Studies on Biology, Pathogenicity and Prophylaxis of Vibriosis in Juvenile Tiger Shrimp *Penaeus monodon* (Fabricius)

by

Pornlerd Chanratchakool

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To the memory of my Father

Declaration

I declare that this thesis represents results of research conducted by me at the Aquatic Animal Health Research Institute, Thailand and the Institute of Aquaculture, University of Stirling, during the period 1989-1992. The literature consulted has been cited, and where appropriate, collaborative help has been acknowledged.

This work has not been submitted for any other degree.

Candidate : P. Chamratchakool

Supervisor : Ronald J. Roberts

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Abstract

Studies were carried out to examine the biology, pathogenicity and treatment of *Vibrio* spp. in cultured shrimp (*Penaeus monodon* Fabricius). The phenotypic characteristics and the sensitivity to oxytetracycline (OTC) of bacteria isolated from diseased shrimp in two areas of Thailand were compared. The areas were Samutsakhon province where shrimp have been cultured for many years and Chanthaburi province, an area in which shrimp culture started after 1988. Thirty-one *Vibrio* spp. isolates were obtained from Samutsakhon and twenty-two *Vibrio* spp. and one *Aeromonas* spp. were obtained from Chanthaburi. The most numerous species from both areas was *Vibrio parahaemolyticus*. Minimal Inhibitory Concentration and Minimal Bacteriocidal Concentration studies were conducted to determine the sensitivity of the isolates to OTC. The isolates from Samutsakhon were found to be more resistant to OTC than those from Chanthaburi.

Batches of shrimp were fed with pellets containing different quantities of OTC and their muscle was subsequently analysed to determine its OTC content. In shrimp fed with pellets containing 3 or 5 gOTC/kg of feed, for up to 5 days, the maximum drug in the muscle was approximately 1 ppm. As the OTC was administered for longer periods the concentration and persistance in the muscle increased.

A study was conducted to examine the interaction between prophylactic administration of in fed OTC and an injection challenge with a pathogenic OTC sensitive *Vibrio parahaemolyticus*. Histological examination revealed that the

prophylactically treated shrimp produced a less marked cellular response and healed more rapidly when injected with bacteria.

The implication of all these results for the treatment of vibriosis in cultured shrimp is discussed.

Table of Contents

List of Tables

List of Figures

Page

Studies on Biology, Pathogenicity and Prophylaxis of Vibriosis in Juvenile Tiger Shrimp *Penaeus monodon* (Fabricius)

General Introduction

As intensive shrimp culture has developed in Thailand since 1987, so disease problems have become very serious, especially in the late stages of the process, in grow-out ponds, where the intrinsic value of the shrimp is high and potential economic loss from disease can readily bankrupt the farm. The bacterial disease, vibriosis, the subject of this dissertation, is generally claimed, often with inadequate data, to be the most significant cause of losses and many farms have already suffered from the disease. Disease outbreaks may be acute, subacute or chronic, and cause mortalities ranging from the inconsequential to total loss of the affected population. Such differences in level of loss will be related to a number of factors, not least of which will be the effects of husbandry factors such as stocking density and water quality, but it is likely that they will be due at least in part to difference in strain or biotype between the different vibrios responsible. Thus a pre-requisite to understanding the problem of vibriosis in shrimp culture is a knowledge about the biology, characteristics and pathogenicity of different vibrios associated with such culture.

In order to prevent the disease or to treat infection in various stages of shrimp culture, usage is currently made of a large number of antibiotics, antimicrobials and disinfectants. However, although these often appear to be effective in controlling and preventing or reducing losses, no scientific data

exists on their usage. One of the most commonly used antibiotics is oxytetracycline, reported as an effective treatment against vibriosis in aquatic animals. It can, however, on occasion, have little effect on mortality even when used at high oral dosage. Thus, an understanding of the biology, characteristics and pathogenicity of various strains of *Vibrio* sp. for shrimps, and the effective treatment, control and prevention of the disease they cause is greatly in need of systematic studies.

The present work was initiated with the intention of providing such a study, with special reference to the cultured shrimp industry of Thailand, which is one of the largest and most advanced in the world, and, where vibriosis is probably the most significant cause of economic loss.

Chapter I. Shrimp Cultures And *Vibrio* Species

A General Review.

Introduction

I. The development of shrimp culture.

Since 1975, when the world crustacean production stood at 2.4 million metric tones, there has been a steady and sustained increase to 3.2 million metric tones in 1985, the last year for which detailed figures are available (Csavas, 1988).

Within these figures, the contribution of aquaculture has been dramatic, rising from 1.2 % of the total in 1975 to 8.2 % in 1985 and probably over 10 % in 1988. Almost 75 % of this cultured product comes from Asia and 96 % of this cultured crustacean product is marine shrimp (Csavas, 1988).

For many years China, Taiwan and Indonesia led the production tables with regard to farm raised shrimp in Asia. In 1987, Taiwan produced 100,000 metric tons of farm raised shrimp from intensive ponds. Unfortunately, with the rapid expansion of shrimp culture, Taiwanese farmers faced serious disease outbreaks in 1988. These were so severe that production was decreased to 30,000 metric tons and continued to drop to an estimated 20,000 metric tons in 1989. At the same time Thailand and Indonesia significantly increased their production and became the worlds biggest farmed shrimp producing countries. It is estimated that Thailand by itself produces 16 % of the world's farmed shrimp production (approximately 90,000 metric tons of head-on shrimp). When comparing shrimp production by species, the giant tiger shrimp *Penaeus monodon*(Fabricius) has proved to be by far the most important species because it was not only the largest but also the fastest

growing (World Shrimp Farming, 1990).

I.I Types of shrimp cultured.

Kungvankij and Kongkeo (1988) have described three general types of shrimp culture.

a.) Traditional or extensive shrimp farming. Extensive shrimp farming is considered to be the simplest method of shrimp culture. It is characterized by the uses of irregularly shaped ponds of varying sizes, usually ranging from 5-20 hectares. Each pond has a peripheral ditch 10-20 m. wide and 30-60 cm deep. Seed (young shrimp) is normally obtained from the wild and therefore the whole system is seasonally dependent. There are two types of method for stocking the seed. The first is simply by opening the pond's gate during the high tide, with or without pumping the water. This allows natural stocks of mixed shrimp seed and fish larvae to be brought in by the incoming water. This type of system occurs in Thailand, Indonesia and Malaysia. The second type of extensive system is that used in the Philippines, where farmers usually stock their ponds with fry collected from the wild. The average stocking density in extensive culture generally ranges from 3,000-20,000 fry/ha.and the yield ranges from 300-800 kg/ha/yr.

b.) Semi-intensive system. As more systematic layouts of ponds have developed, cultured ponds have generally become rectangular in shape with size ranging from about 1-5 ha and depth of 0.8-1.2 m. Each pond has a separate inlet and outlet gate. Inside the pond, a ditch, 5-10 m.wide and 30-50 cm. deep is often constructed in front of the outlet gates in order to facilitate

draining of water and collecting the shrimp during harvest. The stocking density is generally higher. Supplementary feed is normally used and regular water management procedure also performed. Source of seed is usually from hatcheries and the shrimp at the developmental stage post larva 15 (PL-15) are generally stocked at 25,000-80,000 fry/ha.. Semi-intensive shrimp culture of this nature is found in Thailand, Equador, Indonesia, Japan and the Philippines. Production ranges between 1-3 MT/ha/yr.

c.) Intensive culture-system. This type of shrimp culture requires sophisticated facilities, high financial input and has all the features of any high stocking density, intensive food production industry. Fry are produced in large volume from hatcheries and an aeration system and formulated feed as well as intensive water management techniques are used. All of these features distinguish intensive shrimp culture from other systems. Intensive ponds may be earthen or concrete and size may vary from 500 to 5,000 m^2. This type of culture occurs in Thailand, Taiwan, Japan and the Philippines. It is vital in such systems that a separate water inlet and water outlet is used. Stocking density in such systems is relatively high, with over 100,000 hatchery fry/ha being used and successful farm may have a production ranging between 8-12 MT/ha/yr.

I.II The development of shrimp culture in Thailand

Although shrimp culture has been developed since 1940, it has generally been as a traditional extensive system. It only developed slowly, until 1972, when with Royal Thai Government support, it became a major industry.

The area of land under shrimp culture in Thailand has subsequently increased by around 12 percent per year, and production also increased by as much as 22 percent per year (1,589 MT in 1977 and 17,886 MT in 1986). Between 1981-1986, tiger shrimp culture was started and rapidly expanded, the rate of expansion at one stage averaging approximately 70 percent per year. Once mass production of *P.monodon* larvae had been perfected in 1987, the production rose from 17,886 metric tons in 1986 to 64,000 metric tons in 1988 and the average yield was increased from 63 kg/rai/yr.in 1986 to 128 kg/rai/yr.in 1988 due to use of more intensively farmed systems.

From the Sixth National Economic and Social Development Plan (1989-1992),it is expected that shrimp production will rise as follows:

	1989	1990	1991
production (metric tons)	91,000	118,000	145,000
farm areas (ha^{-1})	80,000	80,000	80,000
average yield ($kg.ha^{-1}$)	1,138	1,457	1,800

It is currently proposed to limit the area of land used for shrimp production and to increase production by development of the culture technique. Thus there would be a great extension of use of intensive culture systems. By 1991, the area of land under intensive culture was expected to be increased from 8,000 ha^{-1} in 1989 to 16,000 ha^{-1}, semi-intensive culture to be increased from 16,000 ha^{-1} in 1989 to 24,000 ha^{-1} and the extensive culture area to be decreased from 56,000 ha^{-1} in 1989 to 40,000 ha^{-1}. However this has not yet

taken place.

Whether, in view of the likely expansion of disease out break in intensive culture, this will ever prove possible, remains to be seen.

II. Biology of tiger shrimp *Penaeus monodon* (Fabricius)

II.I Taxonomy

The original description of *Penaeus monodon* was published by John Christ Fabricius in 1798, and presently places its taxonomic status as follows :

Phylum Arthropoda
Class Crustacea
Subclass Malacostraca
Series Eumalacostraca
Superorder Eucarida
Order Decapoda
Suborder Natantia
Section Penaeidea
Family Penaeidae
Subfamily Penaeinae
Genus *Penaeus* Fabricius, 1798
Species *Penaeus monodon* Fabricius,1798

II.II Common name. Jumbo tiger prawn, giant tiger prawn, blue tiger prawn, leader prawn, panda prawn (Australia) Giant tiger prawn (FAO).

II.III Synonymn. *Penaeus caeruleus* Racek 1955, 1959.

Penaeus carinatus Racek 1955, 1959.

Penaeus bubulus Kubo 1949.

(Grey *et al.*, 1983)

II.IV Distribution

The giant tiger shrimp is widely distributed throughout the greater part of the Indo West Pacific region to east and south east Africa, India, Pakistan to Japan, Indonesia, New Guinea and northern Australia. (Grey *et al.*, 1983; Motoh, 1981).

In general, *P. monodon* is distributed from 30°E to 155°E in longitude and from 35°N to 35°S in latitude. (Motoh, 1981)

II.V Habitat

Juvenile and adolescent *P. monodon* are recorded from surface waters in estuaries, while most of the adults inhabit waters down to 162 m. (Racek, 1951 cited by Motoh,1981).

The distribution, in the Philippines was found to be from surface in brackish water rivers down to about 70 m off-shore where the range of water temperature and salinity was between 22 -34°C, and 4 - 35 ppt., respectively. (Motoh, 1981).

III.Biology of vibrios in general.

The members of the bacterial family Vibrionaceae can be found from the aquatic environment, both in freshwater and seawater, as well as from the alimentary tracts of animals. Some species are pathogenic for marine vertebrates and invertebrates. The vibrios are known as an abundant group of micro-organisms in the marine environment and several of them comprise part of the commensal flora of fish and shellfish. Some probably act as saprophytic organisms in their environment. Six species *Vibrio cholerae*, *V. parahaemolyticus*, *V.alginolyticus*, *V.vulnificus*, *V. fluvialis* and *V. metschnikovii*, have been described as potential pathogens in man (West and Colwell, 1984). Austin and Austin (1988) described seven species of vibrios pathogenic to fish, namely *V. alginolyticus*, *V. anguillarum*, *V. carchariae*, *V. cholerae*, *V. damsela*, *V. ordalii* and *V. vulnificus*. They have not included *V. parahaemolyticus* as a fish pathogen but this organism was previously described as a fish pathogen by Colwell and Grimes (1984), and caused diseases in blue crab (*Callinectes sapidus*) (Johnson, 1988) and penaeid shrimps (Lightner, 1988). *V. tubiashii* has been described as shellfish pathogen (Jeffries, 1982; Hada *et al.*, 1984; Sinderman, 1988a; b; c; d; e; f) and this micro-organism was also included as a fish pathogen in reports by Frerichs (1984).

A multiplicity of studies involving isolation and identification of vibrios mainly from the marine environment has been reported in many areas of the world, including Mexico (Vanderzant *et al.*, 1970), Australia (Desmarchelier and Reichelt, 1981), United States (Baross and Liston, 1970; Kaysner *et al.*, 1987a; 1987b), China (Yang *et al.*, 1983). There are many

reports recorded relating to the contamination by bacteria in sea-food in Japan (Fujino *et al.*, 1953; Aiso and Matsuno, 1961), Netherlands (Kampelmacher *et al.*, 1970) and Hong Kong (Chan *et al.*, 1989).

Baumann *et al.*,(1980), proposed the abolition of the genus *Beneckea* and to add them to the genus *Vibrio* with the amended description of vibrios as follows: Gram negative, grow in liquid media, motile by sheathed polar flagella, monotrichous or multitrichous, not forming endospores or microcysts, chemoorganotrophs; facultative anaerobes, fermentative metabolism with the capability of using D-glucose as the sole or principle source of carbon and energy. They do not denitrify or fix molecular nitrogen. Sodium ion is an important growth stimulating agent, most species will not grow in media in the absence of this ion. The G+C contents in the DNA of the genus ranges from 38-51 mol %.

An additional subsequent description of the genus *Vibrio* reported in Bergey's Manual of Systematic Bacteriology by Baumann *et al.*, (1984) included : straight or curved rods, 0.5-0.8 micron in width and 1.4-2.6 micron in length, and may synthesize numerous lateral flagella on solid media, most species are oxidase positive, all grow at 20°C , most grow at 30°C. found in aquatic habitats with a wide range of salinities, very common in marine and estuarine environments and on the surface or in the intestinal contents of marine animals.

III.I Cell morphology.

Species of *Vibrio* consist of straight or curved rods, although some strains exhibit the conversion of rods to spheres during the stationary phase (Baker and Park, 1975). Allen and Baumann (1971) found that some of the marine strains, *V. campbellii* for example, possessed numerous tubular appendages of unknown function which were continuous with the outer membrane of the cell wall.

In liquid media, most vibrios have a single, sheathed, polar flagellum with a wavelength of 1.4-1.8 micron. Occasional cells have been observed with up to three polar flagella (Baumann *et al.*, 1980). The polar flagella of all species of *Vibrio* are 24-30 micron thick and are composed of a 14-16 micron core surrounded by a sheath which is continuous with the outer membrane of the cell wall (Allen and Baumann, 1971; Baumann *et al.*, 1980).

Many species of *Vibrio* show additional unsheathed lateral flagella when grown on solid media (Allen and Baumann,1971). These lateral flagella are essential for swarming on solid media (Shinoda and Okamoto, 1977).

III.II Colonial morphology.

Vibrios can grow readily on a variety of media. Most species give rise to convex, smooth, creamy white colonies with entire edges. Variation in colonial morphology may be detected in some species after reculture or storage on more complex media. (Baumann *et al.*, 1984).

III.III Growth conditions.

Growth of all strains of vibrios is stimulated by Na^+. The minimal concentration required for optimal growth ranges from 5-15 mM for *V. cholerae* and *V. metschnikovii*,to 600-700 for *V. costicola* (Baumann *et al.*, 1984).

Optimal salinity for *V. salmonicida* is 15 ppt.(5-40 ppt.) (Egidius *et al.*, 1986). Singleton and co-workers (1982) reported that moderate salinity of around 15 - 25 ppt.was suitable for *V. cholerae* which confirmed Na^+ requirment for growth of this species.More recently Kaysner *et al.*, (1987a) have isolated *V.cholerae* strains from water,sediment and shell-fish from the California Coast, where the temperature and salinity ranges from 9-31°C and 0-35.6 ppt.respectively. All vibrios can grow at 20°C and most at 30°C; Some can grow at 4°C and 45°C but none of them will grow at 50°C (Baumann *et al.*, 1984).

Many species of *Vibrio* are tolerant to moderately alkaline conditions and will grow at pH 9, and some, notably *V. cholerae* and *V. metschnikovii*, will grow at pH 10. (Baumann *et al.*, 1984).

III.IV Ecology.

Because of the wide range of Na^+ requirement for optimal growth of *Vibrio* species, the distribution of individual species may vary depending on the environmental salinity. *V. cholerae* and *V. metschnikovii* require only 5-15 mM Na^+ for optimum growth so both species can be isolated from freshwater as well as estuarine. But for the member of true marine vibrios, their optimal Na^+ requirement are being 300-700 mM Na^+. These bacteria could be isolated

from the high salinity areas. But almost all of the other species are very common and easy to recover from the marine environment within a salinity range of 10-30 ppt.

Studies of the distribution of luminous species in the water column from the open ocean in the North Atlantic indicated that *V. harveyi* was the predominant organism in the upper 150 metres during the spring. Its numbers decreased in autumn, and it was apparently absent in the winter, while *V. fisheri* was present in low numbers throughout most of the water column (Ruby *et al.*, 1980).

III.V Isolation.

Many strains of *Vibrio* are easy to grow on a basal medium supplemented with Na^+ (Baumann *et al.*, 1984; Austin and Austin, 1988). Direct plating or enrichment methods can be used. The simplest and most widely used enrichment medium for vibrios is alkaline peptone water (APW) which contains 1% (w/v) peptone and 1% (w/v) NaCl, at pH 8.6. The incubation period recommended for *V. cholerae* is 6-8 hrs at 37°C before subculture. For other species a lower temperature is recommended. In food, water and other specimens, it may be necessary to vary the salt concentration, temperature and period of incubation depending on the source or environment of those specimens. (Furniss *et al.*, 1978). Many other enrichment media such as alkaline peptone water tellurite, or trypticase-tellurite-taurocholate-peptone water are suitable for *V. cholerae* (Monsur, 1963). Baumann and co-workers (1984) found that glucose salt teepol broth and salt colistin broth were suitable

enrichment media for *V. parahaemolyticus* but not suitable for *V. cholerae* or other vibrios. Alkaline taurocholate-tellurite gelatin agar and bile-salt agar have also been used for isolation of *V. cholerae* but seemed to be less selective than TCBS. *Vibrio parahaemolyticus* was originally isolated by using SS-agar (*Salmonella shigella* agar) or NA (nutrient agar) containing 4% NaCl (Fujino *et al.*,1953). Kampelmalcher *et al.*, (1970) recommended meat broth with 0.5% NaCl as the best enrichment medium for *V. parahaemolyticus* before plating on Teepol Brom-thymol Blue Agar (BTB) or TCBS. In the same year Twedt *et al.*,(1969) developed a selective medium for this organism by mixing 2% peptone, 0.2% yeast extract, 0.5% corn starch, 3% NaCl, 1.5% agar and penicillin G (5 ug/ml), adjusted to pH of 8.0.

The isolation of luminous vibrios does not involve an enrichment method but the samples are placed directly on a suitable complex medium and luminescence is detected by the dark - adapted eyes of the investigator.

IV. History of Vibriosis

IV.I *Vibrio cholerae*

Vibrio cholerae is one of the most important pathogenic bacteria of human and warm-blooded animals. It is divided serologically into 4 biotypes known as the *Cholera vibrios* composed of biotypes *cholerae* and *eltor* and the *non-Cholera vibrios*, which in the 8th edition of Bergey's Manual of Determinative Bacteriology, were designated as biotypes *proteus* and *albenis*. All of them can cause gastroenteritis or diarrhoea in man (Firehammer, 1980; Furniss *et al*, 1978). Cholera vibrios can be isolated from the

gastrointestinal tract and faeces of human, cattle, dogs and chickens suffering from cholera.

Vibrio cholera biotypes *cholera* and *eltor* can multiply in the lumen of the small intestine of animals which ingest contaminated foods or water. Liberation of the enterotoxin causes the increase of adenyl cyclase activity and results in excessive production of adenosine 3'- 5'- cyclic monophosphate (cAMP) follow by hypersecretion of water and salts. The incubation period from ingestion to development of symptoms varies from 1-5 days. Patients generally show rapid dehydration, diminished skin turgor, cold extremities, high pulse rate and low blood pressure. Fluid loss may exceed 1 l/h.,leading to hypovolaemic shock and metabolic acidosis resulting in death without any pathological signs involving the gut mucosa. This report also suggested that the cholera *Vibrio* is quite sensitive to low pH (Carpenter, 1972). Non-cholera vibrios can cause severe diarrhoea but they are not usually as highly pathogenic as are the classical cholera vibrios.(Firehammer, 1980).

During summer in 1979, Muroga *et al.*, (1979) reporting on a disease out-break in wild ayu in Japan, where diseased fish exhibited petechial hemorrhages on the body surface and congestion in the internal organs, identified the causative agent as a non-cholera *Vibrio*. The bacterium isolated from diseased ayu was injected into healthy ayu and eel to investigate pathogenicity. The results showed that it appeared to be pathogenic and seemed to be more pathogenic at higher water temperatures (Yamonoi *et al.*, 1980).

In China, Gouxing (1986) identified the species and demonstrated the pathogenicity of vibrios isolated from the orbit and haemolymph of diseased shrimp (*Penaeus orientalis*) suffering from "blind disease" during the period July to October, 1983. This organism he also identified as *Vibrio cholera* which he defined as of the 01 serotype. Recently, Kaysner *et al.*,(1987a) reported on the incidence of *Vibrio cholerae* from shellfish, sediment and water samples along the coastlines of California, Oregon and Washington in the United States. It was found in 44.6 % of the 529 samples. Over 55 % of strains isolated were from water samples, 22.0 % from sediments and 6.1 % from shell fish. During the isolation period water temperature ranged from 9-31°C and salinity ranged from 0-35.6

IV.II *Vibrio anguillarum*

Vibriosis was the first fish disease recorded in the European literature. Bonaveri in 1961 refers to historical references dating from 1761 which described "Red Pest" in European eels in the Camachio lagoons of Northern Italy (Austin and Austin, 1988 referring to Bonaveri, 1961). The causative agent as first described by Canestrini in 1893 and named as *Bacillus anguillarum*. This organism was renamed in 1909 by Bergman as *Vibrio anguillarum*.

Clearly, *V. anguillarum* is one of the most common elements of the microflora, not only of the aquatic environment (West and Lee, 1982) but also of marine fish (Mattheis, 1964) and is usually more abundant in periods of higher temperature during the year and less in number when the temperature

is low (Larsen, 1982). Thus, it appears that fish are exposed to the potential pathogen all the time. If fish are subjected to good husbandry and good environmental conditions, diseases are relatively rare, conversely if fish are stressed by any one of a number of stressors, the disease frequently occurs (Roberts and Shepherd, 1974). Anderson and Conroy (1970) mentioned that epidemics in salmonids usually occured when the temperature exceeds 10°C, when there is depletion of dissolved oxygen and when fish are stressed by overcrowding and poor hygiene.

The precise mode of infection is unclear but undoubtedly involves colonization of bacteria on the host and penetration into the tissue. The possible route of entry is by ingestion of organisms or else intake of bacteria through a number of sites on the body surfaces (Horne *et al.*, 1982). This suggestion is confirmed by the report of Baudin-Laurencin and Tangtrongpiros (1980), who found that the pathogen in the diet can infect via the alimentary canal and can kill the fish at the same level as when the bacterium is injected intraperitoneally. The other route of infection is probably through the gill. Basically, the mode of pathogenicity of the bacterium involves uptake and penetration of the host tissue, scavenging for host iron as a result of a plasmid-mediated trait, and damage to the fish by means of haemolysin and proteases (Munn, 1980; Inamura *et al.*, 1984; Kodama *et al.*, 1984; Austin and Austin, 1988). The presence of heavy metals, such as copper can promote the development of infections but it is dependant on the concentration and time of exposure (Baker *et al.*, 1983).

Salmonid fish are common hosts of the infection but it has also been described among non-salmonid fish,i.e. turbot (*Scophthalmus maximus*), eel (*Anguilla* sp.), yellow-tail (*Seriola* sp.), cod (*Gadus gadus*), coalfish (*Pollachius verius*), plaice (*Pleuronectes platessus*), flounder (*Platichys flesus*). (Canestrini, 1893; Buckmann, 1952; Anderson and Conroy, 1970; Hastein and Holt, 1972; Horne *et al*, 1977; Jo *et al*, 1979; Austin and Austin, 1988)

Many species of shellfish have also been recorded as infected with this bacterium including penaeid shrimp (*Penaeus monodon*), lobster (*Homarus americanus*), oyster (*Crassostrea virginica*), hard clam (*Mercenaria mercenaria*), bay scallop (*Argopecten irradians*) and blue crab (*Callinectes sapidus*) (Lightner, 1988; Rosemark and Fisher, 1988; Sindermann, 1988a; b; c; d; e; f)

In diseased penaeid shrimp, Lightner (1988) suggested that *V. anguillarum* was one of the most common bacteria which could be isolated from moribund shrimp at all stages. In juvenile and adult shrimps, gross signs appeared to vary with the type of infection. If infection of cuticle, gill and appendages was apparent as black or brown localised lesions in which the cuticle was eroded, it was called brown spot or shell disease. If infection was septicemic or localized in the internal organs, shrimps displayed general signs of severe stress such as anorexia, expansion of chromatophores, and opaqueness of the abdominal muscle. The haemolymph clotted slowly, was turbid and there was a reduction in the number of haemocytes. In larval and early stages, general signs included melanization and necrosis of appendage tips and/or the presence of large numbers of swarming bacteria visible in the

haemocoel of moribund shrimp. Affected shrimp were also typically off-feed and lacked faecal strands within an empty gut.

In bivalve molluscs, Martin (1976) isolated a *V. anguillarum* strain which proved to be more highly pathogenic to larval mussels (*Mytilus edulis*) than to adults. Tubiash and his colleagues (1973) also suggested that *V. anguillarum* can play a role as an opportunistic pathogen to *Crassostrea virginica*.

