STUDIES ON THE PATHOGENESIS OF *VIBRIO* SPP INFECTION IN *PENAEUS MONODON* FABRICIUS

by

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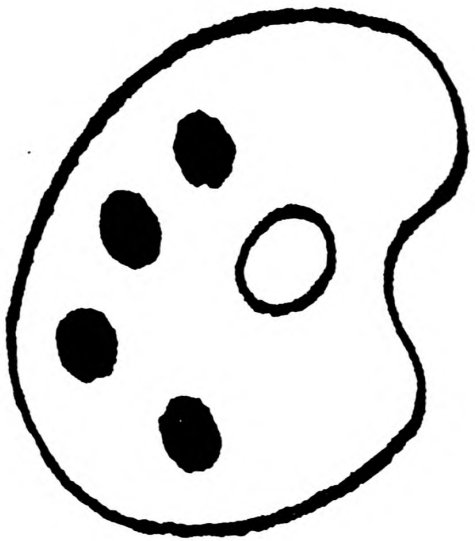
NUMEROUS ORIGINALS IN COLOUR



I would like to dedicate this work to my parents for their devotion to the family, which provided all their children with a model to follow and unconditional support.

DECLARATION

I declare that this dissertation has been composed by myself and that it embodies the results of my own research.

Mª Victoria Alday Sanz

ABSTRACT

Vibrio infections are one of the principal final causes of serious production losses in penaeid shrimp farming. The pathogenesis of experimental *Vibrio* spp infections in *Penaeus monodon* has been investigated using an immunohistochemical technique.

A *Vibrio* spp isolate was obtained from a disease outbreak in Thailand and identified using biochemical tests and SDS-PAGE. The isolate was identified as gelatine negative *Vibrio vulnificus* biotype I with variable urease activity. A polyclonal antiserum was raised in rabbits against the bacterium and its specificity tested by means of agglutination, immunoblot and ELISA tests. This antiserum was used to identify the 31KD protein and a 90-99KD LPS fraction responsible for the immune response using a Western-blot technique. *P. monodon* juveniles were experimentally infected with the bacterium either by injection, ingestion or bath challenge method. In a time course study, an indirect immunoperoxidase technique was employed to trace the presence of the bacterium in the tissue of the challenged shrimps.

The penetration of the bacteria differed between the three challenge methods, the clearance mechanisms of bacteria converged into common routes. Bacterial cells penetrated through damaged gill cuticle, body cuticle or through the insertion of the cuticular setae. Haemocytes were observed to phagocytose the bacterial cells and migrate through connective tissue and be transported with the haemolymph, accumulating around hepatopancreas, midgut caecum, gills and antennal gland. Once the phagocytic haemocytes reached the heart, they were distributed to lymphoid organ. Bacterial material appeared to accumulate in heart and lymphoid organ.

Bacteria entering through the mouth were broken down and only soluble material filtered through gastric sieves passed into the hepatopancreas. This soluble material then diffused through the hepatopancreatic tubules into the haemolymph and then to gills and antennal gland. Once this material reached the heart it was distributed to lymphoid organ accumulating in the heart and lymphoid organ as previously described.

Finally, bacterial material was released to the exterior in two steps. Initially through the gills and later through hepatopancreatic B-cell vacuoles and branchial and subcuticular podocytes.

This mechanism and its implications are discussed.

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CHAPTER I

LITERATURE REVIEW

I. SHRIMP INDUSTRY

Global production of captured shrimp reached its plateau in 1985 and levelled off between 1.9 and 2.0 million tonnes, but an unprecedented economic success in culturing shrimp kept the world supply expanding. The volume of farmed shrimp reached 0.64 million tonnes in 1993 (25% of total shrimp production)(World shrimp farming, 1994), up from about 0.1 million tonnes in 1982, making shrimp farming one of the outstanding commercial success stories in the history of aquaculture (Csavas, 1993).

The exponential growth of cultured shrimp production in the mid-1980s was based on several technological breakthroughs. Shrimp aquaculture was driven by feed and to a certain extent seed suppliers. However, economical and ecological limitations were soon reached and growth slowed down by the end of the decade. Achievements in processing and marketing kept farmed shrimp production growing less rapidly until recently. Shrimp aquaculture development has now reached a critical point, as the environmental deterioration is favouring the onset of diseases and lower production.

Worldwide demand for shrimp is expected to exhibit consistent growth at 2.5% per year until the year 2000 when it is expected to reach 3 million tons. In 1989, the market was influenced by the rapid increase in cultured shrimp output leading to a sharp drop in prices which are not expected to recover to their previous levels in the short term (Infofish, 1991).

Several advantages of cultured shrimp over the wild catch include an outstanding quality due to freshness at processing, considerably less handling compared to common boat operations, and constant year-round supply, these factors may well prevent the collapse of the industry. Future growth, however, depends on further improvements in culture technology and farm management practices, as well as on product and market diversification. Experience accumulated over the past decade has provided a solid basis for addressing past mistakes caused by rapid expansion of the industry (Csavas, 1993).

Such mistakes had a dramatic effect on the Taiwanese shrimp farming industry. In 1987 Taiwan shrimp farming produced 100,000 tonnes from intensive systems, but in 1989 Taiwanese farmers faced serious disease outbreaks. These were so severe that production decreased to 30,000 tonnes and continued to drop to an estimated 20,000 tonnes in 1989 (Infofish, 1991).

It is currently recognised that the industry has to develop sustainable systems that do not cause excessive damage to the ecosystem.

II. ANATOMY AND BIOLOGY OF *Penaeus monodon*

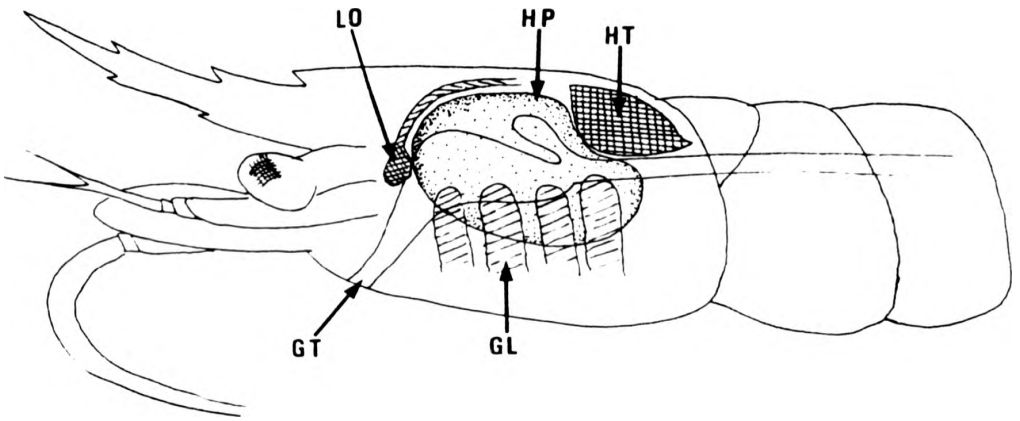
The taxonomic position of *Penaeus monodon* according to Fabricius in 1798 (cited by Chanratchakool, 1992) is as follows:

Phylum:	Arthropoda
Class:	Crustacea
Subclass:	Malacostra
Series:	Eumalacostra
Superorder:	Eucarida
Order:	Decapoda
Suborder:	Natantia
Section:	Penaeidea
Family:	Penaeidae
Genus:	*Penaeus* Fabricius, 1798
Species:	*Penaeus monodon*, Fabricius, 1798

One of the common name used for this crustacean and accepted by FAO is the giant tiger prawn but in this study it will be referred to simply as shrimp.

It is widely distributed throughout the greater part of the Indo-West Pacific region, approximately from 30°W to 155°E longitude and from 35°N to 35°S latitude.

This figure is a diagramatic representation of the anatomy of a normal *P. monodon*



LO: Lymphoid organ

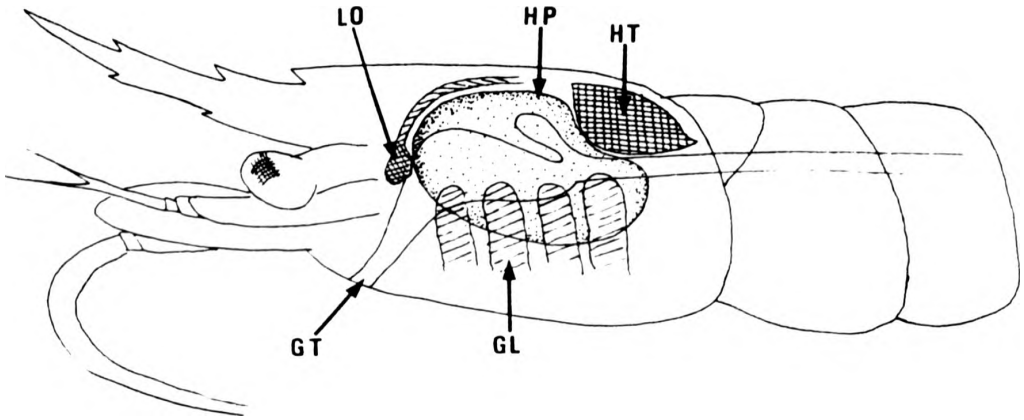
HP: Hepatopancreas

HT: Heart

GT: Gut

GL: Gill

This figure is a diagramatic representation of the anatomy of a normal *P. monodon*



LO: Lymphoid organ

HP: Hepatopancreas

HT: Heart

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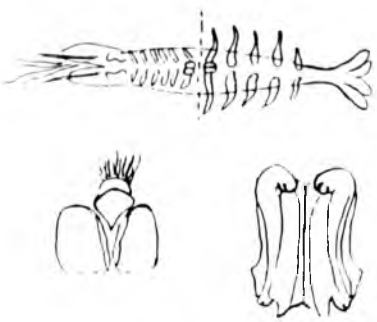
GL: Gill

P. monodon presents sexual dimorphism. Females are slightly bigger than males. The external character of the female is the thelycum situated between the 4th and 5th pereopods. The male is characterized by the presence of the petasma between the 1st pleopods and the appendix masculina on the exopods of the 2nd pleopods (figure 1).

