-	-	-	-	+	+	*Aeromonas hydrophila*
KSGV65	-	G	Rod	-	-	-	+	-	-	-	+	+	+	*Pseudomonas aeruginosa*
KSGV66	-	Y	Rod	+	+	+	-	-	-	-	+	+	+	*Aeromonas hydrophila*
KSGV67	-	G	Rod	-	+	-	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV68	-	Y	Rod	+	+	-	-	-	-	-	+	+	+	*Aeromonas hydrophila*
KSGV69	-	Y	Rod	+	+	-	-	-	-	-	-	+	+	*Aeromonas hydrophila*
KSGV70	-	Y	Rod	+	+	-	+	-	-	-	-	+	+	*Aeromonas hydrophila*
KSGV71	-	G	Rod	-	-	-	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV72	-	G	Rod	-	-	-	+	-	-	-	+	+	+	*Pseudomonas aeruginosa*
KSGV73	-	G	Rod	-	-	-	+	-	-	-	+	+	+	*Pseudomonas aeruginosa*
KSGV74	-	G	Rod	-	-	-	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV75	-	G	Rod	-	-	-	+	-	-	-	+	+	+	*Pseudomonas aeruginosa*
KSGV76	-	G	Rod	-	-	+	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV77	-	G	Rod	-	-	+	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV78	-	G	Rod	-	-	-	+	-	+	-	+	+	+	*Pseudomonas*

No. of isolate	Gram staining	Color on TCBS	Cell shape	Indole	Methyl Red	Voges-proskauer	Citrate	Urease	Catalase	Oxidase	H_2S production	Growth at 37°C	Growth at 42°C	Identification of bacteria
														aeruginosa
KSGV79	-	G	Rod	-	-	-	-	-	+	-	-	+	+	*Citrobacter youngae*
KSGV80	-	G	Rod	-	-	-	-	-	+	-	-	+	+	*Citrobacter freundii*
KSGV81	-	G	Rod	-	-	-	+	-	+	-	+	+	+	*Pseudomonas aeruginosa*
KSGV82	-	G	Rod	-	+	-	+	-	+	-	-	+	+	*Pseudomonas aeruginosa*
KSGV83	-	G	Rod	-	+	+	+	-	+	-	-	+	+	*Pseudomonas aeruginosa*
KSGV84	-	Y	Rod	+	-	-	-	-	-	-	-	+	+	*Aeromonas hydrophila*
KSGV85	-	G	Rod	-	+	-	+	-	+	-	-	+	+	*Pseudomonas aeruginosa*
KSGV86	-	Y	Rod	+	+	-	-	-	-	-	-	+	+	*Aeromonas hydrophila*
KSGV87	-	Y	Rod	+	+	-	-	-	+	-	-	+	+	*Aeromonas hydrophila*
KSGV88	-	Y	Rod	+	+	-	-	-	-	-	-	+	+	*Aeromonas hydrophila*
KSGV89	-	Y	Rod	-	-	+	+	-	+	-	-	+	+	*Stenotrophomonas maltophila*

G = Green, Y = Yellow, + = Positive, - = Negative.

IV. Conclusion

Results revealed that bacteria are the most important pathogens in shrimp culture ponds causing increased mortality and financial loss. Shrimp culture methods must be urgently improved to control pathogenic microbes. Good rearing practices, hygiene and use of antibiotic or probiotic microorganisms as supplemented feed in shrimp rearing ponds are essential for optimal results.

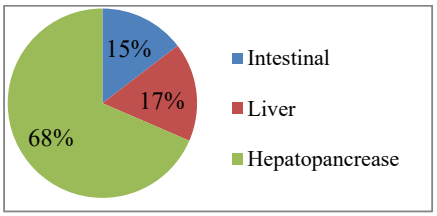
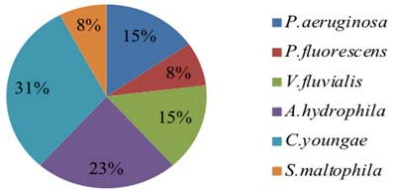
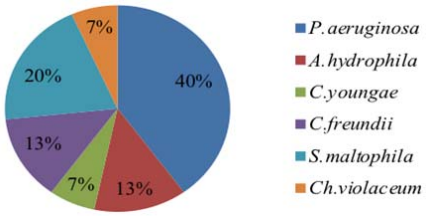


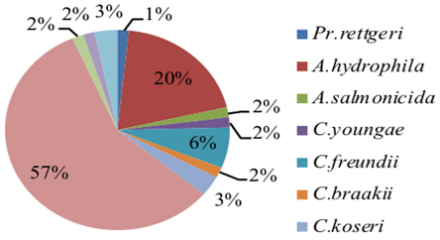
Fig. 3 Percentages of pathogenic bacteria in different organs of giant freshwater prawns



(a)



(b)



(c)

Fig. 4 Percentages of different bacterial species in (A) intestine, (B) liver and (C) HP

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