IV.III *Vibrio ordalii*

Schiewe and his team (1981) proposed the name *V. ordalii* sp. nov. as a new species name for the micro-organism previously known as *V. anguillarum* biotype II. *V. ordalii* has now been well documented in Japan and Pacific North-West of the U.S.A. as a fish pathogen (Colwell and Grimes, 1984). This pathogen appears to have a more host-dependent mode of existance than does *V. anguillarum* because it is rarely isolated from water, sediment, or other abiotic marine samples; *V. ordalii* is recovered only from moribund fish (Schiewe, 1983).

IV.IV *Vibrio salmonicida*

The micro-organism newly described as *V. salmonicida*, the causative organism of Hitra Disease in salmonid fish cultured in Norway, is distinguished from *V.anguillarum* on the basis of biochemical tests. The disease occurs mainly in late autumn, winter and early spring and it is sometimes known as cold water vibriosis. (Egidius *et al*,1986). The disease is

characterized by anaemia and extensive hyperaemia and haemorrhage, especially into the visceral and parietal peritoneum of affected fish.

IV.V *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is well known as a pathogenic organism for fish and at times produces food-borne infections in humans. The first isolation was described by Fujino in 1950, who named it *Pasteurella parahaemolytica*. He isolated it from a gastroenteritis patient who had eaten contaminated food products in Japan. Based on salt tolerance and biochemical characteristics this organism was re-classified into genus *Vibrio* after the studies done by Sakazaki and his co-workers in 1963 (Shewan and veron, 1974)

According to its epidemiology and pathogenicity , *V. parahaemolyticus* appears to be ubiquitous in marine environments, and has been reported in Japan, Germany, United-Kingdom, Netherlands, United States (Miyamoto *et al.*, 1962; Baross and Liston, 1968; Word, 1968; Twedt *et al.*, 1969; Fishbein *et al.*, 1970; Kampelmacher *et al.*, 1970). Miyamoto and his team (1962) studied the seasonal distribution of this organism,they observed a "winter and summer type" distribution. The euryhaline virulent forms were more abundant in coastal areas during the summer months of the year, but stenohaline avirulent forms were commonly found to predominate in winter months. This report confirms the clinical experience that outbreaks of food-poisoning usually occurs in the summer months (Battey *et al.*, 1970; Barker, 1974).

In marine organisms, *V. parahaemolyticus* has been isolated from diseased blue crabs (*Callinectus sapidus*) in Chesapeake Bay (Krantz *et al.*, 1969), penaeid shrimp from gulf of Mexico (Vanderzant *et al.*, 1970), and Pacific oysters (*Crassostrea gigas*)(Baross and Liston, 1970). Lightner and Lewis (1975) described the features of septicaemic vibriosis in moribund shrimp as follows, lethargy, areas of white discoloration of the abdominal musculature, a dorsal flexure of the abdomen and redness of the pleopods, periopods, and increase in number of fouling organisms, mainly *Zoothamnium* spp.at the gills. Later, Lightner (1985) described three general forms of bacterial infections in shrimp, comprising erosions of the cuticle of the general body surface, gills and appendages (bacterial melanosis and shell disease); localized lesions within the body and generalized septicaemia.

More recently, Lightner (1988) confirmed that *V. parahaemolyticus* was associated with shell diseases or other types of vibriosis in shrimp.

IV.VI *Vibrio alginolyticus*.

Formerly *V. alginolyticus* was known as *V. parahaemolyticus* biotype II but it was listed in the eighth edition of Bergey's Manual of Determinative Bacteriology in 1984 as *V.alginolyticus*. In 1968, Sakazaki proposed to separate the two biotypes of *V. parahaemolyticus* into two separate species, using the main difference as follows: *V. parahaemolyticus* is negative in relation to acetoin production, sucrose fermentation, growth in 10% NaCl and swarming on solid media containing 2.7% NaCl. *Vibrio alginolyticus* is positive

to all of those characters. This proposal was confirmed by Hugh and Sakazaki (1975) as to separation of these two taxa as distinct species.

Vibrio alginolyticus is widely distributed in the marine environment and frequently isolated from finfish, shell fish and sea food (Liston and Baross, 1973; Chan *et al.*, 1989). Colorni and his team (1981) reported on mass mortality of farmed seabream (*Sparus auratus*) in Israel. This occurred especially after extensive handling of the fish, which implied that *V. alginolyticus* was a secondary invader.

Vibrio alginolyticus was reported as the causative agent of shellfish diseases in oysters (Sindermann, 1988 b, c), hard clam (*Mercenaria mercenaria*) (Sindermann, 1988 d), bay scallop (*Argopecten irradians*) and juvenile red abalone (*Haliotis rufescens*) (Sindermann, 1988 e, f).

Recently, *V. alginolyticus* has been reported in high incidence in sea food in Hong Kong, always being present in higher numbers than *V. parahaemolyticus* and *V.vulnificus*. It was observed in bivalve shellfish more frequently and in greater numbers than in other types of sea food (Chan *et al*, 1989).

Vibrio alginolyticus, when well established in the human gut, causes acute enterocolitis, and has also been associated with skin infection (Blake *et al.*, 1980; Schmidt *et al.*, 1979). Long and his colleagues (1981) reported that the virulent mechanisms for invading the skin possessed by *V.alginolyticus* may involve collagenase and production of several extracellular proteases.

IV.VII *Vibrio fluvialis*.

This group was originally referred to as the marine - aeromonads because their halo-tolerance and their biochemical characteristics were very close to those of the members of the genus *Aeromonas*. However, Lee and his group (1981) described them as vibrios and defined two different biotypes namely; Biovar I and Biovar II.

Biovar I. was widely distributed through the aquatic environment particularly in brackish and estuarine water, as well as in shellfish and sewage, with many strains readily isolated from human faeces, although enterogenicity has not been firmly described.

Biovar II. can be isolated from the same habitats as Biovar I.and also occasionally from faeces of cattle, pigs and rabbit. It does not appear to be pathogenic.

IV.VIII *Vibrio vulnificus*

In 1976, halophilic vibrios that had not been previously described were reported by Hollis and his colleagues. These strains superficially resembled *V. parahaemolyticus* and *V. alginolyticus* except these two species were not lactose fermenting vibrios and these new strains shared a low RNA-DNA homology with *V. parahaemolyticus* and *V. alginolyticus* (Clark and Steigerwalt, 1977). These organisms were classified as *Beneckea vulnifica* (Reichelt *et al.*, 1976) and are currently known as *V. vulnificus* (Baumann *et al.*, 1980; Farmer, 1980).

Vibrio vulnificus is recognized as a highly virulent, opportunistic human pathogen with a low incidence of disease (Blake *et al.*, 1980; Joseph *et al.*, 1982; Oliver *et al.*, 1983). Oliver and his co-workers (1983) studied the distribution of sucrose-negative vibrios in the seawater environment and in animals from Miami, Florida to Portland, Maine, USA. They concluded that, *V. vulnificus* was ubiquitous, and always found in low numbers. Furthermore, Kaysner and his teams (1987 b) showed the incidence of *V. vulnificus* in the marine environment of Washington, Oregon and California States. They examined water, sediment and shellfish including; Pacific oyster (*Crassostrea gigas*), mussel (*Mytilus edulis, M. californicus*), gaper clam, (*Schizothaerus muttali*) and cockle (*Clinocardium muttali*).

Approximately 6 % of the samples were identified as *V. vulnificus* and most of them were collected from the Oregon coast where temperatures were above 15°c and salinity ranged between 15-30 ppt. They suggested that the low incidence in other areas may be due to the lower water temperatures during the study period.

The organisms recovered from the environment showed cytotoxin production, but this does not indicate the degree of virulence because their titres were varied. Some of these strains showed pathogenicity to injected mice, which died 72 hr post-injection. Iron-treated mice were more susceptible to the pathogen. They confirmed that two different strains of *V. vulnificus* were isolated from mussel and oyster. The presence of this agent in shellfish suggests a potential hazard to shellfish consumers, especially the persons with liver disorders, since they seem to be more susceptible to this diseases.

IV.IX *Vibrio metschnikovii*

Vibrio metschnikovii is an unusual form of *Vibrio* since it is negative in Kovac's oxidase test. This organism was originally isolated in 1884, from faeces of patients suffering from cholera, when it was known as *V.proteus*. It was however synonymous with *V. metschnikovii* which was isolated from a dead fowl with fowl cholera type lesions. Shewan and Veron (1974) assigned these two organisms in Bergey's Manual of Determinative Bacteriology (1974), to the one taxon, as *V.metschnikovii*

This bacterial species is widely distributed throughout the aquatic environment, particularly in rivers, estuaries and sewage. Sometimes it can be isolated from animal intestines, as well as from man, but there is no evidence to suggest it causes enteritis in humans or animals. It has also been isolated from various shellfish, eg. cockles, clams and lobsters.

IV.X *Vibrio carchariae*.

Austin and Austin (1988) reviewed the literature involving *V. carchariae*. They reported the original isolation of this organism from a dead sandbar shark (*Carcharhinus plumbeus*) in Baltimore by Grimes *et al*, (1984) and from lemon sharks (*Negraprion brevirostris*) by Colwell and Grimes (1984). Infected fish showed lethargy, cessation of feeding, were disorientated and developing necrotic subdermal cysts. On post mortem examination, diffuse inflammatory response was observed throughout the internal organs.

IV.XI *Vibrio damsela*

Vibrio damsela was first isolated from damselfish (*Chromis punctipinnis*) from the coast of southern California and it seemed at that time to be only infectious to damselfish (Love *et al.*, 1981). It is now known to infect humans (Love *et al.*, 1981) and sharks (Grimes *et al.*, 1984).

Diseased damselfish showed skin lesions, particularly in the region of the pectoral fin and caudal peduncle. These were up to 5-20 mm. in diameter. Its pathogenicity has been demonstrated by experimental infection, not only in damselfish but also in the spiny dogfish shark (*Squalus acanthias*), and lemon sharks (*Negaprion brevinotris*) (Grimes *et al*, 1984).

IV.XII *Vibrio tubiashii*

Vibrio tubiashii was first isolated from hard shell clams suffering from bacillary necrosis on the East Coast of the United States. It was identified as *V. tubiashii* and given its detailed description by Hada *et al.*, (1984).

IV.XIII Other vibrios.

Pigmented vibrios.

Some *Vibrio* strains have been isolated from the marine environment which produce pigments within their colonies, namely :

Vibrio nigripulchrituda which produces charcoal - coloured colonies on minimal media.

V. gazogenes which appears red on marine agar.

V. logei and *V. fisheri* which produce yellow - orange colonies on

marine agar containing 0.05% yeast extract.

Luminous vibrios.

V. harveyi, V. splendidus biotype I, *V. fisheri* and *V. logei* have been described as luminous marine bacteria, and more recently *V. orientalis* which was isolated from off the coast of China has also been shown to be luminous (Yang *et al.*, 1983).

Hyper - saline vibrios.

Vibrio costicola has been described as a hyper - saline environment *Vibrio*, it requires at least 2 % NaCl for growth, the optimum salt concentration being 5 %. This bacteria is most commonly association with salt-cured food stuffs (Kushner, 1978).

Chapter II. The Microbiology of *Vibrio* sp. in Shrimp Farms in Thailand

Introduction

Shrimp farming in Thailand is not new.It has been practised since at least the early 1940's as an extensive farming method in the rice fields of the mangrove zone, such fields being converted to shrimp ponds during the dry season when the salinity in the irrigation canals was high. Some years later, when salt prices decreased, many salt producers also converted their evaporation ponds to shrimp production. Since around 1947, therefore there has been extensive development of shrimp culture in Thailand. This was helped considerably by the additional support of The Royal Thai Government's Fisheries Department in the late 1970's and it has now become a major component of the fishing industry.

Following the development of techniques for the successful mass production of shrimp larvae, in 1987, the giant tiger shrimp (*Penaeus monodon* Fabricius) became the most important species for intensive culture in Thailand. The industry expanded very rapidly and with the development policy proposals of the Royal Thai Government to greatly increase the production of penaeid shrimp culture in 1988-1989, and the high profits realized by successful shrimp culture, many more farms were built than expected. This resulted in the collapse of giant tiger shrimp prices in the spring of 1989. Because of this, some farms, which could not be operated profitably, failed, but others developed technologies for super intensive farming on the dubious principle of " The higher the stocking rate, the greater the profit". Such technologies however, produced so many problems that the development of shrimp culture rapidly got out of control, with disease problems featuring high in the list of

obstacles to successful production.

The major shrimp diseases in Thailand are usually caused by infectious agents but these are generally associated with stressors such as reduced dissolved oxygen, rapid changes in pH, salinity or temperature, or increase in nitrogeneous waste products. All of these are induced by the inherent problems of managing shrimp ponds with such intensive stocking levels, and are exacerbated by poor husbandry and management. The proliferation of ubiquitous, opportunist bacteria such as *Pseudomonas* sp., *Aeromonas* sp. and especially *Vibrio* sp.in the ponds can readily lead to infection of stressed shrimp and result in serious morbidity and mortalities. Disease outbreaks may be acute, subacute or chronic and result in mortality rates ranging from inconsequential to 100 % of the affected population. Probably these differences are due, at least in part, to the activities of different strains of bacteria.

Vibrio bacteria are generally reported to be the most significant of the opportunist bacteria to be found in brackish water and marine environments and the disease they cause, generally known as vibriosis, is the principal cause of mortality in intensive shrimp culture.

A pre-requisite to understanding the prevention and treatment of vibriosis is a knowledge of the biology, and characteristics of the causative bacteria. The present study intended to define the biology and characteristics of *Vibrio* sp. from two locations in Thailand where intensive culture is practised and where vibriosis is a dominant feature of the industry.

Literature Review.

I. Vibriosis in shrimp hatcheries in Thailand

In the earliest stages of intensive shrimp culture when *Penaeus merguiensis* and *Macrobrachium rosenbergii* were the principal culture species in the hatcheries, the most serious diseases were generally associated with physical obstruction due to growth of the sessile protozoans, *Zoothamnium* sp.or *Epistylis* sp. on the surface of gill or appendages of larval shrimp (Ruangpan, 1981).

However, vibriosis was also recognized as a major cause of mortality at all stages of larval shrimp development, and Ruangpan (1987) reported up to 70 %-80 % mortality of infected larvae within two days of commencement of an outbreak. Because of the importance of the condition to hatchery production, efforts were made to characterize the causative agent, originally defined only as *Vibrio* sp. and to define a suitable regime for treating the affected shrimp. The organism was characterized as *Vibrio parahaemolyticus* and found to be sensitive to an antibiotic level of 1 ppm. of oxytetracycline or chloramphenicol in the form of a bath (Tanthavanish, 1981).

During the winter of 1986 -1987 mass culture techniques for *Penaeus monodon* were developing most successfully, but at the same time, initially in longer established *P. merguiensis* (banana shrimp) hatcheries, a new disease was manifesting itself. This condition was known as " fluorescent disease ". It caused high mortality (over 70 %) over a very short time, and was apparent whenever fluorescence of the sea was observed. This phenomenon is not uncommon in the upper part of the Gulf of Thailand at certain times of

year and is associated with the presence in enriched waters of a variety of fluorescent bacteria.

The condition was caused by contamination of the water of affected ponds by one of these bacteria, *V. harveyi*, which could be isolated from the tissue of moribund specimens. To confirm the pathogenicity of the isolated organism, different stages of shrimp larvae were immersed into bacterial suspensions (10^7 CFU/ml.of sea water) to re-produce the disease. The results showed that the nauplius stages of the shrimps were the most susceptible to this microorganism whilst mysis and post-larvae were less susceptible. Again, sensitivity tests were performed and chloramphenicol given as a bath treatment proved to be the most successful antibiotic for use in the control and treatment of this bacterial infection. Novobiocin was also shown to be effective, but the pathogen exhibited resistance against streptomycin (Sae-Ui *et al*, 1987).

The presence of fluorescence disease was first noted in *P.merguiensis* culture, where it was a major problem, but it soon became a problem also in the newly developing *P. monodon* hatcheries which were so successfully facilitating the expansion of the industry. Again it was particularly prevalent in hatcheries in upper parts of the Gulf, and in areas where water supplies were particular enriched.

Ruangpan (1987) in their updated review of marine shrimp diseases in hatcheries, considered that by this time the bacterial infections of hatcheries were generally caused by the luminous *Vibrio* in newly hatched (nauplius) and mysis stages were considered the most susceptible to the

pathogen. Clinical signs in infected larvae were described as lethargy, milky abdominal muscle and slow swimming. Moribund larvae sank and died at the bottom of the culture tank and mortality rate ranged from 20 % up to 100 % within 1-2 days.

They described how presumptive diagnosis of the disease could be performed by observation of the larval tank during the night when infected or dead larvae demonstrated fluorescence. Isolation of the pathogen confirmed the disease. Bacterial culture of *V.harveyi* was done on the specific medium known as Luminous medium (LM-agar,Difco). After 24 hours incubation at 25-30°c, bacterial colonies of *V. harveyi* are fluorescent in the dark. Control of the condition was based on water supply treatment including filtration and chlorination.

Antibiotic therapy was also described as a measure used to control and treat this disease but good results were rarely obtained.

Studies on the prevention and treatment of this bacterial disease were also reported in 1988 by Rattanavinitkul and his team. They used two antibiotics, oxytetracycline and chloramphenical for bath treatment of shrimp larvae during the post larval stages range from stage 2 to stage 15 (PL 2 -15). Oxytetracycline at 2.68-5 ppm. was found to be reasonably effective as was chloramphenicol at 2.68-7 ppm. but they found that best results were obtained using a bath of 10-15 ppm. formalin with oxytetracycline or alternatively formalin and chloramphenicol, administered for 3 days.

II. Vibriosis in grow-out ponds

As extensive shrimp farming, by conversion from salt pans to shrimp ponds, developed in the early 1970's, so shrimp culture became a major industry and rapidly expanded throughout Thailand's coastal areas. During the early development stages no evidence of serious diseases was reported. This may have been due to very low stocking rates with good hygiene and management. This fortunate situation was confirmed by Leangphibul and her colleagues (1985), who studied the prevalence of bacteria in extensive shrimp ponds at Nakornsrithamarat Province in Southern Thailand and Samutprakran Province in Central Thailand. In both areas there was a large number of closely crowded but extensively stocked shrimp farms. During October 1983 to December 1984, 139 water samples, 144 sediments and 23 normal shrimps were sampled. They isolated 7 species of pathogenic bacteria including *Vibrio parahaemolyticus, V. fluvialis, V. cholerae, Salmonella* spp., *Aeromonas hydrophila, A. sobria*, and *Plesiomonas shigelloides*. However, despite the presence of this wide range of bacteria, they did not seem to produce any serious disease problems at that time.

During late 1986-early 1987, however as more intensive tiger shrimp cultures were expanding through-out Thailand's coastal area, anywhere where salt water could be pumped, farms were densely crowded in any place where a good water supply was available. This together with the lack of knowledge concerning shrimp culture, led to serious disease problems. A very wide spread disease out-break was reported in September, 1988; known as **"Black Splinter Disease"**. Many shrimp farms which stocked their shrimp in

very low salinity water suffered from the disease. Diseased shrimp exhibited a brown to black "splinter"in the muscle, particularly at the tail area or at the joint between the segments. Some showed only brown or black spots or patches on the carapace with a diameter ranging from 0.1-0.5 cm. Others showed the very obvious and characteristic splinter in the carapace. Microbiological examination showed that the condition was associated with the presence of the bacterium *Vibrio vulnificus*, an opportunistic halophilic *Vibrio*. Shrimp specimens were examined histologically, and the black splinter was found to consist of dense focal accumulations of haemocyte and melanin surrounding connective tissue within which numerous colonies of the *Vibrio* could be observed. In some areas the bacteria and necrotic tissue had been encapsulated. It was not confined only to muscle tissue. Bacterial colonies and melanized areas were also observed in hepatopancreas and gills. The pathogenicity of isolated bacteria was demonstrated by intramuscular injection into healthy shrimp. After 1 hour post injection, experimental shrimp showed pink discoloration at the injection area and at the tip of appendages, and some died. Four days after such injections, surviving shrimp exhibited a black spot at the injection site, whilst saline injected controls showed no evidence of any abnormality. Oxytetracycline incorporated in the feed (5 gm./kg.of feed) associated with good management, was demonstrated to control this disease, but only if treatment could be instituted at an early stage of infection. Increasing water salinity also assisted in decreasing the incidence of this disease (Limsuwan, 1988).

Ruangpan and Sae-Ui (1988) also investigated splinter disease and isolated three different species of vibrios from affected shrimp. However in

their cases, also, *V. vulnificus* was the most frequently isolated. They also confirmed the pathogenicity of *V. vulnificus* by experimental injection of approximately 1-4 * 10^4 CFU/ml.intramuscularly. A delicate black line surrounding the injection area was observed within 8-14 days after injection. They performed bath challenges, after the carapace of experimental shrimp had been cut to make a small wound. They immersed the wounded shrimp into sea water containing 3.7-6.1*10^6 CFU of *V. vulnificus*/ml.of sea water. After 4 days such wounded shrimp showed a brown to black line surrounding the wound area with an incidence ranging from 40-70 %. Attempts to treat the pathogen were made by using antibiotics. Chloramphenicol, nalidixic acid and oxytetracycline all showed good results when used in bath treatments.

There is also a single reference to problems associated with the presence of vibriosis in brood stock shrimp. These were experimental stocks being held in deep concrete holding tanks, and the condition developed during studies on eye stalk ablation. Vibriosis was not considered to be the sole cause of the heavy losses of 58 % of the stock, which is unusual in adult specimens. Stressors, parasites, predators and other factors also were believed to have contributed, but *Vibrio* sp. was one of the pathogens isolated from the moribund brood stock (Chonchuenchob, 1988).

The first part of this study investigated some of the bacteria associated with shrimp farming. Two areas were selected, one Samutsakhon province, has a long history of shrimp culture while in Chanthaburi province shrimp culture has only started recently. Anecdotal evidence from farms suggested that disease was both more prevalent and harder to control in

Samutsakhon. The objective was to determine if the apparent differences between the areas were reflected in the bacterial population. The method selected for this investigation involved sampling both healthy and diseased shrimp. The isolated bacteria were subsequently identified by standard methods.

Materials and Methods

I. Collecting the specimens

I.I Source of shrimp

Diseased shrimp specimens (*Penaeus monodon* Fabricius) were collected fortnightly from culture ponds in two different provinces where reports of vibriosis were extant, at Samutsakhon the area where shrimp culture has been established for many years and Chanthaburi the area new to shrimp culture. Dates of sampling from each area are given in table 1-2. At each sampling, specimens from the shrimp populations that showed a healthy appearance were taken as well as clinically affected ones from the same ponds. The sampling period extended from May, 1989 until August, 1989. At each sampling, water temperature and salinity of the pond were recorded as well as the individual weight, condition and age of each shrimp sampled.

I.II Bacterial isolation

Groups of ten shrimps (5 moribund and 5 healthy) from the same pond were collected at each sampling. Their surfaces were disinfected with 70 % alcohol, following which the carapace was removed. The surface of the hepatopancreas was then surface disinfected. In order to ensure aseptic technique, a hot scalpel blade was used to sear the hepatopancreas. Then a sterile loop was inserted through the seared surface to abstract the sample material. The sample was then streaked onto TSA (Tryptic Soya Agar, Difco) medium, incubated at 25°c for 24 hours. Subcultures were then made from typical colonies to obtain pure cultures of the micro-organisms isolated.

I.III Bacterial characterization

Morphological and biochemical tests were performed according to the standard methods described in Cowan and Steel's Manual (Cowan, 1974). To differentiate vibrios from aeromonads, TCBS -agar plates and 0/129 vibriostatic compound were used. The members of the genus *Vibrio* grew preferentially on TCBS agar and were sensitive to the vibriostat O/129, *V.ordalilli* is exceptional in this respect in that it does not grow on TCBS but is nevertheless sensitive to 0/129.

Amino acid decarboxylase were determined in Mueller's basal medium containing 2 % NaCl, arginine, lysine and ornithine. Production of catalase, -galactosidase, H_2S and indole were also tested. Acid and gas production from arabinose, inositol, lactose, maltose, manitol, mannose, melibiose, sucrose, sorbitol and rhamnose were observed during 3 days incubation. The test for degradation of urea, the methyl red test and the Voges-Proskauers test for production of acetyl methylcarbinol or acetoin and tests for nitrate reduction and citrate utilization were performed. Salt tolerance was tested by measuring an isolate's ability to grow in tryptone soya broth containing 0, 3, 6, 8 and 10 % NaCl. All of these determinative tests were incubated at 25°c.

All of the isolated bacteria were grouped as different strains and classified to genus level according to the scheme described by West and Colwell, 1984 and then to species level by reference to Bergey's Manual of Systematic Bacteriology Vol.1 (Krieg and Holt, 1984). In addition, when necessary, attempts were made to identify problem vibrios further, at the

species level by referring to previous reports and by comparison with reference strains of known *Vibrio* species generously provided by The National Collections of Industrial and Marine Bacteria (NCIMB).

Results.

Vibriosis was widely distributed in shrimp farms throughout the coastal area of both Samutsakhon and Chanthaburi provinces. These two provinces contain the most intensive areas of shrimp culture in the entire country. Observations during the study indicated that the disease generally occured in intensive culture farms which stocked shrimp at a higher density than 25 tails/m^2. Disease was most prevalent during those periods of the lunar cycle which led to low tidal movements and losses were particularly high in those farms where management and hygiene were of low standard.

Infected shrimp exhibited white discoloration of the abdominal musculature, redness at the pleopods and periopods, lethargy, off- feeding, and an increase in the number of fouling organisms on the gills and appendages when compared to the healthy shrimp. Heavy infection often led to the erosion of the cuticle of the appendages and body surface, especially at the tail, pleopods and periopods, with development of brown to black spots, or patches on the carapace. Moribund shrimp were easy to observe at the edge of the grow out ponds, as they appeared to slowly swim around the pond, gradually weakening, falling at the ponds' slope and usually dying shortly afterwards. This type of clinical disease pattern was more easy to observe during the night than at daytime, as at night moribund shrimp would migrate to the edge of the pond and show very pale eyes against a light.

When samples were incubated for primary isolation, the plates generally showed a mixed infection. Rarely was there a pure growth of a single species. Usually two or three different colony types could be seen, all generally vibrios,

and at each pond sampling, the same spectrum of biochemically distinct strains would be found in each individual sample from that site. Clinically healthy shrimp from the same pond also had some evidence of internal bacterial infection, generally with the same range of strains as the clinically affected shrimp but never at the same level of bacterial numbers as in the moribund shrimp.

In order to prevent numbers of identical isolates reaching unmanageable proportions, a typical isolate of each bio-type found in a specific sampling site was retained at each occasion, so that for each sampling time and location two or three representative strains were retained for further analysis.