Figure 1: Sexual dimorphism in *P. monodon*

Female	Male
Thelycum	Petasma



Mating and spawning usually occur in offshore waters where the eggs are released into the open ocean where they remain suspended in the water gradually sinking to the bottom. Viable eggs are spherical, yellowish green in colour and somewhat translucent. Hatching generally occurs between 15 and 20h after spawning.

After hatching, the larvae require estuarine conditions to complete larval development. These areas are exposed to wide fluctuations of physico-chemical conditions, such as water temperature and salinity, so they should have high tolerance to those conditions for their survival. The larval stages consists of 6 nauplii, 3 protozoea, 3 mysis, followed by post-larval stages or juveniles and the adult stage. The juveniles and sub-adult stages begin to migrate towards the open sea to complete their life cycle where they inhabit waters down to about 160m (Motoh, 1984).

III. SHRIMP CULTURE

Rosenberry lists the shrimp species more commonly used for farming (World shrimp farming, 1994):

P. monodon constitutes 61% of the world production. With the exception of China and Japan this species dominates production in Southeast Asia. It is the largest and fastest growing of the farm-raised shrimp, but shortages of wild broodstock occur and captive breeding is difficult. The protein requierement is 30% of the diet.

Penaeus chinensis constitutes 15% of the world production and dominates production in China. This species has many positive characteristics, it is second to *P. monodon* in growth rate, it grows at lower water temperature (18 to 26°C) than *Penaeus vannamei* and *P. monodon*, it likes muddy bottoms and it matures and spawns in captivity. On the negative side it has a high protein requirement (40-60%) it is small (maximum length of 183mm) and its "meat" yield (57%) is less than *P. monodon* (65%) or *P. vannamei* (65%).

P. vannamei constitutes 15% of the world production. It is the leading species in the Western hemisphere including Ecuador. Its protein requirement (20-25%) is lower than the species mentioned previously.

Others species are *Penaeus stylirostris* cultured in the Pacific coast of Latin America, *Penaeus japonicus* cultured in Japan, China and Taiwan, *Penaeus penicillatus* cultured in Taiwan and China, *Penaeus merguiensis* and *Penaeus indicus* cultured in Southeast Asia. Together these five species represent 8% of the world production.

Shrimp farming covers the complete cycle. Shrimp seed is obtained either by wild catch for extensive farming or from hatchery for semi-intensive and intensive farming.

Supplies of broodstock are usually obtained from the wild, but farmed broodstock are sometimes used where non-native species are farmed or are successfully reared as with *P. japonicus*, *P. indicus*, *P. merguiensis* and partially *P. vannamei* (Briggs, personal communication). The maturation of these animals in captivity is induced by a controlled environment (temperature, light intensity and photoperiod). Even under these conditions they will not mature with regularity, so the technique of unilateral eyestalk ablation is usually applied. This operation involves the surgical removal of one of the two eyestalks, which contains a complex of glands that may function to inhibit gonad development. Although the viability of artificial insemination has been demonstrated and is commonly used in some *P. vannamei* hatcheries, most maturation units continue to rely on natural impregnation occurring within the maturation tanks since less labour is involved, mortality rates are lower and more consistent fertilization rates are

obtained (Wickins, 1992).

Females ready to spawn are often placed into individual spawning tanks from which they are removed once the eggs are released. However, it is still common to observe batch spawning. Nauplii are hatched and siphoned off later into the larval rearing tanks.

There are two main traditional methods of larval rearing, the oriental and the western methods.

The oriental method typically rears larvae at low densities (30-100 animals/l). The bulk of algal feed is produced by encouraging blooms within the larval rearing tank, for this reason water exchange rates are kept comparatively low (5-10%/day) to avoid flushing away the algae.

The western method stocks 50-200 a./l and uses water exchange rates of 50-200%/day to maintain stricter control over water quality. However, the resulting higher requirement for algae leads to a far greater reliance on independently cultured algae stocks.

Together with these two traditional methods, there are also many intermediate techniques. Larvae leave the hatchery for transfer to the nursery ponds between PL_{10}-PL_{15}. The nursery phase provides hardy juveniles that have been acclimatized to the environment they are likely to encounter during on-

growing. The nursery phase is currently used in a small proportion of asian farms.

The different techniques used for on-growing shrimp culture are categorized into extensive, semi-intensive and intensive systems.

In an extensive culture system, seed is normally obtained from the wild and therefore the system is seasonally dependant and food is naturally available although sometimes it is increased with the addition of fertilizers. The stocking density ranges from 0.2-10 a./m^2, achieving a crop of 300-800kg/ha/year (Chanratchakool, 1992).

In a semi-intensive culture system, the source of seed is usually from the hatcheries and shrimp are stocked at 6-20 a./m^2. Supplementary feed is normally used and regular water management procedures are also performed. Production ranges between 1000 to 3000 kg/ha/year (Chanratchakool, 1992).

In an intensive culture system sophisticated facilities are required, fry are produced in large volumes from hatcheries and an aeration system and formulated feed are used as well as intensive water management techniques. Stocking density reaches 50-100 a./m^2 with a production ranging between 8000 to 12000 kg/ha/year (Chanratchakool, 1992).

Currently, superintensive systems are being run in raceways with stocking densities up to 250 a./m^2.

As density increases, technology required becomes more sophisticated, capital costs increase and there is a potential for higher production. This potential is not always fulfilled because intensive and semi-intensive culture systems encourage the development and transmission of many diseases (Sindermann, 1988) associated with inducing environmental deterioration.

IV. SHRIMP DISEASES

Disease has long been recognized as one of the constraints to the development, expansion and intensification of shrimp farming. In most, if not all, of the major shrimp farming regions of the world, shrimp hatchery and farm production losses due to diseases appear to be increasing (Lightner, 1993).

A variety of agents, both biological and non-biological, have been implicated in the aetiology of shrimp diseases. Viruses, rickettsia/chlamydia, bacteria, fungi, protozoa, metazoa, feed factors (limiting nutrients or toxins), environmental factors (soil and water both physical and chemical, biotoxins and pesticides) and husbandry features may all contribute to shrimp diseases.

Nine viral diseases are presently recognized in the penaeid shrimp (Lightner *et al*, 1992, Asian Shrimp News, 1992). These viruses are displayed in table 1 together with the commercially relevant shrimp species affected.

Table 1: Penaeid viruses and their known natural infected hosts (Lightner *et al*, 1992 and Asian Shrimp News, 1992).

Species	BP	MBV	BMN	TCBV	IHHNV		HPV	LOV	REO	YBV
P.vannamei	+++	+?			++	+	+?	+		
P.stylirostris	++				+++					
P.monodon	++	+++		++	++	++	+	+	+++	
P.chinensis					+	++				
P.japonicus			+++		++			++		
P.merguiensis		+				++				
P.indicus						+				
P.penicillatus	++	+				+				

+: infection observed but without signs of disease.

++: infection may result in moderate disease and mortality.

+++: infection usually results in serious disease.

BP: Baculovirus penaei.

MBV: P. monodon baculovirus.

BMN: Baculovirus midgut gland necrosis.

TCBV: Type C baculovirus.

IHHNV: Infectious hypodermal and haematopoietic necrosis virus.

HPV: Hepatopancreatic parvo-like virus.

LOV: Lymphoid organ parvo-like virus.

REO: Reolike virus.

YBV: Yellow head baculovirus.

Rickettsial and clamydial infections have been recently reported commonly associated with disease or as pathogens (Anderson *et al*, 1987, Brock, 1988, Brock and Lightner, 1990). The economic importance of this group of agents as shrimp pathogens remains largely unknown.

While a number of species of bacteria have been implicated as agents associated with infections and disease in penaeid shrimp, reports of infections by *Vibrio* spp are by far the most numerous (Lightner, 1988). All the reported species of bacterial pathogens of penaeid shrimp (*Vibrio* spp, *Pseudomonas* spp, *Flavobacterium* spp and *Aeromonas* spp) (Sinderman, 1988), have also been recorded as part of their normal microflora. Some recent disease syndromes occurring in penaeid shrimp have been caused by *Vibrio* spp. which behave more like true pathogens than opportunistic invaders (Lightner *et al*, 1992).

Fouling with a number of organisms is a common finding in farmed shrimp. The predominant bacterial fouling organism is *Leucothrix mucor*, other genera reported are *Thiothrix* spp, *Flavobacterium* spp, *Flexibacter* spp, *Cytophaga* spp (Lightner, 1983).

Fungi are important pathogens causing two distinct diseases: larval mycosis caused by *Lagenidium* spp, and *Sirolpidium* spp and fusarium disease caused principally by *Fusarium solani* which is limited to intensive growout culture

(Bell and Lightner, 1987).

Pathology has also been associated with protozoan infections which may be invasive. *Microsporidia, haplosporidia* and gregarines are commonly found in wild and farmed shrimp. Ciliates, flagellates and amoebae are common as epicommensals (Brock and LeaMaster, 1992).

Metazoan parasites (nematodes, cestodes, digenea, trematodes and isopods) are commonly found in shrimp but have not been associated with significant diseases (Brock and LeaMaster, 1992).

IV.I. VIBRIOSIS

The term vibriosis is used to refer all types of infections caused by bacteria of the genus *Vibrio*.

In penaeid shrimps, vibriosis is potentially the most serious of all the disease problems. It has become progressively more important over the last few years and this trend seems likely to continue. It has been suggested that the increasing severity has been associated with degradation of the environment (Nash *et al*, 1990). Sano and Fukuda (1987) reported that in 1984 vibriosis caused an annual production loss of 30.8 tonnes valued at £1.44 million of kuruma shrimp (*P. japonicus*) in Japan.

The *Vibrio* spp reported to cause bacterial disease in cultured shrimp are apparently ubiquitous, being reported from all of the major penaeid culture regions of the world, and from a variety of estuarine and marine crustaceans, as well as fish and shellfish (Sindermann, 1988).

Vibrio epidemics have been associated with additional factors which apparently predisposed shrimp to infection and disease. Principal predisposing factors suggested include handling and injuries to the cuticle of the shrimp (Lightner and Lewis 1975). Prior infections with other pathogens including viruses, rickettsia, *Fusarium* spp and gregarines (Anderson *et al* 1987, Brock and Lightner 1990, Nash 1990 and Lightner 1993), intestinal tissue damage resulting from blue green algal toxins (Lightner 1983), ascorbic acid deficiency (Lightner *et al*, 1977), exposure to physiologically stressful chemical or physiological conditions such as low dissolved oxygen, elevated ammonia, temperature extremes and crowding (Barkate, 1972, Lewis, 1973, Lightner, 1983, 1985 and 1988, Sparks, 1985, Takahashi *et al*, 1985, Baticados, 1988).

Pathology of vibriosis

In larval and early post-larva (PL) shrimp, signs of vibriosis include melanization, necrosis of appendage tips and the presence of large numbers of swarming bacteria visible in the haemocoel of moribund shrimp. Affected shrimp are also typically inappetant or anorexic and therefore, lack faecal

strands and have empty guts.

In juvenile and adult shrimp, the behaviour of affected shrimp may include periods of erratic or disoriented swimming alternating with periods of lethargy. Clinical signs vary with the type of infection. Infections of the cuticle, appendages or gills are apparent as black/brown localized lesions in which the cuticle is eroded. This syndrome is called brown spot or shell disease. In cases of septicaemic vibriosis and localized internal infections, gross signs include those of severe stress, such as opacity of abdominal muscle, anorexia and expansion of chromatophores, causing a darker pigmentation in the affected shrimp with reddish appendages and occasionally a slight dorsal flexure of the abdomen.

Haemolymph of bacteraemic shrimp clots slowly, requiring more than 1 minute at 20-30°C, whereas haemolymph from normal shrimp gels in less than a minute. Haemocyte numbers may be drastically reduced from normal values of approximately 20,000 haemocytes/ml to values of 1,000 to 10,000 haemocytes/mm as they are involved in defense mechanisms.

Lightner (1992, personal communication) described five syndromes associated with vibriosis.

Non-specific bacterial disease syndrome (NSBS). It is characterized by a dramatic reduction in food intake. Shrimp fail to moult or omit at a life-cycle

stage and significant mortalities are observed following a moult. The hepatopancreas and muscle may show atrophy. High levels of bacteria are observed in stomach, hepatopancreas or midgut. No faecal strands are formed as a result of an empty gut. The disease is thought to be associated with toxins produced by the most commonly found bacteria: *Vibrio* spp, *Aeromonas* spp and *Pseudomonas* spp. The syndrome has a worldwide distribution affecting all species of penaeid.

Luminescent bacteria syndrome. Clinical signs are similar to those described for NSBS but with melanized mouth, oesophagus and stomach due to bacterial colonization of these areas. Affected larvae are luminescent due to the presence of luminescent *Vibrio* spp, *Aeromonas* spp and *Pseudomonas* spp bacteria. The distribution of this syndrome is extended to the areas where *P.vannamei* and *P.monodon* are cultured.

"Bolitas blancas" syndrome (White balls syndrome). The clinical signs are similar to those described previously but with a higher mortality rate. There is a sloughing of epithelial cells in hepatopancreas which round up and form characteristic "bolitas" or balls. This pathology has been associated to the toxic effect of vibrio infections.

"Bolitas negras" syndrome (Black balls syndrome). These "bolitas" described previously may contain a dark substance, that has been identified as chlorophyll. Toxins from chitinolitic bacteria associated with mouth,

oesophagus and stomach cause metabolism disorders leading to a poor digestion of algae. This syndrome also has a worldwide distribution of the affecting all species of penaeid.

"Gaviotas" syndrome (Seagull syndrome), (Mahoney *et al,* 1991). This syndrome has been associated with massive epizootics causing mortalities between 40-90%. The name of this condition was adopted when seagulls were observed fishing the moribund shrimp from the edges and surface of the ponds. A series of environmental factors contributed to its onset: high salinity, high temperature, bacterial counts 100 times higher than usual in the water supply, luminescent bacteria in wild larvae, poor shrimp health status as a result of infection with gregarines, baculovirus penaei, runt deformity syndrome associated with IHHNV in *P. vannamei.* Elevated nutrient levels, particularly nitrogen, brought on by a reduced flow of water, due to a severe drought, and by an increase of urea fertilization led to an increase of zooplankton which acts as a reservoir which was easily colonized by *Vibrio* spp. The end result was an acute bacteraemia by *Vibrio* spp, many of them with transient luminescence. This syndrome has been reported twice in Ecuador and Texas, in both cases affecting *P.vannamei.*

Septic hepatopancreatic necrosis. It results in destruction of large areas of the hepatopancreas, reducing it in size and making it dark in colour. The midgut loses its epithelium and can be seen macroscopically. The animals have a bluish appearance and it may also result in tail rot. This syndrome

has a worldwide distribution affecting all species of farmed penaeid.

Some others conditions associated with vibriosis are "black splinter" which is a chronic melanized lesion confined to the muscle of the abdomen. The "one month mortality syndrome" which refers to a *Vibrio* spp infection associated with the deterioration in the pond environment due to benthic algae growth and subsequent decomposition.

Diagnosis of vibriosis

Diagnosis of bacterial infection in larval shrimp can be made by microscopic demonstration of bacterial rods swarming through internal organs or associated lesions including melanized cuticular lesions of appendages (Cook and Lofton, 1973, Cipriani *et al*, 1980) or small, internal melanized nodules in the gills or others organs (Lightner, 1983 and 1988 and Sparks 1985).

In pond reared shrimp, vibriosis is characterized by an abrupt onset and rapid course (several days to 2 weeks) (Brock and LeaMaster 1992). Affected shrimps have disoriented weak swimming and gather along the edge of the pond. The clinical signs have been described previously. Rapid demonstration of the bacteria can be made by microscopic examination of haemolymph specimens with or without gram staining. Bacteria, especially *Vibrio* spp, may be isolated in low numbers from the haemolymph of

apparently healthy shrimp. The stress of crowding, handling, moulting and capture may result in bacteria being introduced into the haemolymph.

Apparently, the shrimp's defense mechanisms are capable of controlling these bacteria when they are in low numbers if the shrimp is not otherwise compromised. Haemolymph drawn from such animals shows normal clotting time and normal numbers of haemocytes. In contrast, the haemolymph of shrimp with a severe bacteraemia (over 10 bacterial cell/mm^3) shows a marked haemocytopenia, and an increasing clotting time.

Isolation of the organism may be achieved by direct plating into any of the bacteriological media listed in table 2 or by using enrichment methods. The simplest and most widely used enrichment medium for vibrios is alkaline peptone water, which contains 1%(w/v) peptone and 1%(w/v) NaCl, at pH 8.6. Identification is based on biochemical reactions by standard procedures and can be used to confirm the identity of the pathogen.