Characteristics of isolates

I. Samutsakhon province

Thirty-one different strains were obtained from 8 farms in this area during the sampling period. *Vibrio parahaemolyticus* was the most frequently isolated pathogen in this area. Twelve different strains (38.7 %) of this bacterium were isolated (Table 3). *V. vulnificus* and *V. alginolyticus* were relatively infrequent, only two, different, strains of each, being isolated (Table 4). Sixteen strains could not be identified to species level, although they were certainly *Vibrio* strains at genus level (Table 5).

The twelve *V. parahaemolyticus* strains were all motile, gram-negative rods, which formed off-white colonies on TSA, and green colonies on TCBS. They all utilized glucose anaerobically and aerobically without gas production. Generally they could not grow in broth without sodium chloride

supplementation and were sensitive to the vibriostatic compound, 0/129. Thus, they were typical halophilic vibrios.

Due to their ability to produce H_2S, indole, oxidase and lysine and ornithine decarboxylase, and their ability to reduce nitrate, coupled with a negative arginine decarboxylase reaction, they were placed within *V. parahaemolyticus* species, distinguished from *V. alginolyticus* by their inability to utilize sucrose as a sole source of carbon and failure to produce acetyl methyl carbinol (Voges - Proskauers' test).

Only two strains isolated from diseased shrimp showed the characteristics of *V. vulnificus*. They were virtually identical in all of their characteristics in physiological and biochemical tests. The only small difference was in terms of their salt tolerance, as indicated in table 4. Both strains produced opaque colonies on TSA plates, and small green colonies on TCBS plates. They were distinguished from the *V.parahaemolyticus*, strains by being capable of fermenting lactose and producing H_2S. Whereas although *V. vulnificus* produced acid from lactose, it produced no fermentation gas and was negative for H_2S production (Table 4).

The other two strains, which showed characteristics very close to those two species described above were classified to *V. alginolyticus*. These two organisms exhibited the typical characteristics of vibrios but differed from *V. parahaemolyticus* by fermenting sucrose and produced acetyl methyl carbinol in the Voges - Proskauers' test. They were separated from *V. vulnificus* by means of lactose and sucrose utilization tests (Table 4).

Fifteen strains were classified as unidentifiable vibrios, and

grouped into 5 clusters based on colonial colour and ability to utilize amino acids as given in table 5.

II.Chanthaburi Province

Twenty-three strains were isolated from the samples taken from 5 farms in this province and were classified into individual species as given below.

Six strains were identified as *V. parahaemolyticus* on the basis of H_2S production and negative Voges - Proskauers' test, lactose and sucrose fermentation were again used to distinguish this species from other vibrios (Table 6).

Five strains of *V. alginolyticus* were defined. This group of pathogens has the ability to grow in very high concentration of salt, up to 10 %. Again the two traits which were used to differentiate *V. alginolyticus* from *V. parahaemolyticus* were sucrose fermentation and VP positivity (Table 7).

Two strains were typed as *V. vulnificus*, again on the basis of lactose fermentation, which was the most important trait for distinguishing this pathogenic organism (Table 7).

One strain was defined as a Non-01 serotype *V.cholerae*, on the basis of its sucrose fermentation and non-halophilicity, but although its biochemistry was identical with this species, its sero-specificity was not determined because it proved impossible to obtain Non-01 serotype antisera (Table 8).

One single aeromonad strain was also identified, this micro-

organism was a motile, gram-negative rod. It was oxidase positive, formed creamy-white colonies on TSA-plate grew only with difficulty on TCBS although it grew readily in salt-free broth and was resistant to 0/129 vibriostatic compound, all of these properties indicating a non - *Vibrio* organism. It was classified as an aeromonad on the basis of gas production from glucose fermentation, inability to metabolize arabinose, sorbitol, inositol, and lactose and inability to produce H_2S or indole (Table 8).

Eight strains all clearly showing the characteristics of vibrios were unable to be identified down to species level. They were grouped into three clusters by means of amino acid decarboxylase tests and ability to utilize sucrose, as given in table 9.

Table 1 Sampling date, age, size, water temperature, salinity and number of shrimp sampled, Samutsakhon province.

Sampling	Date (1989)	age (days)	size (gms)	water temp. (°C)	Sali. (ppt.)	No.of shrimp
S 1	1 May	35	2.2	28	28	10
S 2	16 May	40	2.8	29	26	10
S 3	1 June	32	2.4	30	22	10
S 4	16 June	45	3.5	31	18	10
S 5	1 July	48	4.0	28	15	10
S 6	16 July	37	4.8	27	16	10
S 7	1 August	35	3.0	28	14	10
S 8	16 August	40	3.0	27	16	10

Table 2 Sampling date, age, size, water temperature, water salinity and number of shrimp sampled, Chanthaburi province.

Sampling	Date (1989)	age (days)	size (gms)	water temp. (°C)	Sali. (ppt.)	No.of shrimp
C 1	8 May	38	2.5	28	32	10
C 2	23 May	40	2.8	29	30	10
C 3	8 June	48	4.1	27	25	10
C 4	23 June	59	7.2	30	26	10
C 5	8 July	35	3.1	27	22	10
C 6	23 July	46	6.4	25	20	10
C 7	8 August	61	8.3	26	20	10
C 8	23 August	38	4.0	27	22	10

Abbreviation explanation for Tables 3-9

TCBS	= Thiosulphate citrate bile salt sucrose
ADH,LDH,ODH	= Arginine,Lysine,Ornithine decarboxylase tests
ONPG	= test for beta - galactosidase activity
O/129	= the pteridine derivative, inhibition of vibrios.
H_2S	= hydrogen sulphide production test
O/F-test	= oxidative and fermentative test
+	= typical positive
-	= typical negative
d	= differs among strains
R	= reference strains from The National Collections of Industrial and Marine Bacteria Limited. (NCIMB)

Vibrio alginolyticus NCIMB 1196

Vibrio parahaemolyticus NCIMB 1902

Vibrio vulnificus NCIMB 2046

Vibrio cholerae subsp. *albensis* NCIMB 41

Aeromonas sp. NCIMB 1137

Table 3 : Morphological and biochemical characteristics of isolated bacteria from Samutsakhon Province.

Characteristics.	*Vibrio parahaemolyticus*												
	1	2	3	4	5	6	7	8	9	10	11	12	R
colony on TCBS	G	G	G	G	G	G	G	G	G	G	G	G	G
ADH	-	-	-	-	-	-	-	-	-	-	-	-	-
LDH	+	+	+	+	+	+	+	+	+	+	+	+	+
ODH	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+
ONPG	-	-	-	-	-	-	-	-	-	-	-	-	-
O/129,10/150 ug.	S/S	R/S	S/S	R/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+
H_2S	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	+	+	+	+	-	-	+	-	-	+	+	+	+
MR/VP	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Nitrate	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole	+	+	+	+	+	+	+	+	+	+	+	+	+
0% NaCl	-	-	-	-	-	-	-	+	+	-	-	-	+
3% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+
6% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+
8% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+
10% NaCl	+	+	+	-	-	-	-	-	-	-	-	-	-
O/F-test	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Arabinose	-	-	-	-	+	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+

Characteristics.	*Vibrio parahaemolyticus.*												
	1	2	3	4	5	6	7	8	9	10	11	12	R
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+
Manitol	+	+	+	+	+	+	+	+	+	+	+	+	+
Melibiose	-	-	-	-	-	+	+	-	+	+	+	+	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4 : Morphological and biochemical characteristics of isolated bacteria for Samutsakhon Province.

Characteristics.	*V. vulnificus*			*V. alginolyticus*		
	1	2	R	1	2	R
colony on TCBS	G	G	G	Y	Y	Y
ADH	-	-	-	-	-	-
LDH	+	+	+	+	+	+
ODH	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
ONPG	+	+	+	-	-	-
0/129, 10/150 ug.	S/S	S/S	S/S	S/S	S/S	S/S
Motility	+	+	+	+	+	+
H_2S	-	-	-	-	-	-
Citrate	+	-	+	+	+	+
Urease	-	-	-	-	-	-
MR/VP	+/-	+/-	+/-	+/+	+/+	+/+
Nitrate red.	+	+	+	+	+	+
Indole	+	+	+	+	+	+
0% NaCl	-	+	+	-	-	+
3% NaCl	+	+	+	+	+	+
6% NaCl	+	+	-	+	+	+
8% NaCl	+	+	-	-	+	+
10% NaCl	-	-	-	-	-	+
O/F-test	+/+	+/+	+/+	+/+	+/+	+/+
Arabinose	-	-	-	+	+	-
Glucose	+	+	+	+	+	+

Characteristics.	*V. vulnificus*			*V. alginolyticus*		
	1	2	R	1	2	R
Inositol	-	-	-	-	-	-
Lactose	+	+	+	-	-	-
Maltose	+	+	+	+	+	+
Manitol	+	+	-	+	+	+
Melibiose	-	-	-	+	+	-
Raffinose	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-
Sucrose	-	-	-	+	+	+

Table 5 Morphological and biochemical characteristics of isolated bacteria from Samutsakhon Province.

Characteristics.	*Vibrio* sp.														
	I		II								III			IV	V
colony on TCBS	G	G	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	G	Y
ADH	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+
LDH	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
ODH	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ONPG	+	+	-	+	+	+	+	-	-	+	+	-	+	+	-
O/129	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S
10/150 ug. Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H_2S	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Citrate vti.	-	-	-	+	+	+	+	-	-	+	-	+	+	+	+
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MR/VP	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Nitrate red	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0% NaCl	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-
3% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6% NaCl	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
8% NaCl	+	-	+	-	+	-	+	-	+	+	+	+	+	+	+
10% NaCl	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-
O/F-test	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Arabinose	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Characteristics.	*Vibrio* sp.														
	I					II					III			IV	V
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	+	-	-	-	-	+	+	-	-	-	-	-	-	+	-
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Manitol	+	-	+	-	-	+	+	+	+	+	+	+	+	+	-
Melibiose	-	-	-	+	-	-	-	-	-	+	+	+	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+

Table 6 Morphological and biochemical characteristics of isolated bacteria from Chanthaburi Province.

Characteristics.	*Vibrio parahaemolyticus.*						
	1	2	3	4	5	6	R
colony on TCBS	G	G	G	G	G	G	G
ADH	-	-	-	-	-	-	-
LDH	+	+	+	+	+	+	+
ODH	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+
ONPG	-	-	-	-	-	-	-
O/129, 10/150 ug.	S/S	S/S	S/S	S/S	S/S	S/S	S/S
Motility	+	+	+	+	+	+	+
H_2S	+	+	+	+	+	+	+
Citrate	-	+	+	-	+	+	+
Urease	+	-	-	+	-	-	+
MR/VP	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Nitrate red.	+	+	+	+	+	+	+
Indole	+	+	+	+	+	+	+
0% NaCl	+	+	+	-	+	-	+
3% NaCl	+	+	+	+	+	+	+
6% NaCl	+	+	+	+	+	+	+
8% NaCl	-	+	+	-	+	+	+
10% NaCl	-	+	+	-	+	+	-
O/F-test	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Arabinose	+	+	-	-	-	-	-
Glucose	+	+	+	+	+	+	+

Characteristics.	*Vibrio parahaemolyticus.*						
	1	2	3	4	5	6	R
Inositol	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+
Manitol	+	+	+	+	+	+	+
Melibiose	+	-	-	-	-	-	-
Raffinose	+	-	-	-	-	+	-
Sorbitol	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-

Table 7 Morphological and biochemical characteristics of isolated bacteria from Chanthaburi Province.

Characteristics.	*V. alginolyticus*						*V. vulnificus*		
	1	2	3	4	5	R	1	2	R
colony on TCBS	Y	Y	Y	Y	Y	Y	G	G	G
ADH	-	-	-	-	-	-	-	-	-
LDH	+	+	+	+	+	+	+	+	+
ODH	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+
ONPG	-	-	-	-	-	-	+	+	+
O/129, 10/150 ug.	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S
Motility	+	+	+	+	+	+	+	+	+
H_2S	-	-	-	-	-	-	+	+	-
Citrate	+	+	+	+	+	+	+	+	+
Urease	+	-	-	-	+	-	-	+	-
MR/VP	+/+	+/+	+/+	+/+	+/+	+/+	+/-	+/-	+/-
Nitrate red.	+	+	+	+	+	+	+	+	+
Indole	+	+	+	+	+	+	+	+	+
0% NaCl	-	-	-	-	-	+	-	-	+
3% NaCl	+	+	+	+	+	+	+	+	+
6% NaCl	+	+	+	+	+	+	+	+	-
8% NaCl	+	+	+	+	+	+	+	+	-
10% NaCl	+	+	+	+	-	+	-	-	-
O/F-test	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Arabinose	+	+	+	+	+	-	-	-	-
Glucose	+	+	+	+	+	+	+	+	+

Characteristics.	*V. alginolyticus*						*V. vulnificus*		
	1	2	3	4	5	R	1	2	R
Inositol	–	–	–	–	–	–	–	–	–
Lactose	–	–	–	–	–	–	+	+	+
Maltose	+	+	+	+	+	+	+	+	+
Manitol	+	+	+	+	+	+	+	+	–
Melibiose	+	–	–	–	–	–	+	–	–
Raffinose	–	+	+	+	+	–	–	–	–
Sorbitol	–	–	–	–	–	–	–	–	–
Sucrose	+	+	+	+	+	+	–	–	–

Table 8 Morphological and biochemical characteristics of isolated bacteria from Chanthaburi Province.

Characteristics.	*V. cholerae.* Non 01.		*Aeromonas.* sp.	
	1	R	1	R
colony on TCBS	Y	Y	Y	Y
ADH	-	-	+	+
LDH	+	+	-	-
ODH	+	+	-	-
Oxidase	+	+	+	+
ONPG	+	+	-	-
0/129, 10/150 ug.	S/S	S/S	R	R
Motility	+	+	+	+
H_2S	-	-	-	-
Citrate	+	+	-	-
Urease	-	-	-	-
MR/VP	+/-	+/d	+/+	+/+
Nitrate red.	+	+	+	+
Indole	+	+	+	+
0% NaCl	+	+	+	+
3% NaCl	+	+	+	+
6% NaCl	+	d	+	+
8% NaCl	+	-	-	+
10% NaCl	-	-	-	-
O/F-test	+/+	+/+	+/+	+/+
Arabinose	-	-	-	-
Glucose	+	+	+/G	+/G

Characteristics.	*V. cholerae.* Non 01.		*Aeromonas.* sp.	
	I	R	I	R
Inositol	-	-	-	-
Lactose	-	-	-	-
Maltose	+	+	+	+
Manitol	-	+	+	+
Melibiose	+	-	-	-
Raffinose	+	-	-*	-
Sorbitol	-	-	-	-
Sucrose	+	+	+	+

Table 9 Morphological and biochemical characterisitcs of isolated bacteria from Chanthaburi Province.

Characteristics.	*Vibrio* sp.							
	I				II		III	
colony on TCBS	Y	Y	Y	Y	Y	Y	Y	G
ADH	+	+	+	+	-	-	-	+
LDH	-	-	-	-	+	+	+	-
ODH	-	-	-	-	+	+	-	-
Oxidase	+	+	+	+	+	+	+	+
ONPG	-	+	-	-	+	+	-	-
0/129, 10/150	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S
Motility	+	+	+	+	+	+	+	+
H_2S	-	-	-	-	-	-	-	-
Citrate	+	+	+	+	+	+	+	-
Urease	-	+	-	-	-	-	+	-
MR/VP	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Nitrate red.	+	+	+	+	+	+	+	+
Indole	+	-	+	+	+	+	+	-
0% NaCl	-	-	-	-	+	+	-	-
3% NaCl	+	+	+	+	+	+	+	+
6% NaCl	+	+	+	+	+	+	+	+
8% NaCl	-	-	+	-	+	-	+	+
10% NaCl	-	-	-	-	-	-	-	-
O/F-test	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Arabinose	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	-	+

Characteristics.	*Vibrio* sp.							
	I				II		III	
Inositol	-	-	-	-	-	-	-	-
Lactose	-	+	-	-	-	+	-	-
Maltose	+	+	+	+	+	+	-	+
Manitol	+	-	+	+	-	+	-	-
Melibiose	-	-	-	-	+	+	-	-
Raffinose	-	-	-	-	+	-	+	-
Sorbitol	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	-

Discussion

The level of *Vibrio* infection in the intensive shrimp culture ponds of Samutsakhon and Chanthaburi province was remarkably high at all times of sampling. This was doubtless why, in addition to the abundant growth of bacteria from clinically affected shrimp, there was a general bacteraemia present even in apparently normal shrimps.

The fact that mixed cultures, representing at least two different biotypes were able to be isolated from each specimen, also leads to the conclusion that the vibrios are most probably opportunist and are not primary pathogenic bacteria. This suggests that the bacteria isolated infected the host after they were stressed by other possible causes such as reduction in dissolved oxygen, rapid change in pH, salinity or temperature, as well as an increase in nitrogenous waste produced by poor husbandry and management. It also confirmed the empirical views of Lightner and his colleaques in this regard (Lightner and Lewis, 1975; Lightner, 1985; 1988).

Sometimes culture of affected shrimps failed to reveal any growth of micro-organisms on the culture plates even when material was obtained from moribund shrimp which showed the typical disease symptoms. This is probably because of the use of antibiotic drug treatment on the affected stock.

Some moribund shrimps produced profuse growth, even when stocks had been extensively treated. It is assumed that there were specimens which had a longstanding infection and had not fed since the introduction of treated food. Some other moribund specimens failed to yield any bacteria even when antibiotic usage was not extant and in these cases it is assumed that other

factors such as direct effects of poor water quality without intercurrent bacterial invasion, was responsible for the losses.

In both areas, *V.parahaemolyticus* was the most common pathogen in shrimp, 18 strains (33.3%) were isolated. According to their salt tolerance, these micro-organisms did not appear to have the ability to grow without supplementary salt. This was especially apparent with strains from Chanthaburi Province, which also seemed to be particularly tolerant of high salt concentrations. For example four strains out of six (66.6 %) from this group could grow in 10 % salt, whereas among strains from Samutsakhon, only 3 out of 12 strains (25 %) could grow in 10 % salt (Table 3,6).

This may confirm why this organism is more predominant in a very wide range of habitats, both estuarine and marine environments, when compared to the other isolated pathogens. In 1962, Miyamoto and his co-workers described the winter and summer type distribution of *V. parahaemolyticus* as follows: The euryhaline virulent forms were more abundant in summer months while the stenohaline avirulent forms were found more commonly in winter months. From the results of the present study it has not been possible to demonstrate such a seasonal variation, since the sampling period was restricted to the summer months but certainly stenohaline strains predominated. This also supports the previous reports on the occurence of vibrios in the marine environment of the east coast of the upper Gulf of Thailand, which also found that *V. parahaemolyticus* can be readily isolated.

In Samutsakhon Province in which salinity ranged from 14-28 ppt during the sampling period, *V. parahaemolyticus* comprised up to 38.7 % of

all isolations. It was more abundant than in Chanthaburi province in which 6 strains (26 %) of this species were isolated (salinity 20-32 ppt.). This could possibly indicate that *V. parahaemolyticus* is more common in relatively low salinity areas but a wider survey would be necessary to confirm this.

Vibrio alginolyticus was also isolated from a small number of diseased shrimp. The incidence of this organism seemed to increase when water salinity was higher. Five strains (21.7 %) were isolated from Chanthaburi province while only 2 strains (6.5%) were obtained from Samutsakhon where the salinity was lower.

Vibrio alginolyticus has been recognized as a causative pathogen in fish and shellfish since 1981 (Colorni *et al*, 1981), although it has been mainly reported as a pathogen of molluscs.(Sindermann, 1988a; b; c; d; e; f).

In Thailand, *V. alginolyticus* has been reported as an associated pathogen in Black Splinter Disease, but it was considered less significant than *V. vulnificus* (Ruangpan and Sae-Ui, 1988).

The lactose fermentative *Vibrio*, *V. vulnificus*, was isolated on occasion, but considered not to be the dominant species, certainly during the present study period. Gross examination of diseased shrimp indicated that there was no evidence that any specimen was suffering from the classical "Black Splinter Disease" (Limsuwan, 1988; Ruangpan and Sae-Ui, 1988). The low incidence of *V. vulnificus* infection may support the theory that this pathogen is more virulent in periods of lower salinity, probably lower than 10 ppt. which is the level below which the Black Splinter Disease, is reported to occur (Ruangpan and Sae-Ui, 1988).

By contrast, *V. vulnificus* is reported to be a significant species in the marine environment of the West Coast of the United State of America. Kaysner and his colleagues (1987b) reported that it occured widely in samples of water, sediment and shellfish. They indicated that it was more abundant in areas where temperatures were above 15°C and salinity between 15-30 ppt. It has also been reported to play a significant role as a highly virulent opportunistic human pathogen, but there is a low incidence of the disease. (Blake *et al*, 1980; Joseph *et al*, 1982; Oliver *et al*, 1983)

One strain isolated from Chanthaburi was classified as a Non-01 *Vibrio cholerae* strain because of it ability to grow in salt free broth, sensitivity to 0/129 vibriostat, production of yellow colonies on TCBS agar and demonstration of the other biochemical and physical characteristics described for non 01 *V. cholerae* (Gouxing, 1986). This vibrio has already been described as a shrimp pathogen isolated from the orbit and haemolymph of blind *Penaeus orientalis* in China, during July to October, 1983 (Gouxing, 1986).

All of the shrimp pathogens which have been obtained in the present study are already known as human pathogens, especially *V. parahaemolyticus*, which causes a very well recognised food-borne disease around the world. Particularly in Thailand, bacterial gastro-intestinal diseases such as acute diarrhoea or food poisoning have been widely known to have been associated with shrimp .

In 1979, Maruyama, the Japanese expert and co-workers reported on the incidence of the enteropathogenic bacteria in Chanthaburi Province, where a high incidence of food poisoning had been reported. *Vibrio parahaemolyticus*

was reported as the *Vibrio* with the highest incidence in human faecal specimens between January and December 1978. In 1979 by contrast, this organism was reported as being lower in incidence than *Shigella* strains.

From the marine environment of the east coast of the upper Gulf of Thailand, *V. parahaemolyticus* has frequently been isolated from water and sediment samples. It was found at all sampling stations along the coast, and seemed to be abundant all year round.

The presence of *V. vulnificus* in the study is not surprising and also has connotations for human disease. Three serious cases of *V. vulnificus* infection with skin lesions in humans were reported from Chulalongkorn hospital, Bangkok during one period between August and November 1985. All of the patients had evidence of liver disease; two had primary septicaemia and died within 24 hours of hospitalization. The pathogen was isolated from the blood and from the cutaneous fluid. The route of infection was considered to be the consumption of contaminated sea-food. Patients who suffer from chronic liver disease seemed to be more susceptible to infection by *V. vulnificus* (Pattanaungkul *et al*, 1986).

Due to the prevalence of *V. vulnificus* in the coastal environment and in cultured shrimp, which was confirmed in this study, there is a potential hazard for humans and in particular for persons with liver disorders.

From the view point of public health, these organisms are now more frequently reported in many areas, especially in Thailand. In this study the incidence of *V. vulnificus* in diseased shrimp was low, but that is probably because the sampling was during high salinity periods and it is probably more

abundant and virulent during the low salinity months, as disease outbreaks associated with the agent have generally been reported at this time (Limsuwan, 1988). This is a serious pathogen whose role in both human and crustacean disease is currently ill understood. There is in clear need of a systematic study involving the biology, pathogenicity, prevention and treatment of this pathogen.

Vibrio alginolyticus was another species isolated only in low numbers in the present study. It is however widely associated with contamination of sea-food in Hong Kong (Chan *et al*, 1989), where it was always present in higher numbers than *V. parahaemolyticus* and *V. vulnificus*, and was more frequently isolated from bivalve shellfish than from other types of sea-food. This pathogen has been reported as the cause of acute enterocolitis and is also associated with skin infection in humans (Blake *et al*, 1980; Schmidt *et al*, 1979). There are no reports of *V.alginolyticus* infection in humans in Thailand, but this is not the first time it has been isolated from diseased shrimp (Ruangpan and Sae-Ui, 1988) .

Non 01 *Vibrio cholerae* was only found on a single occasion. Again this species has been recorded as a possible cause of diarrhoea in man. Tanasupawat and Saitanu (1984) reported that the optimum temperature for growth of non-01 *cholerae* isolated from water samples ranged from 30-42°c, confirming that the organism could well establish and grow in the human gut and cause disease.

This isolate of *V.cholerae* is unlikely to be pathogenic for both human and shrimp. It probably represents a contamination of the shrimp by a human pathogen or possibly the opportunist infection of a stressed and susceptible

shrimp. There should have been no direct contamination of the pond with human waste. Therefore the organism probably entered the pond with contaminated water or possibly during fertilization with chicken waste.

The presence of this potential human pathogen emphasises the need to adequatly cook shrimp for human consumption.

Although it has been previously reported, there was no evidence of any *V. harveyi* infection in juvenile or adult shrimp, in the present study. This was probably because it is normally a disease of hatchery larvae and not grow-out shrimp which were the subject of this study.

One strain isolated from clinically affected shrimp was identified as an *Aeromonas* sp. This group of bacteria is very common in the freshwater environment but does on occasion show salt tolerance. It often causes disease in fresh water animals. Its occurance was however probably an isolated instance. Stressed shrimp unable to defend themselves appear to be readily infected by various opportunistic invaders, a role which *Aeromonas* sp. are well recognised to perform. This probably applies also to the number of un-identifiable vibrios, which were also present randomly and in variable numbers in the sampled shrimps, and about which little can be given at this stage in our knowledge of the taxonomy and distribution of vibrios.

As described above, vibriosis in shrimp seemed to be a secondary infection by opportunist pathogenic bacteria from pond system. In an area which has been used for shrimp culture for many years as such Samutsakhon, proliferation of waste as well as opportunist bacteria in environment may readily lead to infection of stressed shrimp. As indicated in this present study, more

strains of the bacteria were isolated from Samutsakhon province.

Chapter III. Studies on the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of Oxytetracycline for *Vibrio* species Isolated During the Study.

Introduction.

Among the antibiotics used as drug treatments against vibriosis in shrimp culture, the tetracycline group and especially oxytetracycline appear to be the most useful and widely used. This latter antibiotic has been successfully used in farmed fin-fish for many years. In shrimp culture in Thailand, both in hatcheries and during the grow-out period, very large quantities of this antibiotic have been used, generally without prescription or any advice from the biologist, and as a result it has often proved unsuccessful in many areas. One possibility for the failure of the antibiotic to be effective is the development of resistance in strains of the shrimp pathogens because of excessive usage of sub-therapeutic dose rates of antibiotic. To obtain the best results in antibiotic treatment, a sensitivity test for the pathogen against the various drugs which might be available, is required and then treatment must always be at the full therapeutic treatment level.

Since although oxytetracycline is the most commonly used antibiotic in Thai shrimp culture, but there is virtually no information available on the question of resistance, studies were devised to enable the determination of the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of oxytetracycline against various *Vibrio* strains isolated from diseased shrimp from the two different shrimp culture areas described previously.

Literature review

Oxytetracycline : History

Oxytetracycline was produced originally as a derivative of the first tetracycline compound, namely chlortetracycline, in 1950. Tetracycline was produced in 1952, and dimethyl chlortetracycline was introduced in 1957, methacycline, a derivative of oxytetracycline was introduced in 1961; doxycycline in 1966 and minocycline in 1972.

The tetracycline-related group of antibiotics was shown to be highly effective against rickettsias, Gram-positive and Gram- negative bacteria, and agents such as the chlamydias, responsible for lymphogranuloma, venereum disease, inclusion body conjunctivitis of humans and psittacosis of birds, and hence they became known as the "broad - spectrum" antibiotics.

I. Source.

Oxytetracycline is elaborated by *Actinomyces rimosus*, a soil mould. The antibiotic is produced in broth by deep-tank fermentation.

II. Chemical name

4-(dimethylamino) - 1, 4, 4 , 5, 5 , 6, 11, 12 a- octahydro-3, 5, 6, 10, 12, 12 a-hexahydroxy-6-methyl-1, 11-dioxo-2- naphthacene carboxamide.

III. Chemical formula

$C_{22}H_{24}N_2O_9$

IV. Molecular weight 460.44

V. Trade name

Glomycin, Terrafungine, Riomitsin,
Hydroxytetracycline, Berkmycen, Biostat
Imperacin (Tablets), Oxacycline,
Oxatets, Oxydon, Oxystevacin,
Terrajecta, Terramycin, Tetramel,
Tetran, Vendarcin and Vendracin.

VI. Chemical properties

Tetracyclines are closely related to the polycycline naphtha cenecarboxamide. The crystalline bases are yellow, odorless and slightly bitter compounds. The base is only slightly soluble in water at pH 7 (0.25-0.5 mg/ml) but the hydrochloride is readily soluble, though some turbidity may result from the unsoluble bases. Both base and hydrochloride forms are readily soluble in dilute hydrochloric acid, fairly soluble in methyl and ethyl alcohol, but insoluble in chloroform and ether. When they are in soluble forms, they lose activity very rapidly.

Oxytetracycline is very stable when compared with chlortetracycline and penicillin. In the dry state this antibiotic is stable for at least two years at room temperature and also relatively stable in acid solution, especially below pH 2.5. However in neutral and particularly in alkaline solutions it deteriorates rapidly. Temperature also affects the stability of

oxytetracycline,its degradation rate being higher at high temperature.

VII.Effects on microbial agents.

Oxytetracycline is bacteriostatic at therapeutic level but in higher concentration may be actively bactericidal. Its effects on micro-organisms, especially on bacteria, are however dependent on the sensitivity of the micro-organism. The drug acts by inhibiting protein synthesis, because it closely resembles the amino glycosides that bind specifically to 30S ribosomes.

Oxytetracycline is synergistic when used with polymixin but it is antagonistic with penicilin.

VIII. Absorption, distribution and excretion.

Oxytetracycline hydrochloride can be administered by any of the normal routes. It is readily absorbed from the intestine. In mammals most absorption takes place from the stomach and upper small intestine. Effective blood levels following oral dosage are achieved in 2 - 4 hrs. Maximum levels are achieved more rapidly with intravenous injection, whereas intramuscular dosage gives peak blood levels after about two hours and levels are not so high, though maintained for a longer time.

The ready absorption of oxytetracycline is most useful in that it readily facilitates distribution thoughout the body, producing therapeutic levels in most tissues and fluids within a short time. The drug is removed from the blood by the liver or the kidney.

IX. Efficacy of drug

Since 1969, intensive studies concerning the efficacy, distribution and toxicity of oxytetracycline have been carried out in various fish species. Bullock and Collis (1969) studied the sensitivity of OTC against Gram-negative fish pathogens including *Aeromonas salmonicida*, *A.liquefaciens*, *Pseudomonas fluorescens*, and *Cytophaga psychrophila*. The results showed that all the test cultures were sensitive to OTC within the range of tissue levels found during the feeding of medicated food (approximately < 0.5 ppm.). This experiment also tested the protection that this antibiotic provided against the pathogen in vivo by force feeding trout with antibiotic for 1 or 2 days before injecting with the pathogenic bacterium *A.salmonicida*. The results not only demonstated the efficacy of OTC in protecting the fish from furunculosis but also that it protected from other infectious diseases as well. Attempts to determine the efficacy of OTC against furunculosis in coho salmon (*Oncorhynchus kisutch*) were carried out by Amend in 1969. Fish were infected with *A. salmonicida* and treated with OTC at different stages of the disease. The disease was controlled but the degree of control depended on the stage in pathogenesis at which the treatment was started. The longer treatment was delayed after infection, the less effective was the treatment. The author, therefore recommended that OTC at 3.5 g/100 pounds of fish per day, for 10 days was appropriate for the control of furunculosis in coho salmon. Herman (1969) studied the toxicity of this antibiotic on trout by force feeding the fish with different dosage of medicated pellets. The drug was found to have a

very low toxicity and under hatchery conditions, it was considered virtually impossible to feed lethal levels. If feeding trout with a single oral dose, more than 1 g/kg of fish can be administered to rainbow trout without noticeable ill effects.

In relation to shrimp pathogens, Takahashi and colleagues (1985) determined the Minimal Inhibitory Concentration of OTC against fifty nine strains of *Vibrio* sp. isolated from diseased Kuruma prawn (*Penaeus japonicus*). The results indicated that the MIC-value of 49 strains ranged between 0.1 and 12.5 ppm. Among the strains, 38 (77.6 %) showed MIC-values of 0.4 ppm or less and 11 (22.4 %) had values of 6.3-12.5 ppm. They also recommended dose concentrations for control of diseases in Kuruma prawn of 25 or 50 mg/kg body weight/day in vivo. By field trials they were able to prove that 50 or 100 mg OTC/kg body weight/day in oral form for 4-6 days showed the best results for treatment of the disease.

In brown shrimp (*Penaeus aztecus*), fed with 100 to 1,000mg OTC/kg diet, they obtained significantly higher growth and improved food conversion ratio (Corliss *et al*, 1977). In a study aimed at preventing of disease, shrimp were fed with 0, 100 and 1,000 mg/kg diets for 3 weeks before challenge by intramuscular injection with 0.02 ml of *Vibrio alginolyticus* suspension (approximately 10^7CFU/ml). The results showed that all the small shrimp (0.1 g) fed with high dosage of antibiotic died within 12 h,but larger shrimp (0.5 g) survived up to 20 hr post infection. This indicated that OTC was only effective in preventing *V. alginolyticus* infection of limited severity and even then only at high dosage levels. The lowest concentration of OTC to show

any therapeutic effect was 360 mg OTC/kg/day which is a level much greater than that necessary to treat infection in fish. The authors speculated that this abnormaly was possibly due to the shrimp not in fact consuming all of the food immediately upon its being placed in the tanks, so that the drug would then leach rapidly and be unavailable to them.

In an attempt to control gaffkemia in lobsters (*Homarus americanus*) (Bayer and Daniel, 1987), fed oxytetracycline-supplemented diets containing 0.5, 1.1 and 2.2mg OTC /g diet to lobsters which were then injected with the bactenum *Aerococcus viridans*. The results demonstrated that lobsters which were treated at the highest dose rate could survive this level of infection while 100 % of infected lobster died in the un-treated group. To confirm the validity of this experimental result, a field experiment was carried out. One diseased lobster was placed with normal lobster in each of 30 crates, containing 450 normal lobsters. The lobsters were then given either non-medicated feed, or medicated feed at 1.1 and 2.2 g OTC/kg of feed for 5 days. Mortality from gaffkemia was 78 % in the group that consumed the non-medicated diet compared with 13 and 2 % for those receiving 1.1 or 2.2 g OTC/kg diet, respectively (Bayer and Daniel, 1987).

X. The development of OTC-resistant bacteria.

Generally, there are many different mechanisms by which microorganisms might exhibit resistance to drugs including : production of enzyme to destroy the active drug, change in the permeability of the bacterial cell wall to protect the drug, structural alterations to the target of the drug or

alteration of the metabolic pathway on which the drug acts.

Three different mechanisms of plasmid-mediated resistance have been identified. The first type, present in Gram negative bacteria, has been found to reduce drug accumulation by active efflux of the drug at the inner membrane. The second type is found in anaerobic bacteria. This mechanism involved drug destruction. The third type has been described in both Gram positive and Gram negative bacteria, and is the mechanism by which resistant to tetracycline arises. Tetracycline and related drugs inhibit bacterial protein synthesis by blocking the ribosomes. Resistant strains of bacteria avoids the drug by coding for a protein which is similar in structure to the ribosomes. This protein binds the drug and thus protects the bacterial ribosomes (see review by Lewin, 1992).

The origin of drug resistance may be genetic or non- genetic but most drug-resistant microbes have emerged as a result of genetic changes and subsequent selection processes. Genetic changes may be chromosomal or extrachromosomal and can be transferred from one species to another by a variety of mechanisms. For tetracycline-groups, R-plasmids , extrachromosomal genetic elements, play an important role as the resistance gene carriers which transfer easily to the recipient bacteria (Lewin, 1992).

As already mentioned, reports from farms suggested that vibriosis in Samutsakhon was much less responsive to antibiotic therapy than similar infections in Chanthaburi. Consequently, the farms in Samutsakhon routinely employed higher dosages of antibiotic when treating shrimp. It was therefore considered appropriate to study the antibiotic sensitivity of vibrio isolates from

the two areas. Experiments were conducted to determine the MIC and MBC of oxytetracycline for the vibrio isolates described in chapter II.

Materials and Methods.

I. Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) determination.

Stock solution of oxytetracycline.

Oxytetracycline HCl, standard grade, obtained from Pfizer Ltd, U.K. was used for preparation of a stock solution.

Half a gram of oxytetracycline was dissolved in 10 ml of 0.1 N HCl and diluted with 490 ml of sterile distilled water to give a final concentration of 1,000 ugOTC/ml. Stock solution was stored in screw-capped vials, 20 ml each, and stored in the refrigerator at 5°C. The stock solution was always used within 14 days.

Bacterial inoculum.

Eighteen hour cultures of 54 strains of isolated bacteria from the two areas, 53 being *Vibrio* sp. of different types and one *Aeromonas* sp. were isolated and grown on TSA - agar. The bacterial suspension was washed from the plate and final concentration prepared by dilution with 2.0 % sterile saline to give a final cell suspension of approximately 1 X 10^7 CFU/ml.

Assay procedure.

Stock solution of oxytetracycline was diluted 1:4 with sterile tryptone soya broth (TSB), to give a working solution of 200 ugOTC/ml. Ten clear, sterile, screw-capped tubes (13 X 100 mm.) were selected and labelled from 1-10. Using aseptic technique, one ml.of steriled TSB was pipetted into the

tubes numbered 2 - 10. One ml.of oxytetracycline working solution (200 ug OTC/ml.) was added into tube 1 and tube 2. The contents of tube 2 was mixed well, then 1 ml of mixed content was transferred to tube 3, one ml of the well mixed content of tube 3 was then transferred to tube 4 and sequentially this was repeated, as a two-fold dilution procedure, to tube 9. The 1.0 ml of mixed solution from tube 9 was discarded and the tenth tube was not injected with antibiotic solution but served as the control. One ml of bacterial suspension, which contained approximately 1 X 10^7 CFU/ml was inoculated into all tubes. The final volume in each tube was two ml, the concentration of drug ranging from 0.39 - 100 ugOTC/ml. After 24 hrs incubation at 25°C, all the tubes were examined for evidence of bacterial growth,which was recognised as a turbidity of suspension. The lowest concentration of OTC in the serial tubes in which no evidence of bacterial growth could be detected was recorded as the Minimal Inhibitory Concentration (MIC).

The Minimal Bactericidal Concentration examination was performed by diluting one ml of solution from each of the tubes which were still clear, into nine ml of 2 % sterilized saline. These were mixed well and then 1 ml of each was poured onto TSA plates for incubation and observation for the growth of bacterial colonies. Again the lowest concentration of drug in the serial tubes which did not yield any bacterial colony growth on TSA after 24 hours incubation at 25°C was taken as the Minimal Bactericidal Concentration (MBC) of the drug.

Results

I. Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) Determination.

The fifty-three strains of the different *Vibrio* species described previously plus the one strain of *Aeromonas* species used in the study, showed results demonstrating a very wide range of Minimal Inhibitory Concentration values (MIC). These ranged from 0.39 ppm of antibiotic up to 50 ppm. The Minimal Bactericidal Concentration value (MBC) also ranged very widely with values from 3.125 ppm up to more than 100 ppm.

Vibrio parahaemolyticus, the most dominant species in Samutsakhon Province, exhibited a very high MIC-value, eight of the 12 strains examined showing MIC-values between 25-50 ppm, while only four were between 0.39-0.78 ppm. Eleven out of the twelve strains also showed a very high MBC-value (>25 ppm) while only one strain showed a low value, 3.125 ppm (Table 10).

Both strains of *V. vulnificus* also showed very high MIC and MBC values. The MIC ranged between 25-50 ppm. and the MBC was 50 ppm. for both strains. *Vibrio alginolyticus* showed relatively lower MIC-values, between 0.78-1.56 ppm. Six of the other un-classified *Vibrio* species showed a MIC-value lower than 12.5 ppm. Five strains out of 15 had a MIC of 0.78 ppm and those strains which exhibited lower MIC-values also showed the lower MBC-value, between 1.56-12.5 ppm. The most sensitive species among the vibrios isolated from diseased shrimp in Samutsakhon Province was *V.parahaemolyticus* (code B4) for which the MIC and MBC were 0.39 and 3.125

ppm, respectively, while the most resistant species, showed both MIC and MBC-values higher than 100 ppm.

Fourteen out of twenty-three of the bacterial strains isolated from Chanthaburi Province showed MIC-values lower than 1.56 ppm. Of these 14 strains, almost all of them showed on MIC-value of between 0.39 and 0.78 ppm. Eight strains from twenty-three were between 25-50 ppm. Almost all of the strains from Chanthaburi Province showed relatively high MBC-values, only 8 strains from 23 strains had a MBC-value of less than 25 ppm while fifteen strains showed a value greater than 25 ppm for the MBC (Table 11).

Vibrio parahaemolyticus strains were the most sensitive strains against oxytetracycline. Most of them had an MIC-value between 0.78-1.56 ppm, while the other species showed a much wider range of MIC-value, between 0.78-50 ppm in *V. alginolyticus*, 0.39- 25 ppm in un-identified *Vibrio* species and 50 ppm in both *V. vulnificus* and Non 01 *V. cholerae*. When MIC-value was related to MBC-value, *V. parahaemolyticus* and *V.alginolyticus* strains showed a very wide range of MBC-value, ranging from 1.56 - 50 ppm and 1.56 up to more than 100 ppm, respectively.

In both *Vibrio vulnificus* and Non-01 *V. cholerae*, MIC and MBC-values coincided at 50 ppm. Again, unidentified *Vibrio* species showed wide variation in MIC and MBC-value, ranging from 0.39-25 and 6.25-50 ppm, respectively, suggesting that this motley collection of very different but unidentifiable strains were linked only by their not fitting the criteria for identification as members of one of the recognised species and should not be considered as in any way closely related. However, the strain of *Aeromonas*

species showed low values for both MIC and MBC.

Vibrio parahaemolyticus, was the predominating *Vibrio* species found in the study in both Chanthaburi and Samutsakhon Province. From the MIC table given, it can be seen that strains of this species isolated from Chanthaburi Province were consistently more sensitive to oxytetracycline than similar strains isolated from Samutsakhon Province, while *V. vulnificus* strains in both Provinces seemed to be equally highly resistant to this antibiotic.

Table 10 MIC and MBC-Value of OTC against vibrios strains isolated from Samusakhon Province

Vibrios strains	Code	MIC (ppm)	MBC (ppm)
Vibrio parahaemolyticus	Uni1	0.78	50
Vibrio parahaemolyticus	Uni2	0.78	50
Vibrio parahaemolyticus	Uni3	0.78	50
Vibrio parahaemolyticus	Uni8	50	50
Vibrio parahaemolyticus	Uni9	50	50
Vibrio parahaemolyticus	A2	25	50
Vibrio parahaemolyticus	A4	50	>100
Vibrio parahaemolyticus	W2	50	50
Vibrio parahaemolyticus	W6	25	50
Vibrio parahaemolyticus	B4	0.39	3.125
Vibrio parahaemolyticus	P3	25	25
Vibrio parahaemolyticus	A7	25	25
Vibrio vulnificus	W4	25	50
Vibrio vulnificus	B3	50	50
Vibrio alginolyticus	S2	0.78	25
Vibrio alginolyticus	S5	1.56	6.25
Vibrio sp.	Uni4	25	50
Vibrio sp.	A3.1	100	>100
Vibrio sp.	W1	50	50
Vibrio sp.	W1.1	25	50
Vibrio sp.	W5	50	50
Vibrio sp.	S1	12.5	12.5
Vibrio sp.	S3	0.78	3.125

Vibrio sp.	S3.1	12.5	25
Vibrio sp.	S3.2	0.78	1.56
Vibrio sp.	S5	0.78	3.125
Vibrio sp.	B1	0.78	6.25
Vibrio sp.	B2	0.78	3.125
Vibrio sp.	P1	50	50
Vibrio sp.	P2	25	50
Vibrio sp.	P5	25	50

Table 11 MIC and MBC-value of OTC against vibrios strains isolated from Chanthaburi Province

Vibrios strains	code	MIC (ppm)	MBC (ppm)
Vibrio parahaemolyticus	N1	0.78	1.56
Vibrio parahaemolyticus	BF1	1.56	25
Vibrio parahaemolyticus	BF2	0.78	25
Vibrio parahaemolyticus	N8	0.78	1.56
Vibrio parahaemolyticus	N9	0.78	50
Vibrio parahaemolyticus	N21	0.39	25
Vibrio alginolyticus	N4	0.78	1.56
Vibrio alginolyticus	N7.1	0.78	6.25
Vibrio alginolyticus	N10	50	50
Vibrio alginolyticus	N12	50	>100
Vibrio alginolyticus	N14	0.78	1.56
Vibrio vulnificus	BF3	50	50
Vibrio vulnificus	N17	50	50
Non O1 *Vibrio cholerae*	N2	50	50
Vibrio sp.	N5	0.39	25
Vibrio sp.	N16	0.78	25
Vibrio sp.	N18	0.39	6.25
Vibrio sp.	N19	0.78	25
Vibrio sp.	N20	0.39	12.5
Vibrio sp.	N22	25	50
Vibrio sp.	N27	25	50
Vibrio sp.	N29	25	50
Aeromonas sp.	N17.1	0.78	3.125

Discussion.

Isolated vibrio strains from diseased shrimp exhibited a very wide range of MIC and MBC-values, 0.39-50 ppm.and 1.56- >100 ppm., respectively. Generally, *Vibrio* strains isolated from Samutsakhon Province seemed to be more resistant to OTC than the strains isolated from Chanthaburi. This was probably due to the development of OTC - resistant strains in Samutsakhon Province where shrimp culture has been extant for many years and therefore where antibiotics have been used for a long time. This result can be compared with the experiment reported by Takahashi and colleagues (1985) who determined the MIC- value of OTC against the strains of *Vibrio* sp.isolated from diseased Kuruma prawns. They found the MIC-values were < 0.1-12.5 ppm., relatively lower than the results from this report. Tanasomwang and Muroga (1989) reported that resistance to oxytetracycline of *Vibrio* sp. isolated from rotifer, the MIC-value was more than 6.2 ppm., again, lower than in this report. Even so they suggested that, what they considered to be a high MIC-value, which from their results appeared higher than in previous reports, may have resulted from the development of OTC-resistant bacteria.

Vibrio parahaemolyticus, the most dominant species in both sampling areas, showed itself to be more resistant to OTC in Samutsakhon Province than from Chanthaburi Province. Only one strain out of the 12 strains isolated showed the lowest MBC, 3.125 ppm. while the others had MBC-values more than 25 ppm. This may account for the lack of success being manifested at the time of sampling by antibiotic treatment of the infection in this area.

The strains from diseased shrimp from Chanthaburi Province were the

most sensitive to OTC, most of *V. parahaemolyticus* had an MIC- value between 0.78-1.56 ppm. and MBC-value between 1.56-50 ppm.

The vibrionic bacterial species which in the past was reported to have a very high incidence and to cause serious disease in shrimp cultured in Thailand was *V. vulnificus*. In the present study, however only two strains were isolated. Both showed relatively high MIC and MBC -values, viz 25-50 ppm. which indicated resistance to OTC. This high level of resistance possibly resulted from the excessive used of subtherapeutic dosage of OTC during disease outbreaks since 1986 (Limsuwan, 1988). It has already been well documented in relation to fin-fish that strains of this latter bacterial species can easily develop resistance and transfer it to the others (Aoki, 1974).

Other species of *Vibrio* from Samutsakhon Province also exhibited higher MIC and MBC-values than strains isolated from Chanthaburi Province. The again almost certainly related to the longer time usage of oxytetracycline in Samutsakhon Province, where intensive shrimp cultures operated earlier and thus faced disease problems earlier than Chanthaburi Province. The farmers in Samutsakhon Province started to use OTC-medicated feed, at 1-3 gms OTC/kg of feed, and showed this to be very effective in 1985. This lasted until 1988, when increasing dosage up to 5-7 gms OTC/kg of feed have had to be used if any positive value was to be gained. To date, in Chanthaburi Province 3-5 gms OTC-medicated feed still shows acceptable result (Limsuwan, pers.comm. 1991)

Oxytetracycline is know to degenerate slowly in solution and it has been shown that its degradation rate in the environment is very slow, with a half-life

of 10 weeks at 4°C-8°C (Jacobsen and Berglind, 1988). In many farms high level of OTC-contaminated sediments occur in the environment as a result of leaching from the medicated feed. These levels may be as high as 0.1 - 4.9 mg/kg dry matter in some long standing intensive farms. All of the micro-organisms in the environment, including the vibrios, will thus be exposed to a relatively high concentration of the antibiotic in such locations. This level will however be lower than the MIC or MBC-value required to inhibit them. Thus for a considerable period of time, potential pathogens may be developing resistance to the drug by genetic selection for change in the permeability of the cell envelope to protect the cell from the dissolved drug.

In addition to the development of resistance within the pond, there are other mechanisms which may be involved. As discussed previously, resistance may move between bacterial species by plasmid transfer. It was also demonstrated that human pathogens may be present in the pond (Chapter II) and animal pathogens may be introduced with pond fertilizer. Therefore resistance resulting from the use of antibiotics in humans and other terrestrial animals may be transmitted genetically to *Vibrio* spp. affecting shrimp. This assumption would suggest that resistance might also be transfered in the opposit direction with obvious public health implication.

Chapter IV. Studies on Oxytetracycline Residues in Muscle of Shrimp Fed with Medicated Feed.

Introduction.

Since oxytetracycline (OTC) has proved to be the most useful of the drugs available against vibriosis in both hatchery and grow-out phases of shrimp culture, it is not surprising that this drug is also the one most liable to be used improperly by farm operatives. Probably because of excessive usage, often at sub-therapeutic dose rates, there has been, following its introduction, a relatively rapid development of OTC-resistant strains of the *Vibrio* pathogens in Thai shrimp culture. To obtain the best result for controlling the disease in Thailand, it has been found increasingly frequently that higher dosages than previously sufficed are now necessary and so the drug is often being applied at considerably more than the recommended 5 g/kg of feed in some areas (Limsuwan.pers.comm. 1990)

In the U.S.A., the Food and Drugs Administration (FDA) approves OTC for use in food fin-fish, and many of the scientific reports concerning diseases in both fish and shell fish recommend OTC both as prophylaxis and as a therapeutic drug against bacterial infection. However, since there is a lack of information on the long term effects on chronic exposure to oxytetracycline via food, on human health, Oxytetracycline is still in the process of approval by the FDA and the Environmental Protection Agency (EPA) as a therapeutic drug for use in penaeid shrimp cultured in the U.S.A. (Williams and Lightner, 1988). Since this is the case, there is great need to gain more understanding of the inter-relationship between this widely used drug and its accumulation and retention time in shrimps. This has become particularly important of late because of the current great concern of countries importing shrimp products

that all antibiotic residues have been eliminated from shrimp muscle before they are marketed.

The present study was therefore carried out in anticipation of American endorsement of the drug for use in shrimp culture and to facilitate the development of reliable guidelines to be followed by producers in order to prevent the export of residue bearing shrimp meat not only to countries where residue levels are strictly regulated but indeed to all consumers of penaeid shrimps. Such information would also improved the effectiveness of antibiotic treatment.

Literature review

As oxytetracycline has become more widely used for treatment of poikilotherms against bacterial infection, so intensive studies have also been carried out in an attempt to determine the absorption, distribution, accumulation and depletion of this antibiotic in different animals, by different routes of administration. By bathing treatment, channel catfish (*Ictalurus punctatus*) were shown to be able to absorb levels of OTC above the MIC-value when they were immersed for 5 hr in solutions containing 4-32 mgOTC/L (Nusbaum and Shotts, 1981). This confirmed the report by Snieszko (1959) who compared the absorption of OTC and chlortetracycline in fish, and found that only OTC could reached therapeutic levels since chlortetracycline apparently was bound too rapidly in the liver. Herman *et al*, (1969) exposed rainbow, brook and brown trout via the oral route with feed medicated with OTC. The results indicated ready absorption of the drug by the gastrointestinal tract, with the highest levels being detected in the liver and muscle tissue, respectively. When the fish were fed with 75 mgOTC/ kg of fish body weight for 14 days at different temperatures, the highest levels of OTC were always found in liver, muscle and plasma in that order. Approximately 10 days were required to clear all brown trout tissues of residues at 12-13 °c. Muscle and plasma of rainbow trout and brook trout were freed from residue in approximately 14 days but the liver took 30 days. At 9-10 ° c, rainbow trout muscle was completely clear within 14 days. However, four weeks were required for elimination of the drug from the muscle and plasma of all three species at 6-7 °c. In channel catfish, the level of OTC was also highest in the liver but tended to be higher in serum than in

muscle. Post-treatment time required for drug elimination was greater for liver than other tissues. This was possibly due to higher initial concentration. When the fish were treated with 50 mg/kg body weight, the liver was free from drug by 10 days at 22.2-25 °c while muscle required only 2 days. In higher dose rate (100 and 200 mgOTC/kg body weight) liver required 21 days and again, muscle and serum showed no measurable residues after 2 days post treatment (Fribourgh *et al*, 1969a).