Table 2: Media suitable for the culture of bacterial shrimp pathogens.

Marine Agar MA

Thiosulphate Citrate Bile Salts Agar TCBS

Trypticase Soy Agar TSA + 1.5-3% NaCl

Nutrient Agar NA + 1.5-3% NaCl

Modern immunological techniques including ELISA are now being used to identify the presence of *Vibrio* spp bacteria. Diagnosis of the clinical condition of vibriosis, however, is still problematic and requieres integration of diagnostic information with clinical and production data.

Treatment and prevention of vibriosis

A variety of strategies have been applied to manage vibriosis in marine shrimp farming.

Hatchery culture practices can vary substantially although there are some common practices such as disinfection between stockings, disinfection of water intake, sanitation of live feeds and equipment before introduction, low larval stocking densities, control of water temperature to avoid fluctuations or reduction of faecal contamination from broodstock in spawning tanks. The use of active, strong swimming, phototactic, non-deformed nauplii going through a clean water rinsing prior to stocking is widely recommended. Periodic water exchange to reduce nutrients and bacterial population, proper storage and handling of larval feeds, use of sucrose (10mg/l in culture tank water, Allan Heres, 1992 personal communication), addition of antibiotics or other chemicals to water or feeds are also common practices in shrimp farming.

The use of antibiotics in shrimp hatchery disease management is

controversial in part because of the induction of antibiotic resistance in bacterial populations and because of the impact on hatchery workers health and the near-shore environment (Brown 1989).

The management strategies most commonly applied in nursery and growout culture to prevent vibriosis include: use of medicated feeds and fertilization of the pond with 20kg/ha of sucrose (Lightner in press). Some other useful preventive measures are a partial harvest to reduce pond biomass, high water exchange and disinfection of the pond bottom between growout cycles by drying, removal of excessive organic sediment and application of quicklime at a dose of 0.5kg/m^2.

In order to optimize the shrimp environment , water quality parameters should be as close as possible to the values shown in table 3 (Chien, 1992) and should always be minimised water quality fluctuations.

Table 3: Optimal water quality parameters for shrimp culture.

Salinity	10-25ppt
pH	7.5-8.5
Dissolved oxygen (minimum)	4mg/l
NH_3.N (maximum)	0.1mg/l
Nitrite-N (maximum)	1.0 mg/l
Unionized hydrogen sulphide	0.005 mg/l

In larval and early postlarval stages, a number of antibiotics and antibacterial substances have been reported to be effective in controlling and reducing losses due to *Vibrio* spp although with a variable success. The most common substances used for vibriosis treatment and the dose-rate used are listed in table 4 (Sindermann, 1988).

Table 4: Most common treatment for vibriosis

At larvae stage	
Ethylenediaminetetracetic acid (EDTA)	10-50ppm
Furanace	1ppm
Furazolidone	1ppm
Nitrofurazone	1 ppm
Oxytetracycline	1-10ppm
Chloramphenicol	1-10ppm
In juveniles and adults penaeids	
Oxytetracycline	1.5g/kg dry feed
Nitrofurans	500 mg/kg of feed for 10-14 days

It is important to remember that antibiotic treatment can be effective only if given early enough and provided the effective dosage is maintained over the requisite period (Nash, 1990). However, treatment of disease is only possible to any significant extent in intensive and semi-intensive culture systems. In

extensive and semi-extensive culture systems, treatment of diseases is usually impractical even if they are diagnosed.

Improving shrimp disease resistance has been the target of recent studies and has been shown that biological and synthetic compounds can enhance the non-specific defense system. Boonyaratpalin (1993) and Itami *et al.* (1993) reported the enhancement of disease resistance of *P. monodon* and *P. japonicus* respectively, after oral administration of peptidoglycans. Immunostimulants may represent an alternative to vaccines in the protection of farmed shrimps against diseases. However, more research is necessary as the protection achieved by this treatments have a short term duration (days or weeks), there is no memory involved and is difficult to quantify.

V. SHRIMP DEFENCE MECHANISMS

Shrimps have primitive defense mechanisms compared to vertebrates. Whether the defense mechanism of shrimp constitute an immune system or simply a group of non-specific defense mechanisms is largely a semantic argument.

The first barrier of the defense mechanism of crustaceans is the cuticle or outer layer of the shell which plays a major role against any potential pathogen or physical injury. It is composed of lipids, proteins and calcium. It lines the gills, oesophagus, stomachs and hindgut, the only area of the shrimp without cuticular protection is the midgut. Any damage on the outer layer predisposes the cuticle and deeper tissues to invasive microorganisms (Brock and LeaMaster, 1982).

The tegmental glands are also involved in the defence system. They are involved in tanning of the integument by the production of phenols and phenoloxidase (Stevenson and Schneider, 1962) and mucus (Johnson, 1980). Bauer (1989) suggested that some tegmental glands may produce an antifouling chemical that would discourage settlement of epibionts and debris.

Shrimps have a primitive immune system. They lack immunoglobulins and T-lymphocytes, relying instead on inflammatory-type responses for the host defense. Among these, phagocytosis plays a major role and can be

considered to be the primary cellular defence mechanism (Bell and Smith, 1992). This reaction is carried out primarily by the haemolymph cells or haemocytes. Foreign particles are phagocytosed by circulating haemocytes and immobilized in nodular aggregates of haemocytes or encapsulated by building into several layers of cells (Ratcliffe, 1985).

Crustaceans generally possess three types of circulating haemocytes, classified based on the presence or absence of granules in the cytoplasm. These cell types are hyalin, semigranular and granular cells which appear to be the various maturation stages in a sequential developmental series of a single cell line (Johnson, 1980, Mix and Sparks, 1980). Hyalin cells are characterized by the absence of granules and represent between 5 to 10% of circulating haemocytes (Martin and Graves, 1985). They are typical phagocytic cells (Bauchan, 1981). Semigranular cells have small granules in their cytoplasm and represent approximately 75% of circulating haemocytes. Granular cells are packed with large granules (Bauchau, 1981). They represent 10-20% of the circulating haemocytes. Both the semigranular and granular cells contain the pro-Phenoloxidase (proPo) system (Soderhall and Smith, 1986).

The proPO activating system is involved in stimulating several cellular defence reactions including phagocytosis, nodule formation, encapsulation (Soderhall *et al*, 1986) and haemocyte locomotion (Lackie, 1988 cited by Johansson and Soderhall, 1989) in addition to its role in melanization.

ProPO is activated in a stepwise process by microbial cell wall components such as B-1,3-glucans of fungi or the lipopolysaccharides (LPS) and peptidoglycans of Gram negative and Gram positive organisms respectively (Soderhall *et al*, 1986, Soderhall, 1982 and Ashida, 1982). The active enzyme, phenoloxidase, oxidizes phenols to quinones which then polymerize non-enzymatically to melanin. Melanin and its intermediates are biocidal. Melanization often accompanies cellular defense reactions. Phenoloxidase, but not its pro-enzyme proPO, is a sticky protein that attaches non-specifically to various surfaces, eg. fungal hyphae. ProPO is activated by a prophenoloxidase-activating enzyme (ppA) which is a serine protease. In crustaceans, ppA is in turn activated by B-1,3-glucans or by LPS (Soderhall *et al*, 1986). There is evidence that the ppA may be triggered into activating by the action of a B-1,3-glucan-binding protein. Conceivably, an LPS-binding protein may exist in crustacean haemolymph (Soderhall *et al*, 1988). In crustaceans, both proPO and inactivated ppA are stored in the secretory granules of the semigranular and granular haemocytes, from where they are released by degranulation (Johansson and Soderhall, 1985).

Another protein associated with the proPO system, has been purified from the haemocytes of the crayfish *Pacifastacus leniusculus*. It is a glycoprotein with a molecular mass of 76KD (Johansson and Soderhall, 1988). This protein is confined to and discharged from the granules of semigranular and granular cells. Its biological activities are generated outside the cells in the presence

of B-1,3-glucans or LPS and are thus activated simultaneously with the proPO system. Like the proPO system, it becomes spontaneously activated in low concentrations of Ca ions and it has been thought to be involved in the wound response and in controlling haemostatic reactions.

All these processes are stopped by protease inhibitors that can inhibit proPO activation and probably restrict its actions to the area of infection (Hergenhahn *et al,* 1987 cited by Johansson and Soderhall, 1989).

Humoral substances in crustaceans consist of the components of haemocyte membrane and secretions or products which are released from cell disruption when the haemocyte is destroyed, so in addition to the already mentioned proPO and 76KD molecule, a series of substances have been demonstrated. Haemagglutinins were found in shore crab (*Carcinus maenas*) by Smith and Ratcliffe (1976,1978) which may serve as opsonins which enhance phagocytosis (Smith and Chisholm, 1992). Agglutinins were reported in lobster (*Homarus americanus*) (Cornick and Stewart, 1968) and were non inducible (Cornick and Stewart, 1973). These factors may also represent opsonins. 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 (Schapiro, 1975). Lectins with the property of binding foreign material have been identified in fresh water prawn *(Machrobrachium rosenbergii)* (Vasta *et al*, 1983).

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CHAPTER II

BACTERIOLOGY AND SDS-PAGE OF *VIBRIO* SPP

INTRODUCTION

Vibrio spp are primarily found in aquatic habitats of a wide range of salinities, very common in marine and estuarine environments and in association with aquatic animals and with diseases of aquatic animals. Baumann *et al* (1980) describes the genus *Vibrio* as follows:

> "Gram negative, straight or curved rods, motile by means of polar flagella, monotrichous or multitrichous, not forming endospores or microcyst, chemoorganotrophs, facultative anaerobes, fermentative metabolism with the capability of using D-glucose as the sole or principle source of carbon and energy. They can 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%."

In 1984, in Bergey's Manual of Systematic Bacteriology, Baumann added a few new characteristics to his previous description:

> "Rods of 0.5-0.8 micron in width and 1.4-2.6 micron in length and many synthesize numerous flagella on solid media, most species are oxidase positive (except for *V. metschnikovii* and *V. gazogenes*), all grow at 20°C, most grow at 30°C. 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."

Six species *V. cholerae, V. parahaemolyticus, V. alginolyticus, V. vulnificus, V. fluvialis and V. metschnikovii* have been described as potentially pathogenic to man (West and Colwell 1984). Eight species of *Vibrio* have been reported to be pathogenic to fish: *V. alginolyticus, V. anguillarum, V. charchariae, V. cholerae, V. damsella, V. ordallii, V. salmonicida, V. vulnificus* and *V. parahaemolyticus* (Austin and Austin 1988).

The main bacterial species isolated from epidemics of bacterial diseases in shrimp are *V. parahaemolyticus, V. alginolyticus, V. anguillarum* (Sindermann 1988), *V. cholerae non 01, V. damsella, V. harveyi, V. splendidus, V. vulnificus* (Brock and LeaMaster, 1992) and *V. fluvialis* (Lavilla-Pitogo, 1993). Certain other *Vibrio* spp have been isolated from diseased shrimp and may occasionally be involved in the disease syndrome (Sindermann 1988).

The biochemical characteristics that differentiate these *Vibrio* spp are described in Bergey's Manual of Systematic Bacteriology (Baumann, 1984).

V. alginolyticus is mainly associated with mortalities in farmed sea bream (*Sparus aurata*) in Israel (Colorni *et al.*, 1981). However, these workers were

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unable to reproduce the infection under experimental conditions, which casts some doubt on the importance of this organism in fish pathology. Occasional isolations have been made from ulcers (Akazawa, 1968) or have been reported as a secondary invader of sea mullet suffering with "red spot" (Burke and Rodgers, 1981). Therefore, the conclusion is that *V. alginolyticus* constitutes an opportunistic invader of already damaged tissues, or a weak pathogen of stressed fish. This organism typically develops a swarming growth on the surface of solid media and it does not grow in 10% NaCl.

V. anguillarum was the causative agent of the first disease reported in Europe, the "Red pest" that catastrophically affected eels in sea water sites within Italy during the XVIII and XIX centuries (Bonaveri, 1761 cited by Austin and Austin 1988). 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. It grew in 0.5 to 3% but not in 0 or 7% NaCl. The complexity in the taxonomy of this organism began with the recognition by Nybelin (1935 cited by Austin and Austin, 1988) of two biotypes A and B. A further group biotype C was recognized by Smith (1961). Type A, known as *V. anguillarum forma typica*, produces indole and acid from mannitol and saccharose. Type B, referred to as *V. anguillarum forma anguillicida*, does not produce indole or acid from mannitol or saccharose. Type C, coined as *V. anguillarum forma opthalmica*, produces acid from mannitol and saccharose, but does not produce indole.

V. carchariae was originally isolated from a dead sandbar shark (*Carcharhinus plumbeus*) (Grimmes *et al*, 1984). Subsequently, a similar organism has been recovered from lemon sharks (*Negraprion brevirostris)* (Colwell and Grimes, 1984). It develops a swarming growth on agar medium. Sensitivity is recorded to 150μg but not 10μg of the vibriostatic agent, 0/129. Growth occurs in 3-8% but not 10% NaCl and at 11-40°C

V. cholerae (non-01) was first reported in an epozootic occurred in a wild population of ayu in Japan. Growth occurs in 0 to 6% NaCl, at 10-42°C and a pH 7-10.

V. damsela was associated initially with ulcerative lesions in blacksmith (*Chromis punctipinnis*), a type of damselfish in California. Surveys of wild fish populations led to the conclusion that the ulcers were restricted to species of damselfish. It might also have a role in human pathogenicity as the organism has been isolated from human wounds (Love *et al*, 1981). Subsequent work by Grimes *et al* (1984) demonstrated its presence in sharks. *V. damsela* grows in 1-6% NaCl.

V. ordalii was the name proposed to accommodate strains previously classified as *V. anguillarum* biotype II (Schiewe, 1981). Disease caused by this organism has been documented in Japan and The Pacific Northwest of the USA (Colwell and Grimes, 1984). *V. ordalii* appears to have a more dependant mode of existence than *V. anguillarum* because it is rarely

isolated from water, sediment or abiotic marine samples, only being recovered from moribund fish (Schiewe, 1983). Growth occurs quite slowly, insofar as 4 to 6 days incubation at 22°C are required for the production of off-white, circular, convex colonies of 1-2mm in diameter on sea water agar. Growth occurs at 15 to 22°C and in 0.5 to 3% NaCl.

V. vulnificus is recognized as a highly virulent , opportunistic human pathogen (Blake *et al.*, 1980) and described in shrimp's outbreak of vibrio infection (Brock and LeaMaster, 1992 and Lavilla-Pitogo, 1993). Kaysner *et al.* (1987) showed the production of cytotoxins, but this did not indicate the degree of virulence because their titres were variable. Some of the strains showed pathogenicity when injected into mice, which died 72h post-injection. Growth occurs between 20 and 37°C, but not at 5 or 42°C and in 0.5 to 5% NaCl. There has been a division into two biotypes. Biotype I is ornithine decarboxylase and indol production positive, while biotype II is negative.

V. salmonicida is the causative agent of Hitra disease or cold water vibriosis in salmonid fish cultured in Norway (Egidius *et al.*, 1986).

V. parahaemolyticus is well known as a pathogenic organism for fish and at times produces food-borne infections in humans. In marine organisms, it has been isolated from diseased crabs (Krantz *et al.*, 1969), shrimp (Vanderzant *et al.*, 1970a) and oysters (Baross and Liston, 1970). Lightner (1988) confirmed that *V. paraemolyticus* was associated with shell diseases or other

types of vibriosis in shrimp.

V. fluvialis was originally referred to as a marine aeromonad because of its halo-tolerance and very close biochemical characteristics to the genus *Aeromonas.* Growth occurs at 40°C.

V. metschnikovii is an unusual type of vibrio since it is negative to Kovac's oxidase test. It was originally isolated in 1884 from faeces of cholera patients. Growth occurs at 40°C.

There are other less well known vibrios associated with fish such as the luminous vibrios: *V. harveyi, V. splendidus* biotype I, *V. fisheri, V. orientalis*, hyper-saline vibrio: *V. costicola* or pigmented vibrios: *V. nigripulchrituda, V. gazogenes* and *V. logei.*

In this study one single *Vibrio* isolate was used. This isolate was supposed to work as a model for the study carried out.

This bacteria was selected on the grounds that it was obtained from a clinical outbreak of disease in farmed shrimps. Today, this is the most reliable method for obtaining potentially pathogenic strains of vibrio. It is necessary to rely on strains isolated from clinical outbreaks because at present there is no effective way for determining the relative pathogenicity of vibrio isolates.

Different infectivity challenges have been used for this purpose. Most commonly injection challenges are ineffective as it is an artificial route of infection which results in mortalities regardless of the type of bacteria used.

A bath challenge would be the most appropriate method to test the pathogenicity of the bacterial strains. Another study was underway concurrently to this one. The aim of the other study was to develop such a challenge. At the time this study was performed no bath model was available. However, the same isolate was used in an injection challenge which provided additional information which suggested its potentially pathogenicity.

In this study, it was necessary to work only with one isolate because of the amount of work involved in the identification, production of the antiserum and the limited number of shrimps available for the infectivity trials. Should a method for assessing pathogenicity be established it would be interesting to repeat this study with a range of isolates.

In this chapter the isolate was identified based on its biochemical characteristics. Electrophoretic separation of bacterial outer membrane on polyacrilamide gels was used to identity the outer membrane proteins and lipopolysaccharides based on their apparent molecular weight relative to known standards.

MATERIALS AND METHODS

1.1. BACTERIAL ORGANISMS

The vibrio isolate was originally from the hepatopancreas of diseased shrimp being farmed at 29°C and 32 ppt salinity in Thailand. Three days before the bacteria was isolated clinical signs of the disease affected 60% of the stocked shrimp. At 64 days post-stocking the average weight of the shrimps was only 4.9g reflecting slow growth. The expected weight would be between 7g and 8g (Funge-Smith, personal communication). Affected shrimp were inappetant and in some cases were affected with protozoan fouling and black gills.