Further work has been carried out in order to compare drug levels in fish tissue by using different methods of administration. Three methods, namely injection, force feeding and free choice feeding with medicated feed were used. The parenteral method consistantly yielded higher serum levels which persisted longer than those resulting from the two methods of oral exposure (Fribourgh *et al*, 1969b). In common carp (*Cyprinus carpio*) fed with a single dose of 60 mgOTC/kg fish body weight, drug was first detected in muscle 11 hr post treatment and the highest level was 0.11 ppm. The tissue was free from residue 96 hr post treatment. Only very low drug concentration (<0.07 ppm.) was detected in plasma (Grondel *et al*, 1987a)

In crustaceans reference studies have been carried out only for juvenile white shrimp (*Penaeus setiferus*). In these, OTC was incorporated in formulated feed and fed at a concentration ranging from 1,000 - 10,000 mg/kg of feed for three weeks followed by two weeks of feeding without OTC-supplementation. The antibiotic was first detected in shrimp muscle after 24 hr of feeding at 5,000 and 10,000 mg/kg of feed, and after 48 hr when 1,000 mg/kg of feed was fed. Peak concentrations for both the 5,000 and 10,000

mg/kg of feed group were reached within 1-3 weeks of feeding and three weeks was required for the 1,000 mg/kg of feed group. After feeding with medicated feed ceased, in the case of the shrimp fed 1,000 mg /kg of feed, the antibiotic could not be detected after three days while the other two groups required two weeks. The recommendation for treatment against the infection in shrimp was 5,000 mg/kg of feed which was considered to protect for 14 days. The recommended withdrawal period was 10 days at 23-27 °c (Corliss, 1979)

I. Factors influencing pharmacokinetics of OTC in fish and shellfish.

Because fish and shellfish are poikilothermic and aquatic, the basic knowledge concerning the pharmacokinetics of the drug in terrestrial animals can not be automatically applied to fish and shellfish. The metabolic rate of chemotherapeutants in fish and shellfish is temperature-dependent. Species variation, types of food, route of administration and size or age of fish are also factors affecting drug metabolism.

I.I Temperature.

The metabolic rate of chemotherapeutants in aquatic animals doubles approximately with each 10°C rise in water temperature (Ellis *et al.*, 1978). After a single oral administration of 75 mg/kg, the level of OTC in the serum of rainbow trout reached the maximum concentration within 1 h at 16°C, 12 h at 10°C and 24 h at 5°C. The half-life of oxytetracycline was 4.8 days at 16°C, 6.1 days at 10°C and 8.9 days at 5°C (Bjorklund and Bylund, 1990).

Jacobsen (1989) recommended withdrawal times for fresh water rainbow trout treated with OTC as follows, 90 days at below 6°C, 70 days between 6 and 12°C and 60 days above 12°C. These coincided to the findings by Bjorklund and Bylund (1990) who found the withdrawal times in rainbow trout treated with OTC varied from 27 days to 135 days depending on the temperature and tissue studied.

I.II Species of the animal

Plasma protein binding of drugs affects their pharmacokinetics, since protein bound drug can not penetrate the blood vessel wall. The extent of antibiotic to protein binding varies with animal species and particular antibiotic. The general differences in blood parameters such as pH, fatty acid composition and/or quantity or quality of plasma proteins, are all factors affecting the different clearance rates. In a comparative pharmacokinetic study conducted in rainbow trout and African catfish by intravenous and intra muscular administration of OTC of 60 mg/kg, the extrapolated zero time concentration obtained in trout was 8 fold higher than that of African catfish (Grondel *et al*, 1987b).

I.III Type of food and route of administration.

Normally the route of drug administration in aquatic animals is oral treatment. In particular cases such as for brood stock or large animals, intramuscular or intravenous injection are also used. Marked differences in bioavailability of OTC were observed after intramuscular and oral

administration with a dose of 60 mg/kg.

I.IV Leaching of Drug from Medicated-Feed

Fribourgh and colleaques (1969c) first recommended the common method of OTC administration to fish namely by mixing the antibiotic with feed. For this method to be successful, the following critera must be met :

1). The drug must be uniformly distributed throughout the feed

2). The drug must be present in such quantity that the therapeutic level is available in the quantity of food normally consumed

3). The drug must be reasonally stable in the finished feed

4). The finished feed must be palatable to the fish and must be consumed

5). The composition of the feed must not effect the availability of the drug

6). The drug should not leach into the water before the fish have consumed the feed.

The principal study on leaching in relation to fish feed was that of Fribourgh and co-workers (1969a), OTC-medicated pellets at 1.83 g/Kg of feed were immersed in the water at different temperature and water pH for studies the leaching rate. During the first 3 minutes, approximately 2 % of the drug was lost. There after, the percentage rate of leaching gradually decreased. Leaching rate was shown to be temperature-dependent, at 23°C the percentage

of drug leaching was more than twice the leaching rate at 13°C. Leaching did not remove more than 20 % of the added drug during any 15 minutes immersion. The surface/volume ratio of feed and drug effected the leaching out. The greater the volume, the less leaching. The maximum rate of drug leaching occurred at pH 3. From their experiment, they suggested that less drug was required at cold water temperature and high water pH.

Medicated-shrimp pellets containing OTC concentration of up to 10,000 mg/ Kg of feed were added to salt water at temperatures around 28 °C to determine the leaching rate. Up to 50 % of the drug may leach from the pellets within four hours (Higuera-Ciapara *et al*, 1990).

I.V Degradation of Oxytetracycline in the Environment.

The mode of biodegradation of oxytetracycline in the environment has not been documented but it seems likely that OTC escapes from the sediment by dissolution and diffusion. Crystalisation and complexation with Ca^{2+} and Mg^{2+} may extend the accumulation period in the sediment (Samuelsen, 1992). Jacobson and Berglind (1988) determined the degradation rate of OTC in sediments by placing OTC-medicated feed (dose rate 7.5-10 g/kg feed) in sedimentation tanks. The results showed OTC to be relatively persistent in anoxic sediments, with an estimated half-life of 10-weeks at water temperatures ranged between 4-8°C. In field examinations, the amount of OTC in the sediment under fish farms varied between 0.1-4.9 mg/kg dry matter which indicated that antimicrobial effects can be expected more than 12 weeks after administration.

Information regarding the persistance of antibiotics in tissue is important for the development of rational disease therapy and avoiding residues in final products. This area of the study attempted to examine the effect of dose, duration of antibiotic therapy and persistance within tissues.

Materials and Methods.

I. Shrimp and testing environment.

Juvenile tiger shrimp (*Penaeus monodon* Fabricius) weighing 3-4 g and 7-8 cm in length were obtained from commercial ponds at Samutsakhon Province, Thailand. The shrimp were maintained as holding stock in 1X 1 X 2 m fibre-glass tanks with sub-sand filtration. The water temperature was maintained at 27-28 ° c and the salinity was 15 ppt. One hundred and fifty shrimp were stocked in each tank. Feeding was with a commercially formulated diet (at least 37 % crude protein). Feeding rate was at a level of approximately 5 % body weight, 3 times daily, the amount of feed being increased or decreased depending upon the amount required at each feeding.

II. Medicated-feeding procedure.

Twenty-eight separate glass aquaria, (45 X 45 X 100 cm), were used. They were each stocked with 30 shrimp per aquarium three days before the start of the experiment. Sub-sand filtration was applied in each aquarium and 10-20 percent of the water was changed every day. During the acclimatisation period, dead shrimp were removed as soon as they were observed and healthy shrimp were substituted. Non-medicated feed was used during the 3 days acclimatisation period. Thereafter the following feeding regime was used. Group replicates of three tanks were used for each treatment, but only one tank was maintained as a negative exposure control. There was no evidence of loss of palatability of feed following substitution of normal feed by medicated feed and all continued to feed well.

group 1 - fed with non-medicated feed.

group 2-4 - fed with 1 g OTC/kg feed, for 3,5 and 7 days.

group 5-7 - fed with 3 g OTC/kg feed, for 3,5 and 7 days.

group 8-10 - fed with 5 g OTC/kg feed, for 3,5 and 7 days.

Medicated feed was prepared by dissolving the drug in water (30 ml.of water per 1 kg.of feed) spraying the dissolved drug onto the pellets, and then drying.

After the shrimp had been fed on the medicated food for the period indicated above, they were then changed to non-medicated food for the remainder of their experimental period. Two shrimp from each aquarium were sampled every day up until the seventh day after feeding with medicated feed had ceased. Sampled shrimp were stored at-10 °c until it was possible to perform the drug residue analyses.

The modified method of Bennett *et al* (1966) for OTC analysis was employed. The medium used was antibiotic medium No. 8 (Oxoid Ltd., England). The assay organism was *Bacillus cereus* (subsp. *mycoides*) which was suspended within the medium. One gram of shrimp muscle was extracted with 4 ml of pH 4.5 KH_2PO_4 buffer in a tissue homogenizer. Samples were centrifuged at 2,000 rpm. and 10 ul of homogenated tissue was placed in a well of 8 mm. diameter cut in the agar to the bottom of the plate. After 24 hrs incubation at 25°C the *B. cereus* had grown within the agar except where inhibited by the presence of a residue of antibiotic at inhibitory levels. Zones of inhibition were measured and compared to the standard curves. Drug concentration in 1 gram of shrimp tissue was obtained by using the dilution

factor 5, multiplied to the observed value.

Results.

Daily tissue concentrations of OTC accumulation after cessation of feeding with medicated feed at different concentrations and periods of administration are shown in figure 1-6. These showed that in all cases the drug was detectable in shrimp muscle at the highest level on the first day after the last feeding with medicated feed. Levels then decreased rapidly, but the rate of decrease depended on the original dose rate.

Groups of shrimp fed with one gram OTC/kg of feed for three days accumulated the OTC in muscle up to a level of 0.24 ppm (Fig.1), whereas the significantly greater accumulation values of 0.51 and 0.68 ppm were achieved if feeding at the same level was continued for 5 and 7 days (Fig.4), respectively. When the dose rate was increased to 3 g OTC/kg of feed, the higher residues, up to 0.44, 0.87 and 0.78 ppm were obtained in shrimp fed for 3, 5 and 7 days (Fig.5), respectively. At the highest dose rate used in the experiment (5 g OTC/kg of feed), high levels of antibiotic residue in shrimp muscle, (0.68-0.89 ppm) were achieved on the first day of determination (Fig.6), that is on the first day after cessation of feeding medicated food.

When the actual tissue drug levels of the groups fed the three different levels of antibiotic supplementation were compared on the first day of sampling after drug treatment ceased, as might be expected, the group of shrimp that were fed one gram OTC/kg of feed showed significantly lower tissue accumulation than those groups that were fed with 3 and 5 g OTC/kg of feed, with slight differences between those fed over the 3, 5 and 7 days consumption periods. However, in shrimp sampled two days after cessation of feeding

antibiotic, there were no significant differences in levels in any groups in relation to periods of administration, except that the group fed with 1 g OTC/kg of feed still showed significantly lower drug concentration than the other two groups (Fig.1). Again, when sampled after three days post treatment, the group receiving 1 g OTC/kg of feed showed lower residues than those fed 3 and 5 g OTC/kg and the group obtaining 1 g OTC/kg of feed for 3 and 5 days showed significantly lower residues, less than 0.16 and 0.22 ppm (Fig.2), while the groups fed for 7 days still showed higher values (up to 0.58 ppm). By the fourth and fifth day of determination, only one group which had been fed one gram OTC medicated feed for 7 days still showed high drug residues in muscle while the other two groups showed residues below the 0.16 ppm which is the level of sensitivity for this type of test, and all groups which were treated at 1 g OTC/kg of feed showed residues below 0.16 ppm by day 6 (Fig.3).

At the higher dose rates of up to 3 and 5 g OTC/kg of feed, all of the treatments showed the same trend of drug retention. The higher the dose rate and the longer the administration, the longer drug was retained. The highest concentration found in muscle and the number of days required for drug clearance from the tissue to a level of less than 0.16 ppm, after cessation of feeding for each group, is given in table 12.

The highest value of drug residue in shrimp muscle, up to 0.89 ppm, was detected in the group of shrimp which was fed with 5 g OTC medicated feed for 7 days (Table 12). The drug was still extant at a concentration higher than the cut off level of 0.16 ppm for 7 days after cessation of feeding.

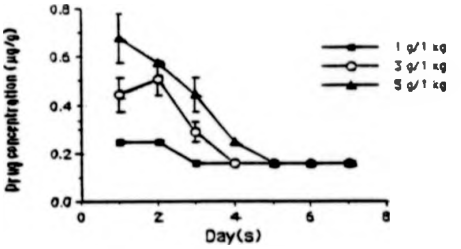


Figure 1 OTC concentration in shrimp muscle, fed with medicated feed containing OTC at 1, 3 and 5 g/kg feed for 3 days.

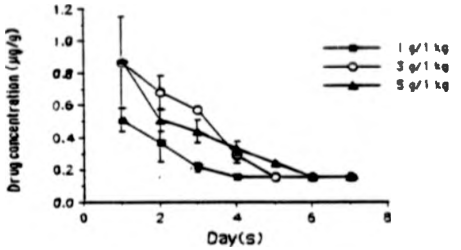


Figure 2 OTC concentration in shrimp muscle, fed with medicated feed containing OTC at 1, 3 and 5 g/kg feed for 5 days.

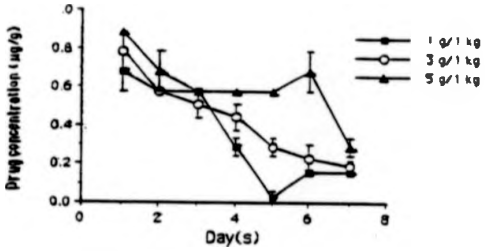


Figure 3 OTC concentration in shrimp muscle, fed with medicated feed containing OTC at 1, 3 and 5 g/kg feed for 7 days.

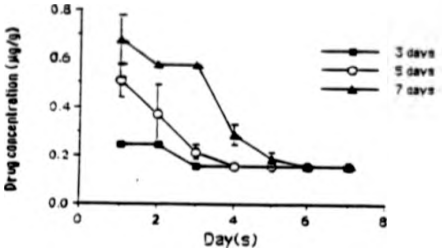


Figure 4 OTC concentration in shrimp muscle, fed with medicated feed containing OTC at 1 g/kg feed for 3, 5 and 7 days.

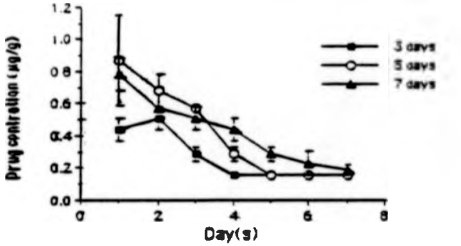


Figure 5 OTC concentration in shrimp muscle, fed with medicated feed containing OTC at 3 g/kg feed for 3, 5 and 7 days.

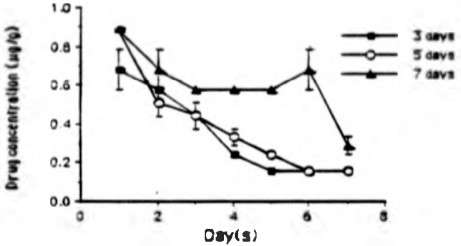


Figure 6 OTC concentration in shrimp muscle, fed with medicated feed containing OTC at 5 g/kg feed for 3, 5 and 7 days.

Table 12 Maximum OTC concentration in shrimp muscle, fed with medicated feed containing OTC at 1,3 and 5 g OTC/kg feed for 3,5 and 7 days and elimination period.

Dosage (g OTC/kg feed)	Administration Period (days)	Drug Concentration (ppm.)	Elimination Period (days)
	3	0.25	3
1	5	0.51	4
	7	0.68	6
	3	0.51	4
3	5	0.87	5
	7	0.78	>7
	3	0.68	5
5	5	0.89	6
	7	0.89	>7

Discussion

It appeared that irrespective of dose level, shrimp fed with oxytetracycline-medicated feed demonstrate the maximal concentration of tissue drug residue on the first day of sampling, after feeding ceases. This highest concentration may indeed be reached before sampling although that was not possible to determine from the present study. In general, the highest concentration of drug in the tissue may be reached within 2-4 hr. after feeding. This rapid uptake would be difficult to explain and does not in any way relate to the problem of residues in farm shrimp stock, so could not be confirmed in this study. However, at the lowest rate, 1 gOTC/kg of feed the retention time ranged from 3-6 days and in the other two groups, 3 and 5 gOTC/kg of feed, the retention times were 4-7 and 5-7 days (Table 12), respectively. Such dependency of retention time on the dosage and the period of administration was similar to that reported in juvenile white shrimp by Corliss (1979). He found that shrimp which received medicated feed at 1,000, 5,000 and 10,000 mgOTC/kg of feed showed the highest OTC concentration in muscle viz.: 0.25 ,0.85 and 1.05 ug/ml of the assay tissue fluid, which approximately equal to 1.25, 4.25 and 5.25 ugOTC/gram of shrimp tissue, respectively. To obtain such drug accumulation in muscle, groups of shrimp which were fed 1,000 mgOTC/kg of feed required 48 hr. after starting feeding whereas shrimp were fed with 5,000 or 10,000 mgOTC/kg of feed needed only 24 hr. When the amounts of drug residue are compared, the white shrimp studied by Corliss appeared to accumulate much more OTC in muscle than the tiger shrimp of the present study, this may have resulted from longer periods of administration,

because the white shrimp were fed with medicated feed up to 3 weeks before stopping for retention studies, the other possible cause may be that different test animals retain different drug levels. In lobster, *Homarus americanus*, held in floating cages and fed with 1.1 and 2.2 mg OTC/g feed for 10 days, drug residues were still detectable in muscle at 14 and 28 days after treatment at 15-19°C and 14-15°C, respectively. If nothing else, this demonstrated that the distribution of this drug was temperature dependent (Bayer and Daniel, 1987).

The retention time demonstrated in the present study, that is approximately 5-7 days after drug feeding regime ceased, supports the findings of the previous reports in white shrimp in which the recommended withdrawal period was 10 days at 23-27°C.

However, in view of variations in temperature, dosage and duration of therapy it would be prudent to advise a minimum 14 days withdrawal period, in order to minimise the possibility of residues in final products.

In fin fish, on the other hand the recommended retention time seemed very different from those in shrimp. In rainbow trout dose with OTC medicated feed at a concentration of 75 mg OTC/kg of fish body weight for 10 days, approximately 14 days were required to clear the drugs from the muscle at 12-13°C, while four weeks were required if the fish was held in the colder water, (approximately 6-7°C, Herman *et al*, 1969). Since it has been proven that the retention periods were temperature-dependent, the establishment of withdrawal times varies greatly from one country to another. Some countries take the temperature into consideration; e.g.the United Kingdom, uses degree days (number of days post treatment multiplied by the water temperature).

Withdrawal periods vary from 30 degree C days up to 500 degee C days dependant on the compounds (Richards, 1992).

The findings of the present study contrast with those of Higuera-Ciapara and co-workers (1992). In tiger shrimp fed with a pellet containing 250 mg/kg of feed for four weeks, they found that levels of OTC were 100-150 ug/kg^{-1} increasing to approximately 200-250 ug/kg^{-1} during the third week and remaining at this level until the end of the experiment. Such a level seems to be much lower than the findings of the present study. It may have resulted from the higher dose rate or different methods of antibiotic analysis.

When comparing the amount of drug retained in the shrimp tissue to the Minimal Inhibitory Concentration (MIC) of OTC reported for most *Vibrio* sp. by Takahashi and colleagues (1985) which ranged from <0.1- 12.5 ppm., it appeared that the level of the drug in the muscle did not reach the inhibition level required for successful treatment of the disease in cases where the bacterium strain had a high MIC.

However, the concentration of antibiotic in the haemolymph and other organs was not examined in this study. Such information is essential to the evaluation of treatment since bacteria in the tissue are usually exposed to haemolymph.

It is also important to take the method of drug administration into consideration. Medicated shrimp feed is routinely prepared by spraying the pellets with a solution of drug dissolved in a water. This coating is easily dissolved and lost due to the drug leaching from the pellets or binding to the cations in seawater. Thus the shrimp do not ingest sufficient antibiotic to gain

adequate drug levels in the tissue. At high temperature, leaching of antibiotic or other soluble compounds from the pellet can be very rapid, as high as 50% of drug can be leaching to seawater within 4 hours at 28°C, pH 7.5 (Higuera-Ciapara, *et al*, 1990). Work conducted elsewhere has suggested that leaching may be minimised by coating the medicated-pellet with oil or starch (Chanratchakool and Pearson, in preparation). Loss of drug efficacy may not only be by leaching from the pellet, or by seawater cation binding but can also be due to binding to the Mg^{2+} and Ca^{2+} in the digestive tract of the animal (Lunestad, 1992). This means that even if high concentrations of drug are ingested, sufficient amounts may not absorbed across the digestive tract to build up an effective dose in the tissue.

In summary, when relating the retention times of drugs in shrimp fed with the same dosage of medicated feed but for different durations of administration, it can be confirmed that the longer the period of administration, the longer the time required to eliminate the drug as well as the higher the drug concentration retained.

Chapter V. Studies on the Efficacy of Oxytetracycline against Vibriosis in Shrimp

Chapter V. Studies on the Efficacy of Oxytetracycline against Vibriosis in Shrimp

Introduction

In penaeid shrimps, vibriosis is potentially the most serious of all disease problems and can readily bankrupt farm operators. In Thailand it has grown progressively worse over the period 1990-1992 and this seems likely to continue still further with the increasing degradation of the environment which is one of the main predisposing conditions (Nash *et al*, 1992). As for most diseases in shrimp, the aetiology of vibriosis not well understood. Where there is some information, the pattern of pathogenesis seems to be complex. In a considerable number of shrimp diseases however there appears to be a bacterial component, and this almost invariablely involves vibrios. There are many others factors involved in the disease complex including poor management and associated poor environmental conditions (Fegan *et al*, 1991; Nash, 1990). Avoidance of diseases by using low stocking rates (no more than 30 shrimp/m^2), providing good water exchange and ensuring adequate nutritional inputs are considered to be more reliable control methods than treatment with antibiotics (Nash *et al*, 1992). However, when an outbreak of disease takes place, antibiotics can be effective if given early enough provided the effective dosage is maintained over the requisite period (Nash, 1990). Treatments with antibiotics against vibriosis have been purely empirical. Little scientifically derived information has been available on which to base treatment regimes and very little is known about the pathogenicity of the disease. The present study was designed to investigate the efficacy of the most commonly used antibiotic, oxytetracycline against experimental infection of the *Vibrio* bacteria in shrimp.

Literature review

The defence system of crustaceans.

Crustaceans have an exoskeleton as their outer limiting tissue and so the cuticle or outer layer of the shell plays a major role as the mechanical barrier against any potential pathogen or physical injury. It is composed of an outer and inner epicuticle, which consists of lipids, proteins and calcium. Any damage on the outer layer predisposed the cuticle and deeper tissues to invasive micro-organisms (Brock, 1983).

In contrast to that of vertebrates, the crustacean internal defence system against infectious agents is not based on immunoglobulins and interactive lymphocytes, but nevertheless it is extremely efficient. It does utilize interaction between cellular and humoral components. These are intrinsically linked since many of the humoral factors are derived from cells while cellular activity itself is influenced by substances within the plasma and the prophenoloxidase activating system (proPO). The proPO is a non-specific enzymic response which is ultimately responsible for responding to foreign material. The consequences of the activation of proPO are effected within the haemolymph and consist of a cellular component involving phagocytosis, nodule formation, encapsulation, and the humoral component. In this review, the basic defence mechanism will be discussed, with emphasis on pathological change.

I. Cellular and humoral defence mechanisms.

I.I The haemocyte and its roles.

Haemocyte Types. The most important cells in the crustacean

defence system are the haemocytes. Many attempts have been made to establish a uniform system of classification of the haemocytes in various crustacean species. The first description of crustacean haemocytes was reported by Haeckel in 1857 (Johnson, 1980). Currently, the classification is based on presence or absence, number, size, shape, staining reaction and ultrastructure of the granules in the cytoplasm. Martin and Graves (1985) identified three basic types of shrimp haemocyte based on these criteria and this is considered to be the simplest and most consistent taxonomy. On this basis they are classified as : hyaline cells (syn. non-granular cells, haemocyte or hyalocytes) which are characterized by absence of granules, granulocytes (syn. granular cells, haemocyte or eosinophilic granulocytes) which contain obvious granules and the third cell with a few or variable number of small granules; semi-granulocytes (syn. semigranulated cells, semi-granular cells, intermediate cells or haemocytes and intermediate granulocytes)

The haemocytes have a very important role in cellular defence mechanisms in shrimps,as they can immediately migrate to the site of any breach or invasion by pathogens. By means of phagocytotic activity, they can readily remove the small particles which have been agglomerated from the circulatory system. Larger particles can be encapsulated by haemocytic aggregation. They are also involved in the clotting process, synthesis and storage for the prophenoloxidase system.

1.II Coagulation and phagocytosis.

When shrimp are traumatized or invaded by pathogens,

hyalocytes and semi-granulocytes are the first and most active cells to migrate to the site of injury, although granulocytes also play a limited part. Haemocytes provide a clotting factor, coagulin, which as a result of its interaction with the plasma fibrinogen, stabilizes the site, trapping any pathogens and helping to seal any wound (Bauchau and de Brouwer, 1974; Bauchau and Mengeot, 1978). This process renders the pathogen immobile so that it cannot migrate from the site and is more easily eliminated by phagocytosis or encapsulation (Sindermann, 1971). In addition to the circulating cells of the haemocyte series, fixed phagocytes very similar to haemocytes are found lining the pericardial sinuses,and are involved in the basic defence strategies in crustacean immune system (Johnson, 1987). Unlike the immune response in the vertebrate, it has not been possible to show evidence of specificity in the crustacean immune response (Paterson and Stewart, 1974; Paterson *et al*, 1976).

Fontaine and Lightner (1974) used experimental injection of carmine into the muscle of penaeid shrimp, to define the inflammatory response. Numerous haemocytes readily migrated to the injection site and had phagocytosed the carmine particles within 30 minutes. Some haemocytes formed into the large multinucleated cells similar to the giant cells seen in vertebrates. They suggested that the carmine particles once phagocytosed, were removed from the circulatory system by the migration of phagocytosed haemocytes to external surfaces of gill, gut and hepatopancreas epithelium, but no free carmine was detected in the gut. To confirm this theory, White and Ratcliffe (1982) investigated the clearance of radio-labelled *Moraxella* from the haemolymph of *Carcinus maenas*, and concluded that the elimination of foreign

particle was probably occured at the gill hepatopancreas and heart.

I.III Encapsulation and nodule formation.

Encapsulation and nodule formation may take place when the introduced particles, whether inert or microbial are present in large numbers or are of large size, and cannot be eliminated by phagocytosis alone. Haemocytic clumping, encapsulation and nodule formation can occur anywhere thoughout the body, but most frequently are observed in the gill, below the carapace, body surface and in heart and hepatopancreas (Smith and Ratcliffe, 1980a; b). The process is very effective in limiting the spread of infectious agents within the haemocoel and brings the foreign material into close contact with the haemocytes for clearing (Smith and Soderhall, 1986). Tyson and Jenkin (1974) observed that haemocyte clumping and nodule formation are initiated by the substances released from the granulocytes (opsonin). After releasing their hydrolytic enzymes, the granulocytes become devoid of granules and thus become agranulocytes (Paterson and Stewart, 1974 ; Smith and Soderhall, 1986).

I.IV Melanization.

Melanization is the process which occurs in most arthopods (Johnson,1980) after nodule formation or encapsulation and was first demonstrated in vitro by Unestam and Nylund (1972) in decapod crustaceans. In mammals and teleosts, melanin is formed in the melanocytes at the integument layer and plays a role as barrier against ultraviolet radiation (Bloom

and Fawcett, 1968). Roberts (1975) referred to the presence of melanin in defensive processes in fin-fish tissue. The brown to black pigments identified as melanin were first shown to deposit in inflammatory areas of diseased penaeid shrimp by Lightner and Redman, 1977. Melanization was stimulated by the prophenoloxidase activating system (proPO) which may play an important role in the recognition of foreign substances in crustaceans (Smith and Soderhall, 1986). The proPO-system will be discussed separately below.

I.V Humoral factors.

Humoral substances in crustaceans consist of the components of the haemocyte membrane and secretions or products which are released from cell disruption when a haemocyte is destroyed. In many crustaceans, the following substance have been demonstrated in the haemolymph as part of the humoral factors.

Haemagglutinins. These substances were found in shore crab (*Carcinus maenas*) by Smith and Ratcliffe (1976; 1978). Haemagglutinins may serve as opsonins for phagocytosis and have a specificity similar to iso-haemagglutinin proteins in vertebrate sera (see review by Smith and Chisholm, 1992).

Agglutinins. These molecules were reported in lobster (*Homarus americanus*) (Cornick and Stewart, 1968) and were non-inducible (Cornick and Stewart, 1973; Paterson *et al*, 1976). These factors may also represent opsonins which enhance phagocytosis. Haemocytes were shown to be the source of this factor which is present free in the haemolymph or bound to the

plasma membrane of haemocytes. (Tyson and Jenkin, 1974).

Cytolysins and haemolysins are naturally present in crustaceans (Sindermann, 1971).

Precipitins have also been recognised in lobster haemolymph by Stewart and Foley (1969).

Bactericidins have been shown to be inducible in lobster, (Paterson *et al*, 1976), West India spiny lobster and American lobster (Schapiro, 1975).

Lectins with the specificity property of binding foreign materials have been identified in freshwater prawn (*Macrobrachium rosenbergii*) (Vasta *et al*, 1983).

Prophenoloxidase activation is important during inflammatory responses in crustaceans. The roles of the factors involved are described seperately below.

Recently, Adams (1991) demonstrated the inflammatory responses of penaeid shrimp to the bacteria *Vibrio alginolyticus* in both cellular and humoral mechanism and found that bactericidins appeared to be induced within 1 day after injection with heat-killed bacteria. Peak titres were achieved within 2 days and persisted in the haemolymph until day 5. They showed partial specificity. Lectins were also detected in bacteria-treated shrimp but were short-lived. Haemagglutinins were induced and detected at a variety of titres in both treated and control shrimp (Adams, 1991).

II. The prophenoloxidase activating system.

The prophenoloxidase (proPO) activating system is an important defensive process in crustaceans since it provides the recognition capacity to define foreign material. It comprises a cascade of a number of enzymes (mostly serine proteases) proteins and other factors (Soderhall and Smith, 1986a; b) which are involved in the conversion of inactive prophenoloxidase into active phenoloxidase, which plays an important role in initiating encapsulation and melanisation (Ratcliffe, 1985).

The precise mechanism by which the system works in penaeids has not been defined but in other crustaceans, it has been shown that microbial molecules such as lipopolysaccharide (LPS) found in bacterial cell walls and B-1, 3-glucans and carbohydrates found in fungal hyphae can activate the proPO system (Soderhall and Smith, 1986a; b). Other substances including proteases, lipids, detergents, organic solvents and chloroform also elicit the non-specific activation of proPO (Soderhall, 1982; Soderhall and Smith, 1983; 1986a; b)

Other stimuli which activate of the proPO system was known as low calcium level.In this reaction, low calcium levels cause an inactive serine protease to become active by a limited proteolysis involving removal of a peptide. Once the proPO system is activated, coagulation is stimulated and the wound closed, preventing the further loss of tissue fluid (Soderhall and Smith 1983; 1986a; b)

Since the phenoloxidases are located as prophenoloxidase proenzymes, within the semi-granulocytes and granulocytes (Soderhall and Smith, 1983), when the prophenoloxidase containing haemocytes come into contact with

activating factors, degranulation or exocytosis and subsequent lysis take place, initiating the system.

Controlling factors for the proPO cascade are present in the haemolymph in order to prevent widespread activation of the system. These are known as proteolytic inhibitors (Soderhall, 1986; Soderhall and Smith 1986a; b).

III.Inflammatory response in shrimp

Attempts to study the basic inflammatory response in a variety of crustacean species have been made by various methods. Fontaine (1971) and Fontaine and Lightner (1973) studied wound repair mechanisms in penaeid shrimp and Fontaine and Dyjax (1973) investigated scar formation. Subsequent studies on the response of penaeid shrimp to injection of foreign particles have also been carried out using carmine (Fontaine and Lightner, 1974) and the fungus, *Fusarium* sp. (Solangi and Lightner, 1976).

The cellular response to physical injury in penaeid shrimp was reviewed by Fontaine and Lightner (1975). Haemocyte infiltration at the damaged or infected area was the first reaction to take place. Phagocytosis of smaller particles such as micro-organisms or non-living materials occurred, followed by encapsulation of larger particles in the multilayered capsules which formed around them. These were composed of fusiform haemocytes toward the centre and larger haemocytes at the periphery. These surrounded any non-phagocytosable materials including fungal hyphae, parasites or cellular debris.

Fibroblasts have also been shown to participate in cellular infiltration and encapsulation in damaged tissues, when a dense network of fibroblasts and collagen-like fibres is formed which persists, to produce a well organized and relatively permanent scar (Fontaine and Dyjax, 1973). Brown to black pigmentation due to melanization quickly forms at the site of encapsulation and is a noteworthy clinical sign of the chronic inflammatory response to infection in many decapod crustaceans (Fontaine and Lightner, 1975).

Adams (1991) studied the response of black tiger shrimp to *Vibrio* spp. infection. Heat-killed *Vibrio alginolyticus* was injected intramuscularly. Haemolymph was taken and examined for evidence of the bacterial invasion using ELISA - technique. More than 99% of heat - killed bacteria were cleared from the haemolymph within 4 h. High - mortality was also observed within 2 h post injection, due to toxic shock rather than to the stress of injection regimes. Toxins from *Vibrio* sp. have been reported to cause mortalities in penaeid shrimp (Leong and Fontaine, 1979).

In order to study the efficacy of oxytetracycline against bacterial infection in shrimp, oxytetracycline-medicated feed at selected dosage was fed to healthy juvenile shrimp for 7 days before they were injected intramuscularly with a *Vibrio parahaemolyticus* suspension at a level which preliminary studies had shown would produce a 20-30% mortality rate. Histology was carried out to compare the response of pre-treated and un-treated shrimp.

Materials and Methods.

I.Pilot study

A pilot study was carried out to determine the required inoculum of the test strain which would produce a 20-30 % specific mortality rate when used by a standard defined exposure method.

Bacterial suspension

Vibrio parahaemolyticus Strain B_4, the pathogenic strain which in previous studies (Chapter III.)showed the lowest MIC and MBC-value to oxytetracycline (0.39 and 3.125 ppm., respectively) was grown on a TSA - slant for 18 hrs.After an incubation period, bacterial colonies were harvested washed and diluted in 2% sterile saline to give a series of inocula with final number of bacteria in suspension of approximately $1X10^8$,$1X10^7$,$5X10^7$ and $1X10^9$ CFU/ml.

Shrimp and environment

Healthy juvenile shrimp with an average weight of 2 g (1.7-2.5 g) and 6.5 cm. (6.2-7.3 cm.) in length were obtained from commercial culture ponds. The shrimp were stocked in 80X100X50 centimetre fibre-glass tanks containing 250 litres of 15 ppt. sea water. Sub-sand filters were used in each tank. One hundred experimental shrimp were stocked in each tank and commercial pellets containing 37 % protein (pellet No. 3, CP - Company) were fed three - times daily at approximately 6 % body weight. The amount of feed was increased or decreased daily depending upon consumption. The

acclimation period was 1-2 weeks before studies commenced. Ten percent of water was changed daily to ensure good water quality.

Challenge procedure

Three days before the experiment started, 5 experimental aquaria were cleaned and filled with 15 ppt. sea water. Shrimp in aquarium No.1 were injected intramuscularly between the 3 th and 4th abdominal segment with 0.05 ml.of 2 % sterile saline and served as control group. Shrimp in aquarium No.2 were injected at the same site as the control with 0.05 ml of a bacterial suspension containing approximately 1X10^{6} CFU/ml, and shrimp in aquarium 3,4 and 5 were injected with 0.05 ml of 1X10^{7},5X10^{7} and 1X10^{8}CFU/ml of bacterial suspension respectively. Dead shrimp were removed as soon as observed and recorded as mortality, observation periods were 7 days after injection.

I.I Results

Accumulated mortality of infected shrimp with different numbers of *Vibrio parahaemolyticus* revealed that the bacterial suspension which contained 5 x 10^{7} CFU/ml caused an appropriate level of mortality for using in a prophylactic experiment while the others showed too high and too low mortality as indicated below

Bacterial Number	Accumulated Mortality (days)							Total
	1	2	3	4	5	6	7	
control	-	-	-	-	-	-	-	-
1 x 10^8 CFU/ml	-	-	-	-	-	-	-	-
1 x 10^7 CFU/ml	1	-	-	-	-	-	-	1
5 x 10^7 CFU/ml	3	2	-	-	-	-	-	5
1 x 10^8 CFU/ml	13	2	-	-	-	-	-	15

Moribund shrimp in the 1 x 10^7 CFU/ml infected group were severely stressed, they swam erratically around the aquarium and sank to the bottom with slower movements and then stopped and died within a short period. Surviving shrimp appeared normal.

I.II Discussion

From this preliminary test,it was apparent that mortalities occurred within 48 hrs post exposure in all test groups. Almost all of the shrimp injected with the highest rate of bactena (1 x 10^8 CFU/ml) died on the first day, mostly within 6 hr of exposure. This was possibly due to toxic shock as described by Leong and Fontaine (1979) and Adams (1991). Two days post infection, the infected shrimp did not show any sign of the disease and the mortality had stopped. This may indicate that the inocula of pathogenic bacteria were being eliminated by the shrimp before they were able to multiply to provide lethal amounts of toxin. The relatively rapid clearance of the bacteria

by the defence mechanisms of shrimp has been described by Adams (1991) who demonstrated that more than 99 % of heat-killed *Vibrio alginolyticus* were cleared from the haemolymph of the injected black tiger shrimp within 4 hr.

II.Prophylactic study

Having established, in the pilot study, the level of bacterial suspension required to kill approximately 20-30 percent of exposed shrimp within 7 days, this level, $5X10^7$ CFU/ml was used for the prophylactic study. Shrimp were acclimatised as described for the pilot study above.

Shrimp were divided into 2 groups, pre-treated with antibiotic and un-treated, with 3 replicates in each. Forty shrimp were stocked in each 50x100x80 cm. fibre-glass tank with sub-sand filter. Each replicate used two aquaria. Shrimp in group 1 were given normal feed and served as the control. Medicated feed containing 5 g oxytetracycline (OTC)/kg of feed was given to the the shrimp in group 2, with feeding regimes described in chapter 3. After 7 days, experimental shrimp in both groups were challenged by intramuscular injection between the 3^{rd}-4^{th} abdominal segments with 0.05 ml of $5 X 10^7$ CFU/ml of the designated bacterial suspension. Mortalities were recorded daily until the end of the experiment. Dead shrimps were removed as soon as observed. After cessation of medicated feed, the normal pellets were being used to substitute the medicated feed in group 2, for the remainder of the experimental period.

Sampling procedure.

Three shrimp were removed from each tank for histological examination at fifteen, thirty and sixty minutes then at 3,4,6,12,24, hrs. after infection. Sampling times were continued thereafter at 2,3,4,7 days then every week until one month after injection. Sampled shrimp were fixed in Davidson's Fixative as described by Bell and Lightner (1988). The fixative was injected into the anterior and posterior hepatopancreas and also into the posterior abdomen of the living shrimp. The shrimp was then bisected at the junction of the cephalothorax and abdomen and placed in Davidson's fixative for 24-36 hrs, transferred into 50 % ethanol, and kept cool until embedded. Sampled shrimp were not included when percent mortality was calculated.

Histological procedure.

Well fixed cephalothorax and abdominal portion was prepared for embedding by following the procedure described by Bell and Lightner (1988). Appendages were removed from the cephalothorax, and the remainder was bilaterally disected. The cuticle of the left cephalothorax was removed to expose the branchiostegal region. The abdomen was cut behind the 4th segment to remove the excess tail portion and then bisected. Tissues were embedded in paraffin wax using a AO automatic tissue processor TP 8000. Processed tissues were placed in a cold mold and melted paraffin. The blocks were trimmed and cut at 5 um. thickness and sections were stained with a routine haematoxylin and eosin (H&E) or a special stain for particular purposes. The sections were examined by light - microscopy.

Results.

Some shrimp those injected with bacteria from both groups,(pre-treated and un-treated with OTC-medicated feed) were died within the first 48 hrs. No more died after that period. There was however a significant difference in losses. More shrimp died in the un-treated group (22.5%) than in the pre-treated group where the mortality rate was 15 percent. In the pre-treated group all of the dead shrimp died within the first 6 hrs. In the un-treated shrimp approximately 18 % died within the first 6 hrs. 5 % of infected shrimp continued dying at day two post injection and then the mortality stopped.

i. Histological observation

Histological examination revealed significantly different responses in the tissues of the pre-treated and un-treated shrimp, as well as in the saline injected control. These pathological changes were principally noted in and around the area of injection and are described below and summarized in table 13.

Injection site

Immediately after injection an area of muscle destruction due to traumatic injury was observed both in bacterial and saline injected shrimp (Fig.7-8) but at this stage, there was no evidence of a cellular response. By 15 minutes post injection, saline injected controls showed only slight tissue destruction without any cellular response (Fig.9). However, in both pre-treated and un-treated groups, extensive destruction of muscle fibres had occurred

Table 13 Summary of the pathological responses of experimental shrimp at the site of injection

Time post injection	Saline injected shrimp	Bacterial injected shrimp	
		pre-treated	un-treated
Immediately after injection	destruction of muscle due to traumatic injury in all groups without any evidence of cellular response (Fig.7-8)		
15 mins.	slight muscle destruction, no cellular response (Fig.9)	haemolymph accumulation and haemocytic infiltration in the area (Fig.10)	
1 hr.	limited haemocytic infiltration (Fig.11)	abundant haemocytic infiltration with coagulated haemolymph (Fig.12)	
3 hrs.	limited haemocytic infiltration (Fig.13)	increased haemocytic migration to the area (Fig.14) with some nodule formation (Fig.15-16)	
6 hrs.	small area of muscle necrosis (Fig.17)	less haemocytic response and relatively smaller disrupted area was noted in pre-treated group (Fig.19) when compared to the un-treated group (Fig.18)	
24 hrs.	more haemocytic response than at 6 hrs.(Fig.21)	small nodule formation (Fig.24)	large nodule with multiple layers (Fig.23)
2 days.	slightly increased response (Fig.27)	small melanized nodules (Fig.25)	large melanized nodule (Fig.26)
7 days.	semi-organized regenerating tissues (Fig.33)	small melanized nodules	large melanized nodule
14 days.	increasing tissue regeneration. (Fig.35)	small melanized nodules in small disrupted area	large melanized nodule with much larger destroyed area (Fig.38) (Fig.37)
21 days.	healing nearly completed (Fig.39)	small disrupted area with healing (Fig.41)	large disrupted area with various size of melanized nodules (Fig.42)
28 days.	healing completed (Fig.43)	healing nearly completed (Fig.45)	various sizes of melanized nodule were still noted in healing area (Fig.46)
35 days.	healing completed	healing nearly completed (Fig.47)	melanized scar tissue was noted (Fig.48)

throughout the bacterial injection area. A cellular inflammatory response was initiated at the damaged site, with migration of haemocytes from the surrounding area into the vicinity of the injection, associated with haemolymph infiltration. Many of the muscle fibres were disrupted, with infiltration of haemocytes. Pyknotic muscle fibre nuclei were present alongside the myonecrotic fragments (Fig.9). It was not possible to identify clusters or bacterial colony at the injected sites.

At 30 minutes post injection, the area of muscle destruction in both groups of bacterially injected shrimp had expanded, with an apparent increase in the number of phagocytic haemocytes. Migration of the inflammatory cells to the destroyed areas was especially evident during this time. The saline-injected shrimp still did not show any inflammatory response. At 45 minutes post injection, migration of some haemocytes was observed in the control group, mainly along the muscle septum, associated with haemolymph accumulation in the area. After the same period, a much greater haemocytic response was observed in the bacteria injected shrimp. The area of muscle necrosis spread to the adjacent tissues. Haemocytic aggregation around the necrotic centre or bacterial colony was present mainly along the sloughing myoseptum.

One hour after injection, extensive muscle necrosis was readily apparent. Haemocytic aggregation surrounding the disrupted area was observed with a noticeable increase in the number of granulocytes. Pyknotic muscle cell nuclei were present in the damaged area. Myophagia of tissue debris by phagocytic haemocytes was also noted in all three groups of shrimp

(Fig.11-12).

At 2 and 3 hours post injection, the saline injected control shrimp showed considerably less haemocytic infiltration and destroyed tissue and showed no indication of encapsulation (Fig.13). Migration of the haemocytes toward the injection site was still continuing, particularly in the haemal space adjacent to the necrotic area of bacterial injected shrimp (Fig.14). Damaged muscle fibres, haemolymph accumulation and nodule formation were found throughout the injection site (Fig.15). In comparison with shrimp which were pretreated with OTC-medicated feed, the untreated, bacterially injected, shrimp displayed a more severe diffuse area of myofibrillar necrosis. Karyorrhectic and karyolytic nuclei from the dead haemocytes or from necrotic muscle tissue, were observed amongst the tissue debris in both of the groups injected with bacteria. The nodule formation was observed during this stage, indicated by aggregation of haemocytes surrounding the damaged tissues and were themselves undergoing slow necrosis. This small nodule formation was more obvious at 3 hrs post injection (Fig.16).

At 4 hours post injection, migration of haemocytes was still occurring in the shrimp injected with the bacteria. Necrotic muscle fibres exhibited more prominent nuclei. Some areas showed lytic tissue fibres and cellular debris of destroyed haemocytes. Aggregation of haemocytes in the haemal space was noted, with some melanized nodules, at 6 hours after injection. Again, saline injection shrimp was showed relatively small area of muscle destruction with some pyknotic muscle fibre nuclei (Fig.17) whereas a marked increase in haemocytic infiltration between the muscle fibres was still

observed in bacterial injected un-treated shrimp (Fig.18). In comparison, a relatively less haemocytic response and smaller distroyed area was noted in pre-treated shrimp (Fig.19). Large numbers of dead haemocytes which exhibited pyknotic nuclei were identified at the disrupted area where some encapsulations of the tissue debris were observed (Fig.20). Possible giant cell formation was also observed in the haemal space adjacent to the necrotic site.

At 12 hours post injection, cellular response to the injury was progressively taking place. Migration of fibroblast-like cells to the encapsulated necrotic site could still be observed and the subsequent nodule formation was also particularly evident at this time. There was encapsulation of the necrotic myofibre debris which was scattered in many areas. The focus of myo-necrosis, characterized by the change of the staining characteristics of the sacroplasm, was significantly infiltrated by darkly nucleated haemocytes, both within the lesion itself and peripherally.

At 24 hours post injection, control shrimp which were injected with saline, still showed much smaller destroyed area with less cellular responses (Fig.21) when compared to the shrimp injected with bacteria (Fig.22). The nodule formation by the infiltrating fibroblasts had progressed further. Some nodules became melanized. This was thought to be the result of changes associated with the degradation of the haemocytic enzyme system after cell death. Necrotic haemocytes were widely present amongst the damaged tissues. Twenty four hours after injection,an increased number of small nodules had developed and was readily seen within the destroyed area, in both groups of bacteria injected of shrimp. Comparison between the haemocytic response of

the OTC-treated and the un-treated bacterially injected groups, revealed smaller but more widely dispersed nodules in the drug-treated shrimp (Fig.23-24).

By day two post injection, the scattered nodules were still noted and these had virtually all become melanized. The replacement of necrotic muscle tissue by partial-organization of the modified haemocytes and muscle fibre was observed. A dark cell resembling a giant cell of the epithelial lining was also present. The area of reaction was still significantly larger and more active in untreated shrimp (Fig.25-26). In contrast, in saline control shrimp a much less significant haemocytic response and smaller tissue destruction was noted (Fig.27-28).

At 3 days, small melanized nodule formation was widely present throughout the necrotic area of both bacterially exposed groups (Fig.29). The melanized nodules were also noted in the haemal sinus. Proliferation of numerous fibroblast-like cells was still acting as a limiting boundary to the necrotic areas. Sarcolytic fibres present at the injection site and subsequent nodule formation were the principle observations at 4 days post injection (Fig.30). Phagocytosis of damaged muscles continued with some organization of the regenerated tissues.

In saline-injected controls, at 5 days post injection, un - organized fibrous connective tissue with prominent active nuclei was present within the traumatically damaged area (Fig.31). Myo-necrotic cells however were rarely observed. Melanized nodules around necrotic muscle fibre and surrounded by multilayers of modified- haemocytes were still seen in pre-treated group, but in smaller numbers than in the un-treated groups. The new muscle fibres had

replaced the necrotic tissues (Fig.32).

Examination of control shrimp at 7 days, revealed still further replacement of demaged tissue at the injection site with new muscle fibres (Fig.33). Very little haemocytic response was observed in the area of healing, whereas in both groups of bacteria injected shrimp, many more haemocytes were still present surrounding the necrotic foci. Melanization in larger necrotic centres was noted (Fig.34). From day 5-7, the extensive replacement of necrotic muscle at the injection site by new fibrous connective tissue was very conspicious but sarcolysis of the muscle fibres was now rarely observed. Melanized nodules were still present in the inflammatory site and haemal space. Haemocytes were markedly decreased in numbers, whereas there was an increasing number of fibroblasts encapsulating the foci of necrotic tissue debris.

At 8 days post injection, melanization of the encapsulated areas was more prominent. Tissue surrounding the encapsulated areas became virtually normal. Regeneration of muscle fibres had taken place to largely replace much of the connective tissue.

From day 9-10, melanized nodules were more condensed with a large deposit of dense haemocytic nuclei. Markedly less cellular response was noted at the peripheral area.

At 14 days post injection, only a small area of tissue disruption could be noted in control shrimp, primarily defined by the presence of a mild haemocytic response and re-arrangement of regenerating muscle (Fig.35). By this time no melanized nodules were to be observed in control shrimp whereas

very dense multinucleated haemocytic infiltration in melanized nodules was still present amongst the more normal surrounding tissue of the shrimp injected with the bacteria (Fig.36). However, smaller melanized nodule was noted in pre-treated shrimp (Fig.37) when compared to un-treated shrimp (Fig.38).

At 18 days post injection, melanized nodules were present in bacterially exposed shrimp with some haemocytic infiltration. Fibroblasts were markedly reduced. Regeneration of new muscle bundles was taking place.

In saline-injected controls at 21 days post injection, the site of disruption was apparently healed with very little cellular response (Fig.39). New muscle fibres with prominent nuclei were noted only at the centre of damaged tissue. Melanized nodules occurred extensively in both groups of shrimp that had been injected with the bacteria but there was a slight difference between the pre- treated and un-treated shrimp in term of the degree of destruction,and smaller and more diffuse melanized nodules were present in the OTC pre-treated shrimp (Fig.40-42).

By day 28, it had become very difficult to locate the affected area in the saline-injected shrimp (Fig.43) while various sizes of melanized nodules were still present in untreated shrimp (Fig.44). However, healing of the disrupted area in the pre-treated shrimp was very advanced. The peripheral area had become normal with virtually no extraneous cellular response. Small melanized nodules were only present at the centre (Fig.45). Multinucleated haemocytic infiltration was still widely spread amongst the disrupted area of tissue in untreated shrimp (Fig.46).

By day 35, healing of the disrupted area in pre-treated shrimp was nearly completed. Only a very small area of obvious regenerating tissue was present (Fig.47). In untreated shrimp, well organised fibroblasts were observed surrounding the large areas of melanized nodules, which appeared to form an obvious scar (Fig.48).

Other tissues

Heart

At 15 minutes post injection, haemocytic aggregation in the heart was observed (Fig.49-50) and increased markedly from this time. Haemocytic aggregation in the haemocoel of infected shrimp was first observed at 3 hours post infection.Shrinkage of heart muscle with the presence of small foci giving the appearence of small thrombi was observed at 24 hours post injection (Fig.51). Further haemocytic aggregation was present between the cardiac muscle fibres as well as in the pericardial chamber (Fig.52). Haemocytic nodules were also observed in the haemal sinus. Small haemocytic nodules were more abundant throughout the heart at 2 days post injection (Fig.53-54), and melanization took place by day 5 post injection. From day 6, melanized nodules were noted in the myocardium.These small nodules were still present in the heart upto 14 days post injection, but no cellular changes appeared to take place.

Hepatopancreas

Hepatopancreatocytes were shrunken and more prominent at 30 mins post injection. By 1 hr, a reaction in the haemolymph sinus between the hepatopancreatic tubules was observed,associated with inflammation and haemolymph congestion in the hepatopancreas. Tubular degeneration occurred 24 hours post injection (Fig.55). Pyknotic nuclei were markedly increased by one day post injection. By day 2, melanized nodule formation was taking place in the hepatopancreas (Fig.56).