This organism was later identified as gelatin negative *Vibrio vulnificus* biotype I (*V. vulnificus* b. I) following the procedures described in section 3.

The biochemical and structural characteristics of the isolate were compared with a group of organisms. The selection of these organisms was based on their previous description as shrimp pathogens such as: *V. parahaemoliticus, V. alginoliticus, V. fluviali, V. vulnificus, Aeromonas, Pseudomonas* (Brook and LeaMaster, 1992) and *Pleisomonas shigelloides* (Lightner, 1988). In addition, because they are commonly found in water: *Escherichia coli* and *Pasteurella piscicida*.

The origin of each selected organism and its animal source, when known, are detailed in table 5.

Table 5: Organisms used in this study.

Organism	Origin	Specie origin
V.vulnificus b. I	Thailand	shrimp
V.anguillarum	Scotland	salmon
V.parahaemoliticus	NCIMB1902	human faeces
V.vulnificus	NCIMB2136	eel
V.alginoliticus	NCIMB1339	hard clam
V.fluvialis	NCIMB2249	human faeces
A. salmonicida	Scotland	salmon
A. hydrophila	Scotland	salmon
E.coli	Inst. Aquaculture	
P. piscicida	Inst. Aquaculture	
P.shigelloides	NCIMB9243	human faeces

2.1. BACTERIAL CULTURE

Bacterial organisms were supplied as bead culture, freeze dried or inoculated plates. All of them were then cultured in tryptic soy agar (TSA), supplemented with 1.5% sodium chloride (NaCl) for *Vibrio* spp and *P. piscicida* then incubated at 22°C. Purity was checked and bacteria subcultured again from single colonies in new TSA plates, supplemented with salt when required.

3.1. BACTERIAL PHENOTYPICAL IDENTIFICATION

The vibrio isolate was studied through the following procedures:

3.1.1. **Gram's staining** (see appendix 1 for description)

3.1.2. **Motility test**:

Motility was observed directly under the microscope using a suspension of living bacteria in sterile saline in a hanging drop from underside of a coverslip, mounted using soft paraffin on a microscope slide. Direct observation of the slide under the X40 objective lens revealed whether the bacteria were motile.

3.1.3. **Oxidase test**

This test demonstrate whether the bacteria possesses certain oxidase enzymes which are involved in electron transfer from electron donors. When the redox dye tetramethyl-p-phenylenediamine is used as the electron acceptor, it will be reduced and turn a blue colour.

Growth from a TSA agar culture of the organism was smeared onto an oxidase strip (BioMeriaux). The colour of the smear was noted after 30 seconds. Cultures must be smeared using a platinum wire as other

bacteriological wires may contain traces of iron which could catalyse the reaction and give false positive results.

3.1.4. Growth in cholera medium TCBS

TCBS media plates were inoculated with the organism and incubated for 24 hours at 22°C. Yellow colonies were interpreted as sucrose fermentative organisms whereas blue or green colonies were interpreted as no reaction with sucrose.

3.1.5. Sensitivity to the vibriostatic 0/129

0/129 (2,4-Diamino-6,7-di-iso-propyl pteridine phosphate) is a bacteriostatic agent effective against *Vibrio* spp. This sensitivity differentiates them from *Aeromonas* spp which are resistant to 0/129.

Sensitivity was determined by placing filter paper discs impregnated with 0/129 (10μg and 150μg) on a TSA plate (1.5% NaCl supplemented) which had been spread with a suspension of the isolate under test. After 24h incubation at 22°C, sensitivity to 0/129 was read as follow:

150μg	10μg	Interpretation
No zone	No zone	Resistant
Zone	No zone	Partially sensitive
Zone	Zone	Sensitive

3.1.6. O-F test

This test demonstrates whether bacteria can break down glucose aerobically (oxidation) or anaerobically (fermentation).

A culture of the organism was inoculated into two O-F medium tubes by a single stab with a straight wire. One tube was incubated in the presence of air (open tube) whereas the other one was covered with a thick layer of liquid paraffin to exclude air (close tube). The medium contains bromothymol blue pH indicator to show the formation of acid from the breakdown of glucose. After 24 hours incubation at 22°C, results were read and interpreted as follow:

Open tube	Close tube	Results
Green	Green	No reaction
Blue at top	Green	Alkaline reaction
Yellow	Green	Oxidative
Yellow	Yellow	Fermentative

3.1.7. Growth in different NaCl concentrations

Duplicate TSA plates supplemented with 0, 4, 6, 8 and 10% NaCl were inoculated with the isolate under study. After 24h incubation at 22°C growth was observed.

3.1.8. Antibiotic sensitivity test:

Sensitivity to a range of antibiotics was determined by placing filter paper discs impregnated with the antibiotic on Mueller-Hinton agar plates which had been spread with a suspension of the organism. After 24h at 22°C plates were examined for the presence of zones of inhibition.

The antibiotics tested against the bacteria were:

Antibiotic	Symbol	Disc content (μg)
Oxytetracycline	OT	30
Oxolinic acid	OA	2
Co-Trimoxazole	S+T	25
Nitofurantoin	F	100
Furazolidone	FR	50
Amoxycillin	AML	10

3.1.9. API 20E Microbial identification test

The API 20E strips (BioMeriaux) are a microtube system enabling 22 biochemical tests to be carried out on a bacterial culture. These tests used are as follows:

Beta-galactosidase activity (ONPG),
arginine dihydrolase activity (ADM),
lysine decarboxylase activity (LDC),
ornithine decarboxylase activity (ODC),
citrate utilization (CIT),
hydrogen sulphide production (H_2S),
urease activity (URE),
tryptophane desaminase (TDA),
indol production (IND),
acetoin production (VP),
gelatinase activity (GEL),
nitrate production (NIT),
acid and gas production from glucose and sugar oxidation:

- glucose (GLU),
- mannitol (MAN),
- inositol (INO),
- sorbitol (SOR),
- rhamnose (RHA),
- saccharose (SAC),
- melobiose (MEL),
- amygdalin (AMY)
- arabinose (ARA).

The bacteria were tested in duplicate but since different results of some of the API biochemical reactions were observed, a selection of colonies from different plates and grown in different conditions were tested again with API:

- Four fresh cultures in TSA that had not been subcultured (colonies 1,2,3,4), sometimes in duplicate (a and b).

- A colony isolated from the edge of a 10μg 0/129 disc (colony 5).

- A colony isolated from a TSA plate which had previously proved to be urease activity positive in urea media (colony 7).

- Colonies which appeared as a regrowth after 15 days (colonies 8, 9 and 10).

Incubation was performed at 22°C (except colony 4b at 37°C) for 24h (except colonies 4a and 4b for 48h).

3.1.10. Urea hydrolysis test

Due to inconsistent results obtained for the urease activity with the API system, urea media was prepared in some trials with the addition of 1.5% NaCl. Twenty single colonies, were inoculated. Reaction should be completed after 3h to 5h at 35°C for *Proteus* spp but hydrolysis by some

other bacteria need a much longer incubation period (24-48h). In this case, reading of the results was performed at 3, 5 and 24h incubation at 22°C and 37°C.

Controls of base urea-free media were also inoculated because prolonged incubation may produce an alkaline reaction not caused by urease activity.

3.1.11. Degradation of the gelatin test

Ability to degrade gelatin was determined by placing charcoal gelatin discs (Oxoid) onto agar plates which had been spread with a suspension of the organism. After 24h and 48h at 22°C plates were examined.

4.1. STANDARD GROWTH CURVE

A standard curve of *V. vulnificus* b. I colony formation units (CFU) versus OD was necessary in order to estimate the concentration of CFU in a suspension by reading its optical density.

Bacteria were grown in TSB (supplemented with 1.5% NaCl) at 22°C for 24h. The broth was centrifuged at 2200g at 4°C for 20 minutes. The supernatant was poured off and the pellet resuspended in sterile saline. This wash was

repeated twice and the pellet finally resuspended again in sterile saline. This suspension was diluted to approximately match the following optical densities read at 610nm in a spectrophotometer: 1.0, 0.8, 0.6, 0.5, 0.4, 0.3, 0.2. Each of these suspensions (0.1ml) were plated in TSA (2% NaCl) in triplicate and incubated at 22°C for 24h.

The number of colonies grown was counted and an average CFU/ml calculated. These results were plotted against the OD read at 610nm.

5.1. BACTERIAL STRUCTURE IDENTIFICATION

5.1.1. Outer membrane protein extraction

Outer membranes proteins from the 9 different bacterial organisms listed in table 5 were extracted for later use in gel electrophoresis. A modification of the method by Chart and Trust (1983) was employed.

1. **Materials**:

1.1. Equipment:

.MSE 150 Watt Ultrasonic disintegrator

.MSE Mistral 3000 i centrifuge

.Beckman L8-M ultracentrifuge

1.2. Reagents and solutions:

.20mM Tris-HCl buffer pH 7.2

.EDTA sodium salt

.Deoxyribonuclease I (Sigma)

.Ribonuclease type III-A (Sigma)

.1.5% Solution of n-laurylsarcosine sodium salt in distilled water

2. **Method**:

2.1. **Sonication of cells**:

Each bacterial sample was cultured in 200ml of TSB and harvested by centrifuging at 3000g for 15 minutes at 4°C. The pellets were washed twice by resuspending them in 20mM Tris buffer and bacteria collected again. Pellets were suspended in Tris buffer containing 10mM EDTA and 0.2μg each of DNAase and RNAase per ml in order to degrade the released nucleic acid.

Cell disruption was carried out on ice by sonication. Bacteria were sonicated 6 times, with a 3 minute interval between each sonication to avoid any significant temperature increase of the probe. Bacteria were sonicated the first 3 times for 15 seconds with the power in the intermediate position, and with amplitude set at position 3. Then the bacteria were sonicated a further 3 times for 15 seconds but with the power set at maximum and the amplitude

in position 4.

2.2. **Dissolution of inner membranes**:

Sonicated cells were centrifuged at 2000g for 30 minutes at 4°C to spin down whole unbroken cells. Supernatants were centrifuged at 40000g for 1h at 4°C to pelletise both the inner and the outer membranes. To dissolve the inner membranes the pellets were treated with 1ml of n-laurylsarcosine solution for 30 minutes at room temperature. Samples were pipetted gently during the treatment to dissolve the pellets. Another 7ml of Tris buffer were added to each tube and the samples centrifuged at 2000g for 10 minutes at 4°C to spin down any particulate matter and to eliminate the foam.

Supernatants were then centrifuged at 40000g for 1.5h at 4°C to spin down the outer membranes. Pellets were resuspended in 20ml of Tris buffer, divided into two samples of 10ml each and centrifuged again at 40000g for 1.5h at 4°C.

Supernatants were discarded and for each bacterial sample one pellet was resuspended in 200μl of Tris buffer and frozen at -70°C for future analysis by electrophoresis.

5.1.2. OUTER MEMBRANE LIPOPOLYSACCHARIDES (LPS) EXTRACTION

1. **Materials**

Proteinase K

20mM Tris buffer pH 7.2

2. **Method**

A stock solution of proteinase K enzyme (Sigma) was prepared (10mg/ml) in 20mM Tris-HCl buffer. 10μl of this stock solution was added to 100μl of each outer membrane extraction sample suspended previously in 200μl of 20mM Tris-HCl buffer.

Samples were incubated at 60°C and the reaction stopped by adding 400μl of sample buffer.

5.1.3. SODIUM DODECYL SULPHATE (SDS)- POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

This technique allows the separation of the different molecules, including possible antigens, according to their charge and size.

1. **Materials**

1.1. Equipment:

.Mini Protean II System Bio-rad

.Microlitre syringe (Hamilton Company)

1.2. Reagents and solutions

.N,N,N',N'Tetramethylethylenediamine (TEMED)

.10% Ammonia persulphate solution in distilled water. It had to be prepared fresh.

.1% Bromophenol blue in distilled water

.Iso-butanol

.Separating gel buffer: 1.5M Trizma base n-Lauryl sulphate sodium salt (SDS), pH was adjusted to 8.7 and stored at 4° C no longer than 4 weeks.

.Stacking gel buffer: 0.5M Trizma base

0.4% SDS

pH was adjusted to 6.8 and stored at 4°C no longer than 4 weeks.

.Sample buffer (2x concentrated):

30ml of 10% SDS

12.5ml of stacking buffer

10ml glycerol

pH was adjusted to 6.8 and stored no longer than 4 weeks.

.Acrilamide stock solution:

30% Acrilamide

0.8% Bis-acrilamide

Solution was filtered through Whatman paper number 1 and stored in a dark bottle at 4°C no longer than 4 weeks.

.Reservoir buffer (5x concentrated):

0.125M Trizma base

0.96M Glycine

0.5% SDS

pH was adjusted to 8.3 and store at 4°C no longer than 4 weeks.

.SDS-page low molecular weight standard (Bio-rad)

.Staining solution: 0.1% Coomassie Blue R250 CI 42660

16.9% Glacial acetic acid

41.5% Methanol

41.5% Distilled water

Dye was dissolved in water and solvents added, then filtered through Whatman paper number 1 and stored no longer than 8 weeks.

.Destaining solution: 10% Glacial acetic acid

40% methanol

50% distilled water

.10% Glacial acetic acid in distilled water

2. **Method**:

The description for this standard technique is detailed in the appendix II.

Molecular weight of the unknown proteins was determined by using a curve generated by plotting the molecular weight of the standard versus the distance migrated from the interface of the stacking and separating gel. This curve was plotted for each gel.

5.1.4. GEL STAINING

a. **Coomassie blue stain**

Staining of the outer membrane proteins was achieved by placing the gel in Coomassie Blue staining solution for 30 minutes and then in destaining solution for 15 minutes. The gel was kept in glacial acetic acid solution.

b. **Silver stain**

Silver staining (Bio-rad) was used to visualised the LPS and protein. Glassware and all the materials that could contact the gel including the gloves had to be cleaned carefully and rinsed with nanopure water.

The gel was stained by treating it with the following solutions and method:

1.	Fixative (40% methanol/10%acetic acid)	200ml	1h
2.	Fixative (10% ethanol/5% acetic acid)	200ml	30min
3.	Fixative (10% ethanol/5% acetic acid)	200ml	30min
4.	Oxidiser (10ml reagent/90ml H_2O)	100ml	10min
5.	Nanopure water	200ml	10min
6.	Nanopure water	200ml	10min
7.	Nanopure water	200ml	10min

Repeat until the yellow colour is removed from the gel.

8.	Silver reagent (10ml reagent/90ml H_2O)	200ml	30min
9.	Nanopure water	200ml	2min
10.	Developer (9.6gr reagent/300ml H_2O)	100ml	30sec

Develop until yellow colour is obvious or brown precipitates appears, then pour off and add fresh developer.

11.	Developer (9.6gr reagent/300ml H_2O)	100ml	5min
12.	Developer (9.6gr reagent/300ml H_2O)	100ml	5min
13.	Stop reaction with 5% acetic acid	200ml	5min

RESULTS

3.2. BACTERIAL IDENTIFICATION

3.2.1. Gram's staining

The bacterium was visualized as a medium size Gram negative rod.

3.2.2. Motility test

Bacteria appeared to be motile.

3.2.3. Oxidase test

The result of the oxidase test was positive.

3.2.4. Growth in cholera medium TCBS

Bacteria grew as medium size green colonies.

3.2.5. Sensitivity to the vibriostatic O/129

The organism was found to be resistant to 10μg, but sensitive to 150μg.

3.2.6. O-F test

Bacteria fermented glucose as media turned yellow in the open tube as well as in the closed tube.

3.2.7. Growth in different NaCl concentrations

Colonies grew between 4 and 6% NaCl , but not in 0, 8 or 10% NaCl.

3.2.8. Antibiotic sensitivity test

The bacterium was sensitive to all the antibiotics tested except for amoxycillin (10μg). The diameters of the inhibitory zones for each antibiotic are listed in table 6.

Table 6: Results from the antibiotic sensitivity test.

Antibiotic	**Disc content(μg)**	**Zone diameter(cm)**
Oxytetracycline	30	3
Oxolinic acid	2	1.9
Co-Trimoxazole	25	2.6
Nitrofurantoin	100	1.9
Furazolidone	50	2.3
Amoxycillin	10	0

3.2.9. API 20E Microbial identification test

Results from API 20E biochemical tests were variable, especially those concerning urease activity, gelatinase activity, citrate utilization and melibiose oxidation. The results read after 24h or 48h incubation at 22 or 37°C, as previously described, are displayed in table 7.

The results from the API tests were inconsistent. It is possible that this was related to the conditions under which the bacteria were grown.

Table 7: Results from the API 20E biochemical tests.

	1a	1b	2a	2b	3	4a	4b	6	7	8	9	10	11
ONPG	+	+	+	+	+	-	-	+	+	+	+	+	+
ADH	-	-	+	d	-	+	+	d	-	-	d	d	d
LDC	+	+	+	+	+	+	+	+	+	+	+	+	+
ODC	+	+	+	+	+	+	-	+	+	+	-	-	-
CIT	d	d	+	+	-	+	-	d	-	+	-	-	-
H_2S	-	-	-	-	-	-	-	-	-	-	-	-	-
URE	+	+	-	-	-	+	-	-	-	-	-	-	-
TDA	d	d	d	d	d	-	-	d	d	d	-	-	-
IND	+	+	+	+	+	+	+	+	+	+	+	+	+
VP	-	-	-	-	-	-	-	-	-	-	-	-	-
GEL	-	-	-	-	-	+	+	-	-	-	+	+	+
GLU	+	+	+	+	+	+	+	+	+	+	+	+	+
MAN	+	+	+	+	+	+	+	+	+	+	+	+	+
INO	-	-	-	-	-	-	-	-	-	-	-	-	-
SOR	-	-	-	-	-	-	-	-	-	-	-	-	-
RHA	-	-	-	-	-	-	-	-	-	-	-	-	-
SAC	-	-	-	-	-	-	+	-	-	-	-	-	-
MEL	d	d	+	d	+	+	-	d	d	-	-	-	-
AMY	+	+	+	+	+	+	+	+	+	+	+	+	+
ARA	+	-	+	+	-	-	-	-	-	-	-	-	-
NIT	+	+			+	+	-	+	-	+	+	+	+

+:positive reaction, -: negative reaction, d: unclear reaction. Read after 24h incubation (except colonies 4a and 4b after 48h), at 22°C (except colony 4b at 37°C).