By 7 days post injection, in some shrimp,the hepatopancreas still showed tubular degeneration and the presence of haemocytes infiltrating between the tubules. These changes were however also to be seen in saline control shrimp and therefore assumed to be unrelated to the injection of the bacteria.

Gill

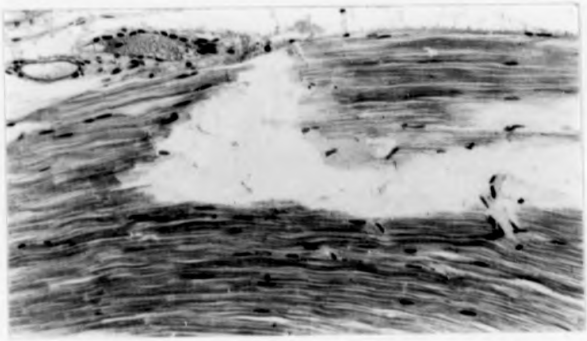
The presence of podocytes was observed in the afferent blood vessel associated with some haemocytic infiltration in the secondary lamellae at 2 hours post injection. No significant cellular response in the gills was observed throughout the study period,except for the presence of podocytes.

Lymphoid organ

At 15 minutes post injection, hydropic degeneration of lymphoid tissue was observed, associated with some haemocytic aggregation in the haemal space (Fig.57). Granulocytes were increasing by 30 minutes. Necrosis

of the lymphoid tissues was identified by the presence of pyknotic nuclei (Fig.58). Nodule formations occurred 6 hours after injection. This change appeared up to 8 days post injection and by that time, the nodules had become melanized. No major difference was noted beyond 8 days.

Fig.7-8.Traumatic destruction of muscle fibres at the injected site was observed immediately after injection, in both saline (Fig.7) and bacterial injected shrimp (Fig.8) (H & E) (X 264)



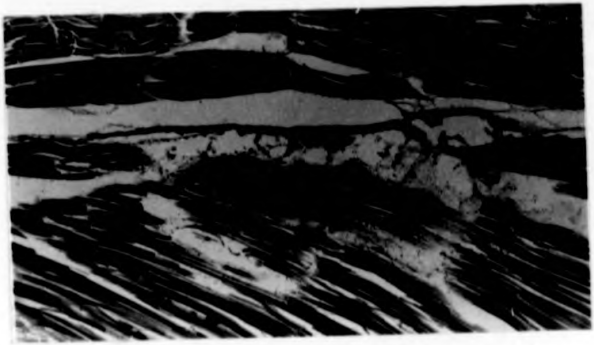
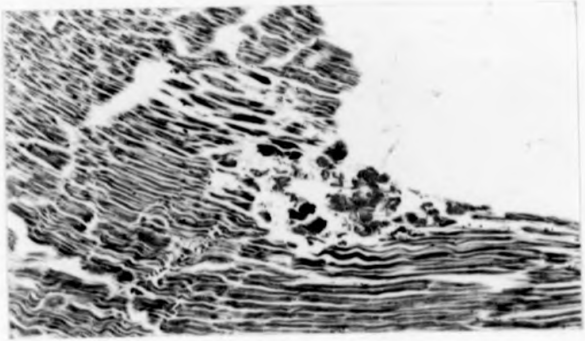


Fig.9 By 15 minutes after saline injection only small area was destroyed (H & E) (X 132)

Fig.10 Haemocytic infiltration (HI) was observed at the bacterial injection area at 15 minutes post injection (H & E) (X 132)



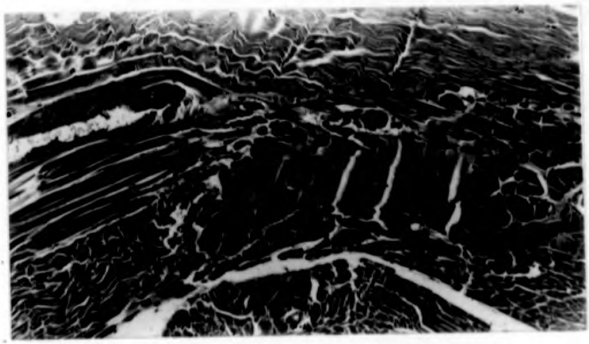


Fig.11-12 By 1 hour after saline injection a few haemocytes (H) presented at the injection site (Fig.11) whereas much more haemocytic response was observed along the necrotic muscle fibre in bacterial injected shrimp (Fig.12) (H & E) (X 264)

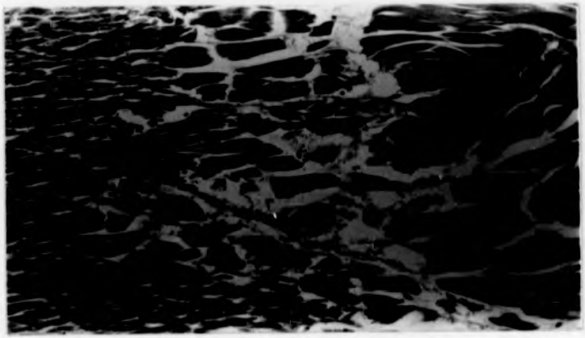


Fig.13 By 3 hours post injection more extensive destruction of muscle fibres was observed in saline-injection control associated with more marked haemocytic response.
(H & E) (X 132)

Fig.14 Massive haemocytes (H) migrated to the disrupted area in bacterial injected shrimp at 3 hours post injection
(H & E) (X 132)

Fig.15 Encapsulation of tissue debris or dead haemocytes taking place at the affected area; accumulation of haemolymph (HL) was still observed at 3 hours after injection (H & E)(X 264)

Fig.16 Elongation of modified haemocytes (MH) surrounding the damaged tissues at 3 hours after bacterial injection (H & E) (X 528)

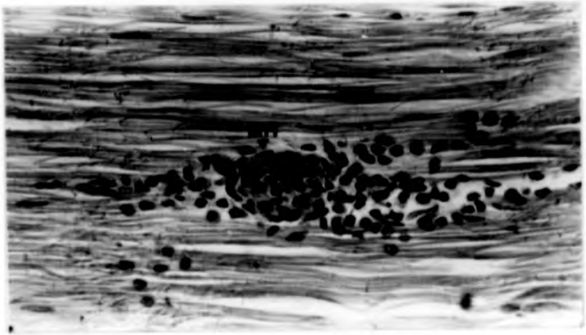


Fig.17 Pyknotic muscle fibre nuclei (PN) were extensively presented among the necrotic muscle fibres in saline injected shrimp at 6 hours post injection (H & E) (X 264)

Fig.18 Haemocytic infiltration (HI) between muscle fibres adjacent to the disrupted area was still observed in bacterial injected, un-treated shrimp at 6 hours post injection. (H & E) (X 264)

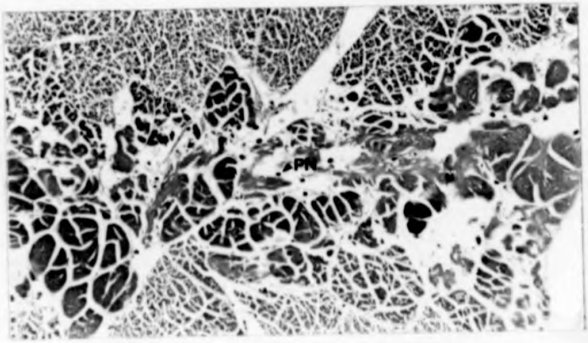


Fig.19 A limited haemocytic response and relatively small necrotic area was observed in pre-treated shrimp when compared to the un-treated shrimp (Fig.18) at 6 hours post injection (H & E) (X 264)

Fig.20 Encapsulation of tissue debris between muscle bundles of bacterial injection shrimp at 6 hours. (H & E) (X 528)

P21 - 54A
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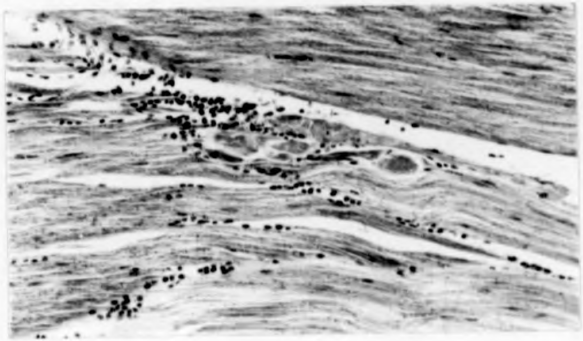


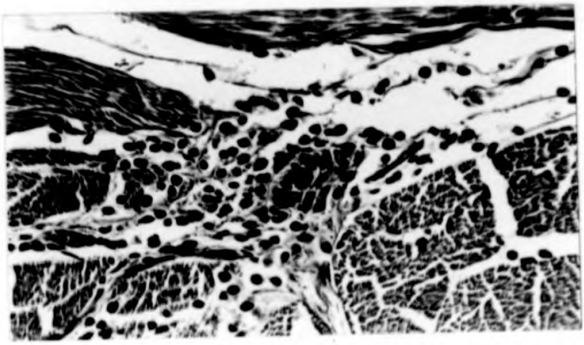
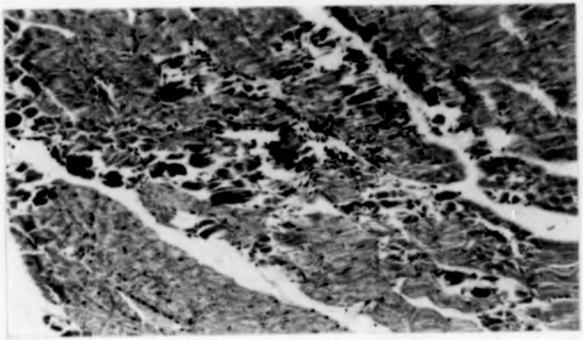


Fig.21-22 Again, at 24 hours post injection, more cellular response was observed in shrimp with bacterial injection. (H & E) (X 132)



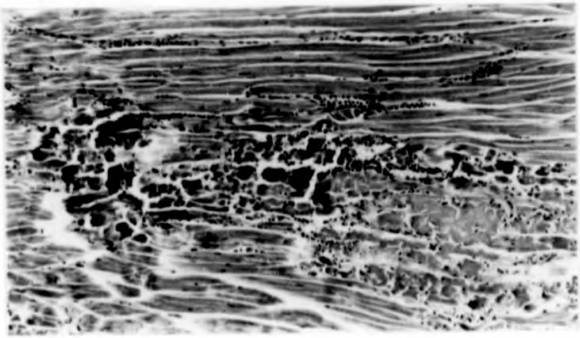
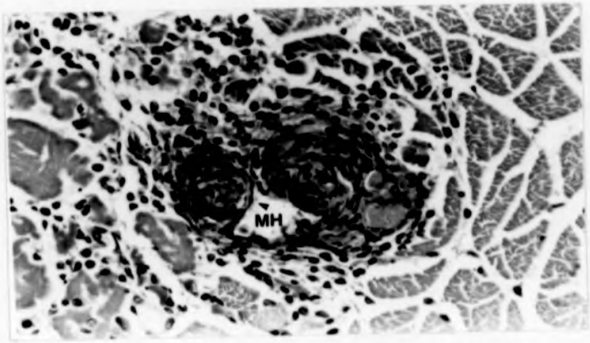


Fig.23-24 Encapsulated nodule in un-treated shrimp at 24 hours after bacterial injection (Fig.23) showed relatively larger and more modified haemocytes (MH) presented at site of injection than in pre-treated shrimp (Fig.24) (H & E) (X 528)


MH

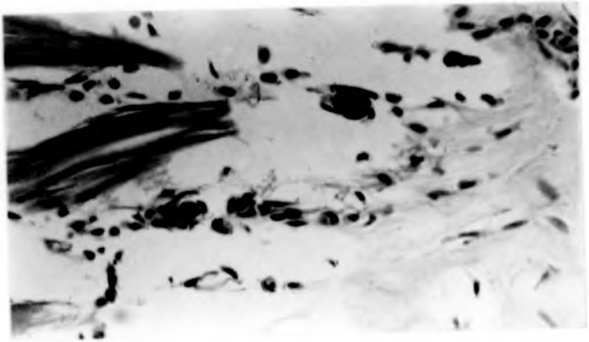
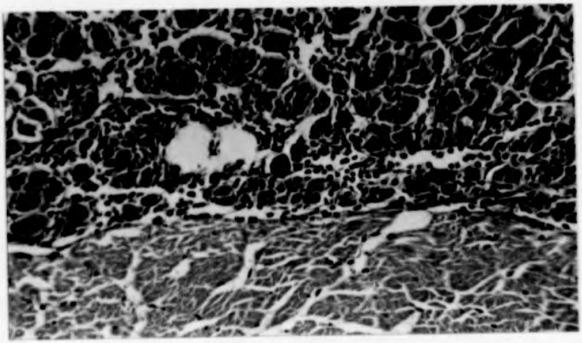


Fig.25 By 2 days post injection, replacement of the semi-organization of the modified-haemocytes with the melanized nodules (MN) was seen in pre-treated shrimp (H & E) (X 264)

Fig.26 In un-treated shrimp injected with bacteria,a larger area of encapsulation by the modified haemocytes was noted by 2 days post injection (H & E) (X 264)



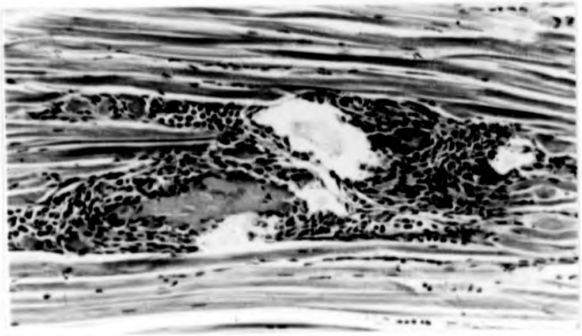
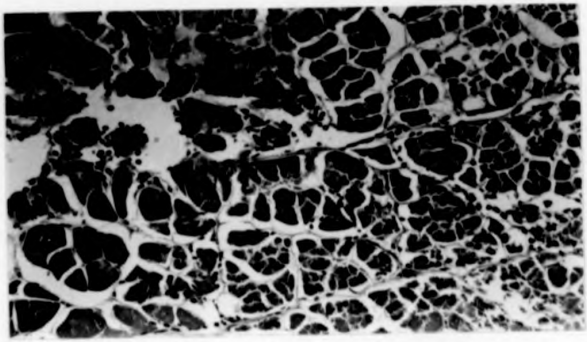


Fig.27-28 By 2 days after injection, very few haemocytes were observed in saline injected control (Fig.27) ; a greater response and a larger area of tissue destruction was still to be observed in the necrotic tissue of the bacterially injected shrimp (Fig.28) (H & E) (X 264)



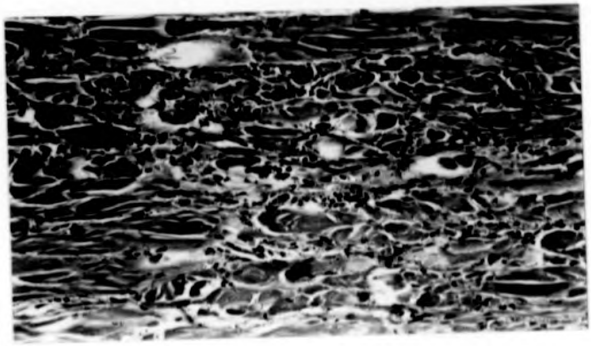
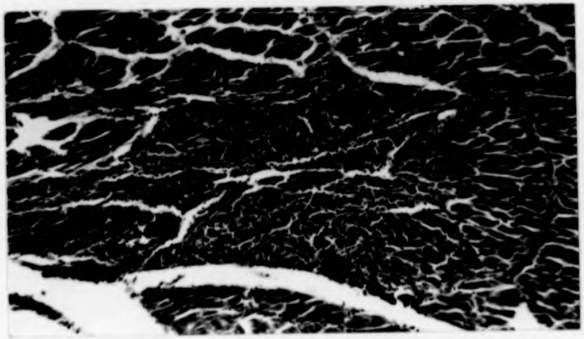


Fig.29-30 In un-treated shrimp injected with bacteria, melanized nodules with proliferation of fibroblast-like cell (F) was observed during day 3 (Fig.29) (X 132) and day 4 (Fig.30) (X 264)



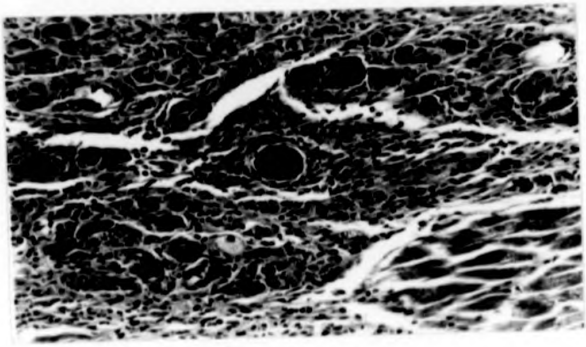
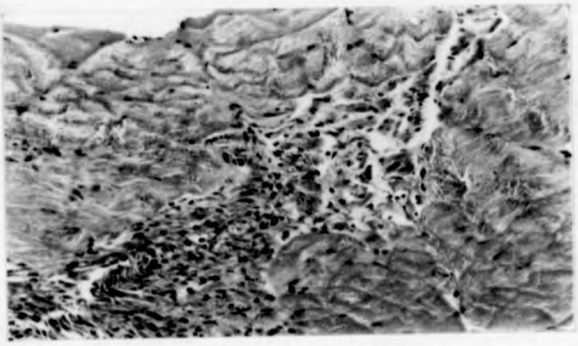


Fig.31 By days 5 after injection, replacement of the damaged area by un-organized fibrous connective tissue was noted in saline injection shrimp. (H & E) (X 264)

Fig.32 Haemocytic infiltration at the damage tissues was still present in bacterial injected shrimp at 5 days. (H & E) (X 264)



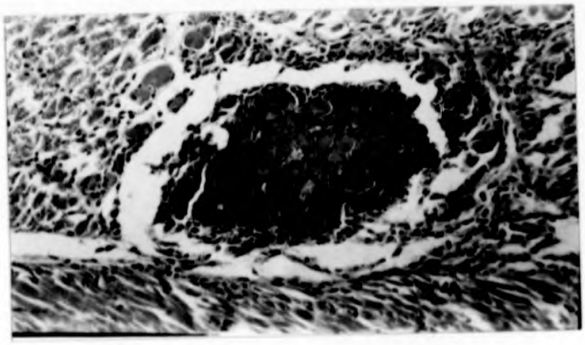
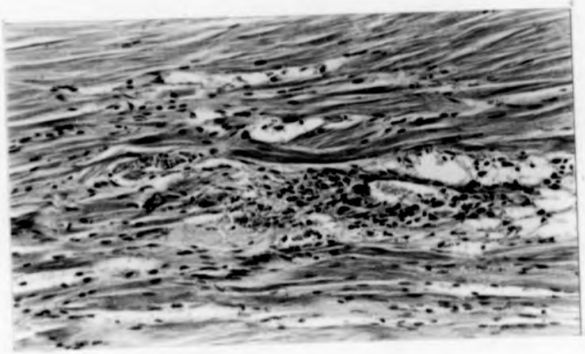


Fig.33 One week post injection, more organized connective tissue and regenerated tissue to replace the necrotic area was observed in saline injection shrimp. (H & E)(X 264)

Fig.34 Various sizes of melanized nodules with semi-organized regenerated tissue was observed in bacterial injected shrimp at 7 days. (H & E) (X 264)



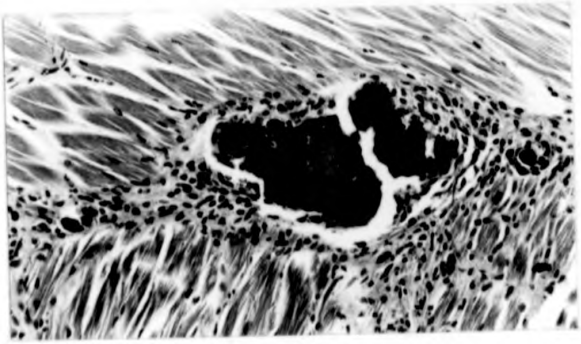


Fig.35-36 Two weeks after injection, replacement of necrotic tissue by the fibrous connective tissue and modified haemocytes was nearly complete in saline injected controls (Fig.35) whereas some melanized nodules and un-organized fibrous connective tissue were still present in bacterial injected shrimp (Fig.36) (H & E) (X 132)

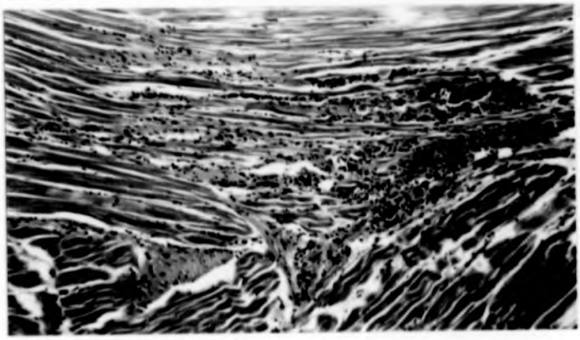
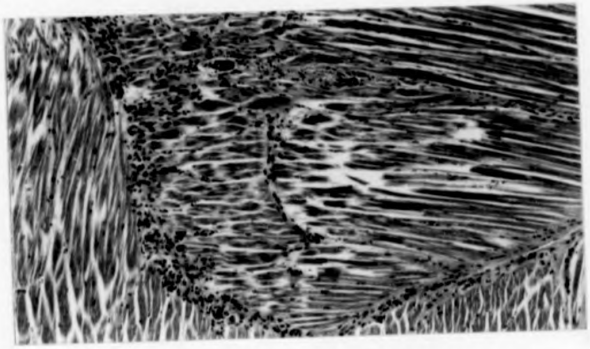


Fig.37-38 At 14 days post injection much smaller nodule formation (arrow) was noted in pre-treated shrimp injected with bacteria (Fig.37) whereas in un-treated group very prominent haemocytic nuclei presented within the large nodules (arrow) (Fig.38) (H & E) (X 132)



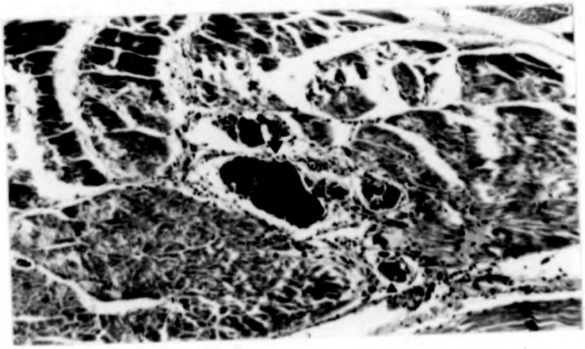
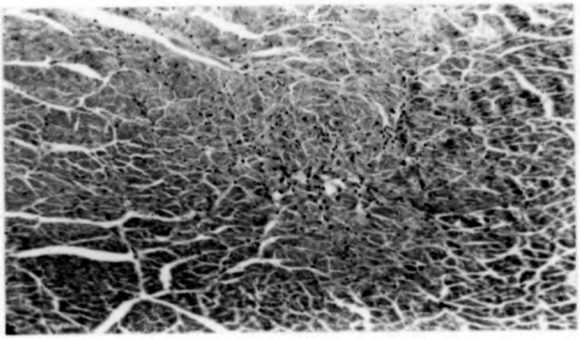


Fig.39 By week 3,the necrotic area in saline injected control shrimp became virtually normal (H & E) (X 132)

Fig.40 Melanized nodules (arrow) with multiple layers formed from modified haemocytes still noted in necrotic area of un-treated shrimp injected with bacteria at 21 days post injection (H & E) (X 132)



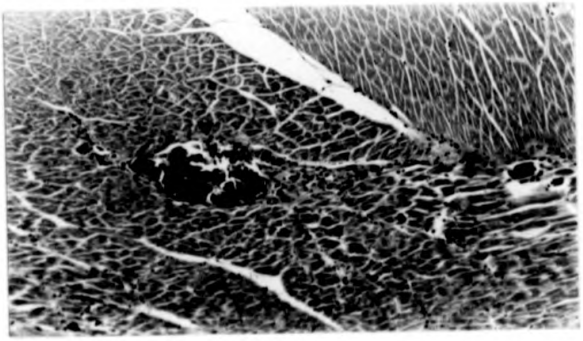


Fig.41 By days 21 after bacterial injection, pre-treated shrimp showed small and scattered nodules among the necrotic area (arrow), the centre of the necrotic tissue being replaced by the fibrous connective tissue and regenerated tissue (H & E) (X 132)

Fig.42 Very small nodule (arrow) with some haemocytes present amongst the regenerated tissue in pre-treated shrimp injected with the bacteria at 21 days post injection. (H & E)(X 528)

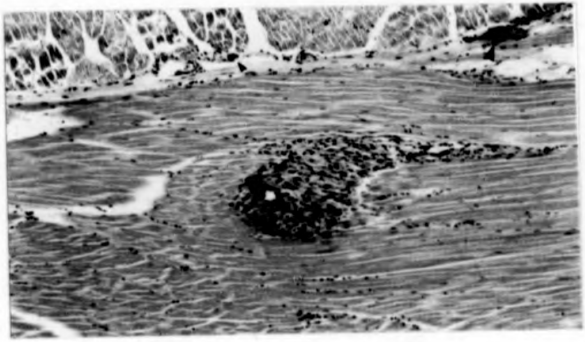
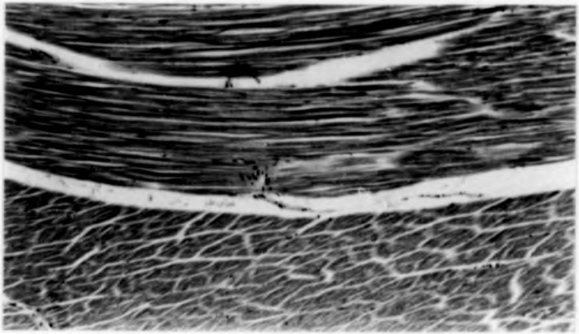


Fig.43 Healing of the necrotic area was completed in saline injected shrimp at 28 days post injection (H & E) (X 132)

Fig.44 By day 28 after bacterial injection, some melanized nodules of various sizes (arrow) still present amongst the necrotic area in un-treated shrimp (H & E) (X 132)



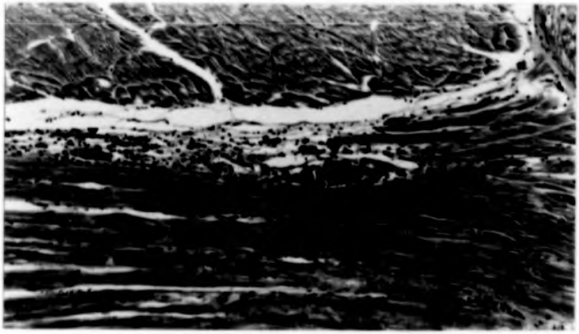
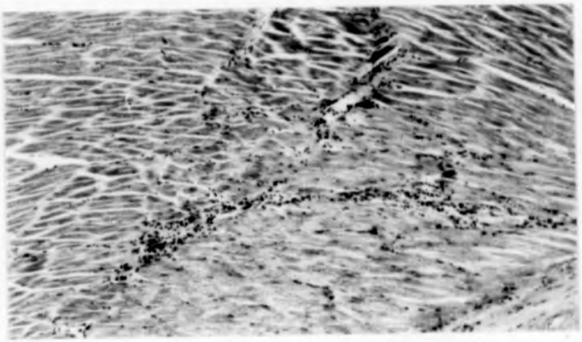


Fig.45-46 In comparison at 28 days post bacterial injection, a few very small nodules with some haemocytes were noted in pre-treated shrimp (Fig.45) whereas larger and more numerous nodules presented in un-treated shrimp (Fig.46) (H & E) (X 132, X 264)



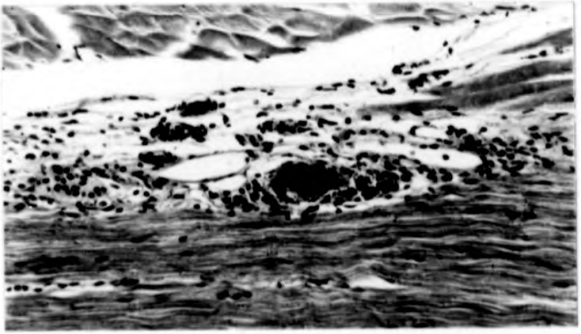
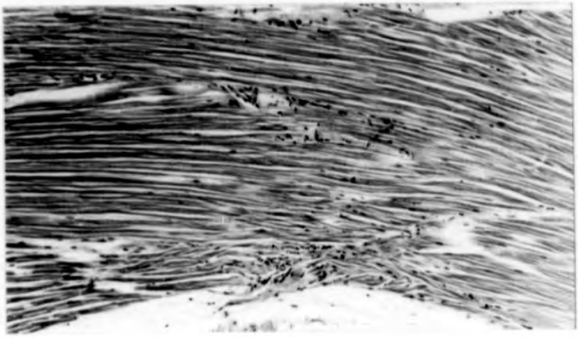


Fig.47-48 By days 35 after bacterial injection, healing of the necrotic area in pre-treated shrimp was nearly completed (Fig.47), a large area of melanized nodule was still present and seemed to be the permanent scar at the injection site of the un-treated shrimp (Fig.48) (H & E)(X 132) (X 52)



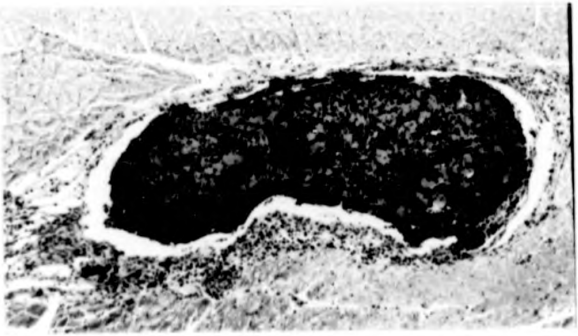
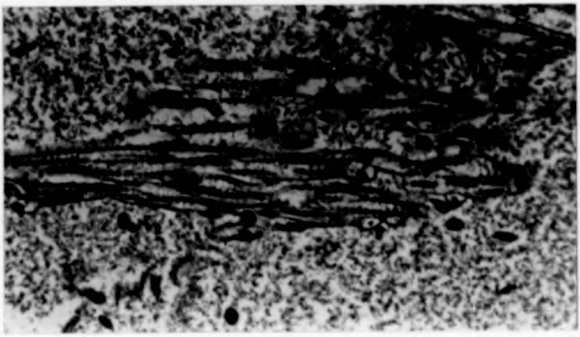


Fig.49 Normal heart tissue in saline-injected control, 15 minutes after injection. (H & E) (X 528)

Fig.50 Aggregation of haemocytes (H) in heart of bacterial injected shrimp at 15 minutes post injection (H & E) (X 528)



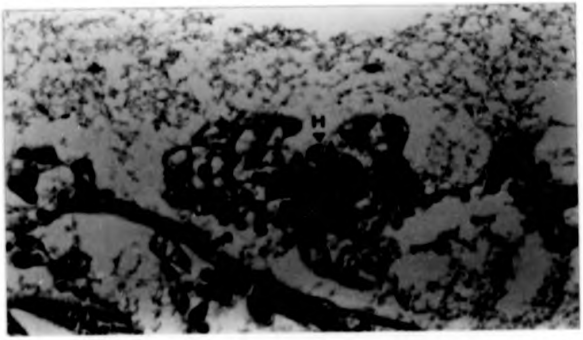

H

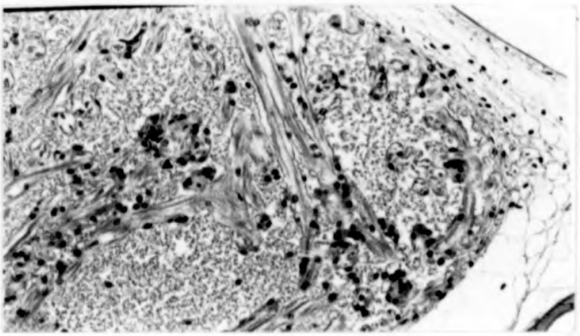
Fig.51 More haemocytes were accumulated in heart of bacterial injected shrimp at 24 hours post injection (H & E)(X 528)

Fig.52 Encapsulation (E) of debris in haemolymph sinus and between the cardiac muscle occured extensively in bacterial injected shrimp, at 24 hours post injection (H & E) (X 264)


E

Fig.53 By day 2 post bacterial injection, small nodules (arrow) forming from modified haemocytes were developing throughout the heart. (H & E) (X 264)

Fig.54 Modified haemocytes lining to form a nodule (arrow) in the heart of bacterial injected shrimp up at 2 days post injection (H & E) (X 528)



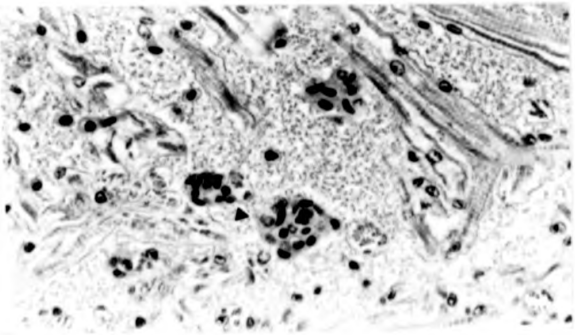
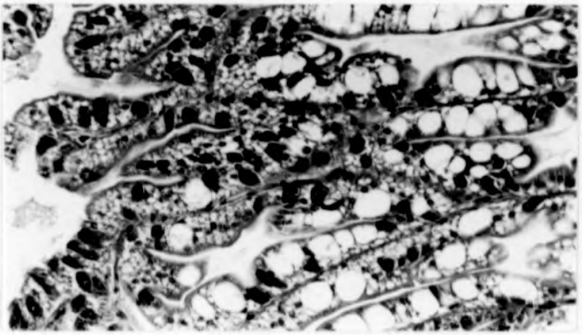


Fig.55 By hours 24 post bacterial injection, Some hepato-pancreatic cells underwent necrosis with slightly swelling with more prominent nuclei (H & E) (X 132)

Fig.56 By days 2 after bacterial injection, aggregation of the haemocytes (H) between necrotic tubules (arrow), was observed forming the nodule. (H & E) (X 528)



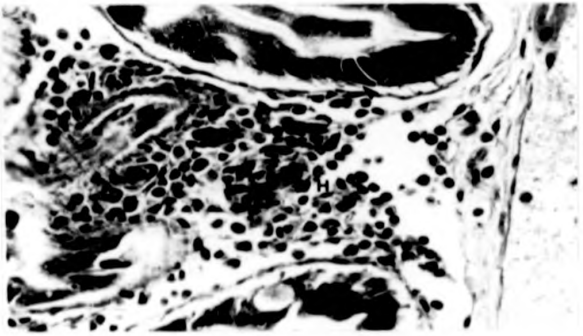
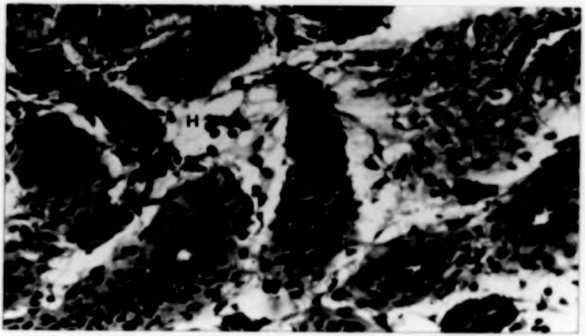
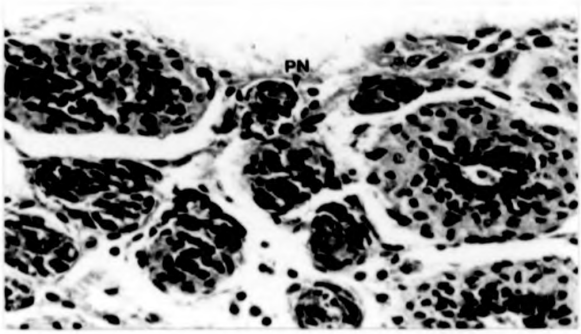


Fig.57 Some haemocytes (H) accumulated in the lymphoid organ at 15 minutes post injected with the bacteria (H & E) (X 528)

Fig.58 A lymphoid cell in Oka's organ undergoing necrosis, indicated by pyknotic nuclei (PN) ; 24 hours post injected with bacteria. (H & E) (X 528)


H


PN

Discussion

According to the preliminary studies described previously, in pilot study, *Vibrio parahaemolyticus* was considered to be highly pathogenic to shrimp, killing significant numbers of them within 6 hrs post injection with approximately 2.5 x 10^6 cells/shrimp. The comparative histological examination of shrimp injected with the bacteria after pre-treatment with OTC and un-treated, manifested a number of points of relevance to an understanding of the cellular inflammatory response and treatment of the black tiger shrimp (*Penaeus monodon*) associated with this opportunistic pathogenic bacterial species.

In contrast to that of vertebrates, the shrimp internal defence system against invading materials does not involve a range of various types of white blood cell each with its own specific role, but rather involves the haemocyte, a phagocytic cell which contributes to both cellular and humoral inflammatory responses. The mechanism, in contrast to the higher animals, is highly non-specific (Cornick and Stewart, 1968), which has again been shown in the present studies. Two haemocyte types have been identified, the hyaline cell and the semi-granulocyte or granulocyte which correspond to the description of these two cells given by Fontaine and Lightner (1973) in the review of inflammatory response to injury in penaeid shrimp and in lobster (Paterson and Stewart, 1974).

As early as 15 minute post-injection, in this study the accumulation of the haemolymph was observed at the injection site of the bacterial injected shrimp. The degree of muscle destruction was more severe in the shrimp injected with the bacteria than in saline injected controls. The cellular response manifested

by the accumulation of haemocytes was also present at first only in bacterially injected shrimp. These responses took place around an extracellular fibrinous clot which played an important role in limiting the movement of the injected bacteria and rendering them more susceptible to phagocytosis by migrated haemocytes from the surrounding areas (Sindermann, 1971). No evidence of cellular response in saline-injected controls occurred until up to 45 minutes post injection, indicating a much slower response.

In both pre-treated and un-treated shrimp injected with bactena, it was hard to identify clusters or even colonies of bacteria post injection, due to the excellent elimination by the haemocytes. This clearance mechanism, described as the most important crustacean response in disease resistance (Schapiro, 1975), comprises haemocytic migration, aggregation and subsequent encapsulation with nodule formation throughout the body, especially localized in the gills, heart and hepatopancreas. The mechanism plays a vital role in clearing foreign material such as bacteria and fungi from the circulation and tissue (Johnson, 1980; Smith and Ratcliffe, 1976; 1980a; b). Smith and Ratcliffe (1980a) demonstrated that more than 70 % and 90 % of the injected bacteria was cleared from the haemolymph of the crab (*Carcinus maenas*) within the first 5 minutes and first hour post-injection respectively. And this extremely rapid clearance of the bacteria was recently confirmed, in black tiger shrimp injected with heat-killed *Vibrio alginolyticus* by Adams (1991). This bacteria was intramuscularly injected to the shrimp and more than 99 % of the labelled bactena were cleared from the haemolymph within 4 hrs.

Granule containing haemocytes migrated to the damaged area in larger

numbers than the hyalocytes which indicated that the granulocytes gave a more specific response to the bacterial injection. The migrating granulocytes came into close contact with the necrotic tissue and the antigen and then most became necrotic due to autolysis. Released products from degranulation of the granulocyte then promoted the inflammatory mechanism both cellular and humoral. The activation is rapidly and the active proPO proteins achieved by this activation thus generated stimulate further degranulation of proPO component from the adjacent granular cells. Haemocytes adherence to the foreign material then commences and continue to form the capsules of affected site (Soderhall and Smith, 1983; Smith and Soderhall, 1986). By 24 hours post injection with the bacteria it appeared that some unknown humoral substance such as has been reported in shrimp by Adams (1991) was released and played a role in controlling the establishing or multiplying of the injected bacteria in shrimp. These substances were known as bactericidins, lectins and haemagglutinins. The results from these released substances have been observed as the necrotic cells with prominent nuclei as well as karyorhectic and karyolytic debris amongst the disrupted areas.

Encapsulation of the bacteria and organic debris which could not be phagocytosed was the subsequent process observed at two hour post-injection. Many haemocytes migrated to surround the necrotic foci and elongated themselved along the center of the encapsulated area in order to prevent the spread of infectious agents and enhance the phagocytosis which was previously described by Smith and Ratcliffe (1980b). Subsequent melanization of the encapsulating nodule was soon initiated. This process has been found

to be activated by gram-negative bacterial cell walls which the products from the process can also initiated the prophenol oxidase activating system (proPO)(Soderhall and Smith, 1986a, b). The melanized nodules were still observed amongst the injected area up until 21 days in pre-treated shrimp where as the larger nodules in un-treated shrimp was stable and eventually form a permanent scar with well organized fibroblast and collagen-like fibres as previously described by Fontaine and Lightner, 1973; 1975; Fontaine and Dyjax, 1973. This black permanent scar had also been reported as "Black Splinter" in shrimp diseases named "Black Splinter Disease" in black tiger shrimp cultured in Thailand during low salinity season in 1988 (Limsuwan, 1988).

No evidence of encapsulation and melanization in the saline injected controls was observed, as the proPO response was not stimulated. The composition of the injected saline is an agent not normally encountered by internal defence mechanisms of shrimp, therefore it was not recognised and thus did not initiate the proPO cascade.

Chapter VI. General Discussion

The studies which have been described in the previous chapters of this thesis were established with the intention of obtaining at least some understanding of the mechanism and control of vibriosis in commercial cultured shrimp, where that condition is currently the most significant cause of losses. From the comparative studies of the pathogenic bacteria, isolated from diseased shrimp specimens in two different culturing areas, viz Samutsakhon and Chanthaburi it was apparent that the most prevalent species of *Vibrio* bacteria in both sites was *Vibrio parahaemolyticus* whereas *V. vulnificus*, which had been reported from these two areas as the cause of a well recognised and frequent condition known as "Black Splinter Disease" was rarely isolated. This unexpected finding may have been due to the particular period of time and salinity of this study, where the salinity ranged between 14-32 ppt. Reports suggest that black splinter disease usually breaks out when the salinity is below 10 ppt for long periods (Ruangpan and Sae-Ui, 1988).

Most of the moribund shrimp in the survey showed multiple infections, with at least two different species of bacteria being isolated. This tends to support the view that the isolated bacteria were, in the majority of cases, secondary infections which infected the host after the host had been stressed from other disease agents or environmental stressors and was thus more susceptible to the infection. The results obtained from this survey coincided with those report on the characteristics of vibriosis in penaeid shrimp described by Lightner (1988) where again *Vibrio* sp. were consistently involved in shrimp disease but usually played a role as the secondary invader.

A wider range of bacterial strains were isolated from affected shrimp in

Samutsakhon area, where shrimp culture had first been established, than from Chanthaburi. The level of losses in the stock was also higher in Samutsakhon area probably, because of unsuccessful control by antibiotic treatment or any management measures.

Vibrio alginolyticus, which was a very common pathogen involved in vibriosis in marine shrimp in the studies of Lightner and Lewis (1975) and Lightner (1985; 1988) was also isolated from moribund shrimp in this study and seemed to occur in higher salinity. This bacteria was isolated more frequently in Chanthaburi where the salinity was above 20 ppt.

Ruangpan and Kitao (1991) reported on the isolation of the *Vibrio* bacteria from diseased shrimp in nine different areas in Thailand during the period February 1988 to January 1990. As in the present study, they also concluded that *V. parahaemolyticus* was the most common opportunistic pathogen of shrimp. It represented about 47% of their total isolates. This was also reported by Luangtongkum *et al* (1983) who indicated that *V. parahemolyticus* was the dominant bacterial species isolated from a total of 52 samples of fish, shrimp, squid and blue crabs.

For the study of therapeutic control of the pathogenic bacteria which cause disease in shrimp, oxytetracycline (OTC) was chosen because of its regular use in the field. Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of OTC against the isolated bacteria were determined and these two values were compared between the two different sources of the pathogen.

From the studies, it was evident that both MIC- and MBC-values were

very wide ranging and were both bacterial species and source-dependent. Among the strains, the MIC-value ranged from 0.39 - 50 ppm. and MBC-value was between 1.56->100 ppm. This result confirmed the reports of Ruangpan and Kitao (1992) who provided similar findings for the MIC-value of OTC against 205 isolated *Vibrio* sp. from diseased shrimp in different areas of shrimp culture in Thailand. Their value ranged between 0.2 - 100 ppm. Generally, *Vibrio* strains isolated from Samutsakhon seemed to be more resistant to OTC than the strains isolated from Chanthaburi, which can again be related to the development of OTC-resistant strains in Samutsakhon where shrimp have been cultured for many years and where OTC has also been used repeatedly. It has been suggested by some scientists that repeated use of drugs was probably the cause of this due to development of resistance in the bacterial population due to the transferring of R-plasmids in their DNA. (Aoki, 1974; Lewin, 1992).

When comparing the MIC-value obtained from the test for *V. parahaemolyticus*, the most dominant pathogen in the two areas, it was obvious that the more OTC-resistant strains were the strains isolated from Samutsakhon.

The most sensitive strains to OTC in this experiment were those of *V. parahaemolyticus* isolated from Chanthaburi and it was one of these that was chose for the challenge experiment and for the prophylactic study.

During the studies, *V. vulnificus*, which in the past was reported as the cause of serious diseases in many areas throughout Thailand also showed resistance to OTC, with MIC-value of 25 ppm. Again, there is a probability that the OTC resistant strain developed in response to the large volumes of OTC

which have unfortunately been repeatedly applied in attempts to control the black splinter diseases in 1988.

Other un-identified *Vibrio* species, which seemed to be part of the normal microflora in pond environment and infected the stressed shrimp like an accidental pathogen were also isolated. These showed relatively low MIC and MBC-values and seemed relatively easy to control by OTC.

As indicated in tables 10-11, the pathogenic strains showed a very wide range in the inhibition concentration of OTC required to control them *in vitro*. In practice, OTC is delivered to shrimp by incorporation of the drug within the feed. In such circumstances it is impossible to know exactly what concentration is achieved within the shrimp. To prove this, OTC-medicated feed at the rate of 3 and 5 g OTC/kg of feed was prepared by dissolving the desired amount of drug in water prior to spraying the dissolved drug on to the feed,which was then dried and kept in the freezer. This medicated-feed was used to feed the shrimp for 3, 5 and 7 days, to compare the retention of the drug in shrimp tissue.

The results of the retention experiment gave data which is best summarized by the statement that the more drug used and the longer the administration period, the higher the level of drug in tissue and the longer the retention time. The maximum drug level which could be induced in shrimp muscle in the present study was approximately 1 ppm. This was a relatively low concentration, which seemed to be inadequate in relation to the inhibition level as seen *in vitro* (MIC) to successful treatment of some of the pathogens isolated in the study. This level, however was the level measured in muscle,

which is usually much lower than in the other tissues such as haemolymph and hepatopancreas, at least in the reports for fish tissue (Fribourgh *et al*, 1969b, c), so that the effective level may well have been being achieved in tissue fluids. Some resistant strains showed MIC-value higher than 25 ppm. and it was not possible to treat these strains in the pond outbreaks, with antibiotic. Most of those strains were isolated from Samutsakhon. This is probably why OTC-medicated feed is not particularly successful in controlling the diseases in this area.

This emphasises the need for monitoring antibiotic sensitivity in Vibrio spp. isolates, in order to ensure the efficacy of treatments. Use of ineffective antibiotics not only fails to help the problem and therefore wastes money but also encourages the development of resistance.

For more information, it would be very useful to determine the drug concentration in different tissues of shrimp after being fed with known dosages of the drug and to compare the resultant level in different organs. In addition the rate of loss of drug from medicated feed during preparation, feeding and consumption is an important figure that must be known in order to understand how to make a good medicated feed. This is essential if the drug is to be delivered to the shrimp successfully at the desired dosage without loss. Equally, this is also an important factor in helping to prevent contamination of the environment by the drug.

It is important to minimise leaching and maximises the amount of antibiotic ingested by the shrimp. Therefore, additional work is required to determine the most effective and economical method of attatching the antibiotic

to the feed.

In order to study and confirm the efficacy of OTC-treatment against sensitive *Vibrio* sp. infection in shrimp, the most OTC-sensitive strain of the isolated *V. parahaemolyticus* was chosen for prophylactic studies. Injection of this bacterium into pre-treated shrimp which had been fed with medicated feed containing 5 gm OTC/kg of feed for 7 days was carried out. Comparative studies were performed to compare the mortality and the histological changes between the pre-treated and un-treated shrimp.

In this experiment, an inoculum of approximately $2.5x10^8$ CFU of the bacteria was injected into each shrimp. The results showed that almost all of the shrimp in both groups died within 6 hrs, more in the surviving un-treated group died at days 2 post injection. This rapid mortality of infected shrimp was probably due to toxic shock, which has been previously described by Leong and Fontaine (1979) who used a bacterium-free filtrate extracted from a 24 hrs. culture of *V. parahaemolyticus* ($5.1x10^{11}$ CFU/ml) injected into white shrimp.

When the mortality of non-treated and pre-treated shrimp is compared, higher mortality (22.5%) occurred in the un-treated shrimp compared with pre-treated shrimp (15%). These results are not as clear cut as might be wished but a proportion of early mortality in both groups is likely to be again a result of the high endotoxin level induced by the inoculum will before any antibacterial effect is able to be exerted. More meaningful results in term of protection relate to the losses after two days, when the initial toxic shock effect is over, and mortalities can be more closely related to bacterial multiplication. In the case of un-treated shrimp there were losses which were not echoed in the stocks

pre-treated with OTC medicated feed. In pre-treated shrimp, which contained the antibiotic in the body at an inhibitory level, the injected bacteria were controlled .

From the histological examination, it was apparent that the most effective mechanism in the shrimp inflammatory response was the cellular response, demonstrated by the reaction of the haemocytes against the injected bacteria during the study period.

At later stages, cellular response at the injection site in injected shrimp was very obvious; haemocytic aggregation, encapsulation of the tissue debris by modified haemocytes and subsequent nodule formation were observed in both pre-treated and un-treated shrimp, but very much smaller nodules presented in pre-treated shrimp, suggesting that the drug acts by controlling the number of the injected bacteria, with many being cleared from the injection site by the phagocytosis mechanism leaving only a few to be harmlessly sequestered in small areas of encapsulation. In un-treated shrimps the large number of injected bacteria could not be cleared by the haemocytes and in those which survived the bacteria were encapsulated by a mass of modified haemocytes and induced a lesion which appeared as a big nodules much larger than in pre-treated shrimp, which ultimatedly became melanized. In pre-treated shrimp, the injection area healed within 35 days but in un-treated shrimp, black nodule formation was still present in muscle as a permanent scar. This scar tissue consisted of connective tissue and modified haemocytes and looked very similar to the splinter exhibited in "Black Splinter Disease". From this result, one may conclude that the black or brown splinter in shrimp muscle

is not only caused by *V. vulnificus* as previously described (Limsuwan, 1988; Ruangpan and Sae-Ui, 1988) but may be caused by any species of *Vibrio* bacteria which has got into the shrimp and then activated the inflammatory response and become melanized.

Additional information regarding the initiation of the host reaction might be obtained by comparing pre-treated shrimp injected with either live or killed bacteria.

In conclusion, has been shown that it is possible to prevent or treat vibriosis in shrimp culture by using oxytetracycline-medicated feed. It appears however that some strains of the pathogen have already developed resistance to this drug, in these area where the drug have been repeatedly applied for a long period. It appear however that oxytetracycline can be only used to control vibriosis in cases where the shrimp are not severely infected by massive bacterial infection. The drug-treatment must be performed immediately after the disease is first diagnosed in a few members of the population. Otherwise the period of time necessary for the drug to act to the bacterial from establishment in the remaining shrimps is not going to be available. The last thing that must be of concern is how to deliver the drug at the therapeutic level to the diseased shrimp as soon as possible without any loss of drug into the environment. The level of leaching of drug into the environment, where it may persist and promote the development of OTC-resistant bacteria in the whole area, is one of the major concerns for the future of shrimp culture. These bactena or their related cohorts may also be responsible for diseases in other aquatic species and even, on occasion, for humans.

Future Work

As mentioned previously information concerning the leaching of antibiotics from medicated-feed and persistance in different shrimp tissues would allow more rational treatment strategies. If this were combined with a study of developing resistance patterns, including prevalence of R-plasmid, the long term efficacy of treatment would be greatly enhanced.

It is also important to emphasise that vibriosis in cultured shrimp is an opportunist infection precipitated by environmental conditions. Therefore, control has to involve all aspect of management. Additional useful information for controlling the diseases might be obtained by monitoring the number of potentially pathogenic bacteria both within the pond and the shrimp.

It might also be possible to prophylactically stimulate the defence responses of the shrimp to reduce the rate of infection.

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