The definitive biochemical profile was concluded as:

+ - + + + v - v d + - - + + - - - - v + - +

(+: positive reaction, -: negative reaction, d: unclear reaction, v: variable reaction).

3.2.10. Urea hydrolysis test

Urease activity varied greatly. Three of the 20 samples incubated at 22°C were positive within 3h, two of them became positive in 5h and another eight in 24h, while seven of them remained negative.

The samples incubated at 37°C became positive in a narrower range of time (7h) but some of them remained negative.

Controls remained negative for the mentioned lengths of time.

3.2.11. Degradation of gelatin test

The organism was unable to degrade the charcoal gelatin.

Finally, the isolate received the name of Gelatine negative *Vibrio vulnificus* biotype I.

4.2. STANDARD CURVE FOR *V. VULNIFICUS* B. I

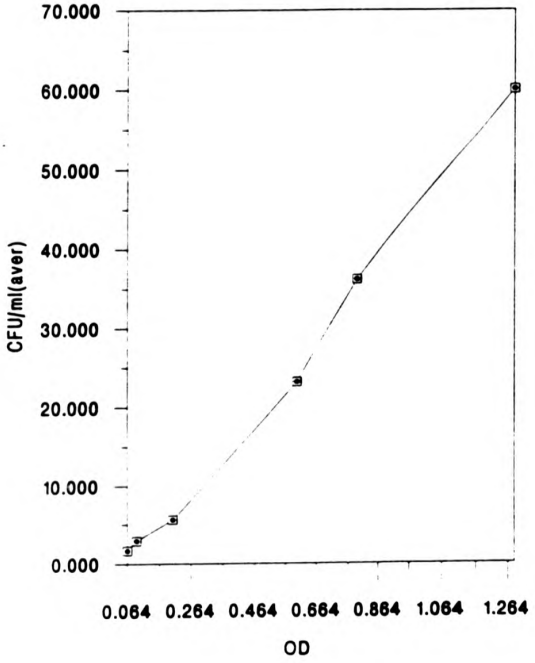
The number of colony formation units (CFU)/ml grown from bacteria suspensions at certain optical densities (OD) are shown in table 8.

Table 8: Number of CFU of *V. vulnificus* b. I at a certain OD read at 610nm.

OD	CFU/ml (average)
1.284	60.0
0.794	36.20
0.602	23.2
0.206	5.61
0.093	2.91
0.064	1.7

The plotting of the values obtained after culturing bacteria at different concentrations gave the graph displayed in figure 2.

Figure 2: Standard growth curve for *V. vulnificus* b. I



5.2. BACTERIAL STRUCTURE IDENTIFICATION

5.2.1. Analysis of the outer membrane protein samples stained with coomasie blue

The protein pattern of the different bacteria studied revealed differences in their structure.

The outer membrane extracts of *V. vulnificus* b. I isolate showed two major bands of 23 and 25 KD and some minor ones of 74, 70, 65, 40, 38 and 13.5 KD (figures 3, track 2 and figure 4, track 7).

V. vulnificus outer membrane extracts showed a single and major band of 27 KD (figures 3, track 3 and figure 4, track 6).

V. parahaemoliticus outer membrane extracts showed one major band of 29 KD and a minor one of 28 KD (figure 3, track 4 and figure 4, track 3).

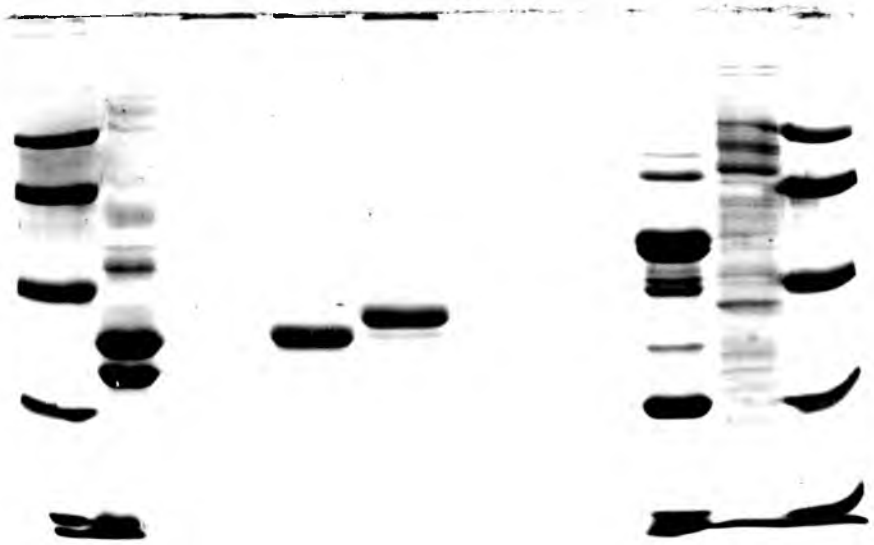
P. fluorescens outer membrane extracts displayed a rich pattern with three major bands of 20, 34 and 40 KD and six minor ones of 55, 52, 33, 32, 25 and 14 KD (figure 3, track 5).

V. fluvialis outer membrane extracts showed a single band of 27-28 KD (figure 4, track 4).

V. anguillarum outer membrane extracts displayed a major band of 27-28 KD and a minor one of 35 KD (figure 4, track 5).

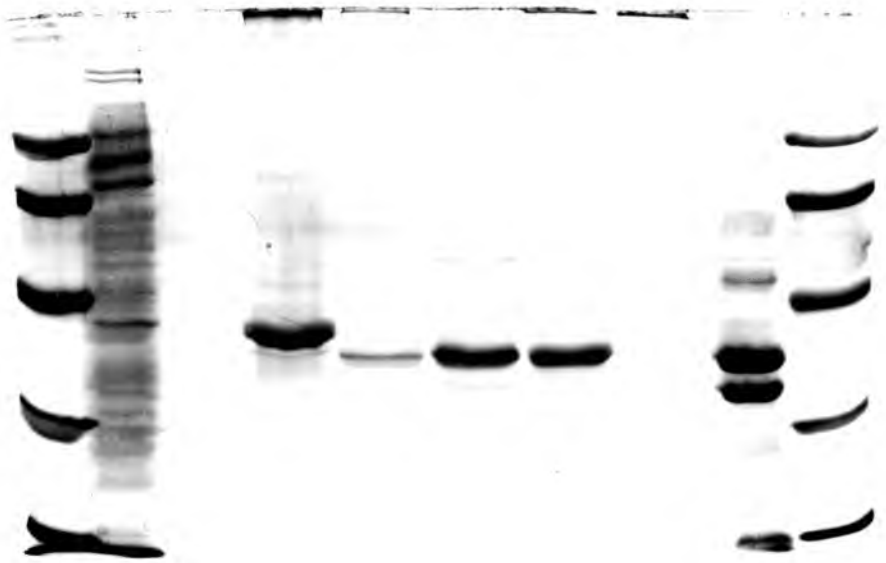
Whole cell extracts of *V. vulnificus* b. I isolate showed an almost continuous succession of bands between which 82, 80, 65, 60, 55 and 30 KD bands could be distinguished (figures 3, track 6 and figure 4, track 2).

The molecular weight standard was run at both sides of the gels and showed five bands of 66.2, 45, 31, 21.5 and 14.4 KD (figures 3, tracks 1 and 7 and figure 4, tracks 1 and 8).



Tracks

Figure 3: SDS-PAGE stained with Coomassie blue of the outer membrane extracts of *V. vulnificus* b. I isolate (track 2), *V. vulnificus* (track 3), *V. parahaemolyticus* (track 4) and *P. fluorescens* (track 5). Whole cell extract of *V. vulnificus* b. I isolate (track 6). Tracks 1 and 7 represent the molecular weight standard.



Tracks

Figure 4: SDS-PAGE stained with Coomassie blue of the whole cell extracts of *V. vulnificus* b. I isolate (track 2), outer membrane extract of *V. parahaemolyticus* (track 3), *V. fluvialis* (track 4), *V. anguillarum* (track 5), *V. vulnificus* (track 6) and *V. vulnificus* b. I isolate (track 7). Tracks 1 and 8 represent the molecular weight standard.

5.2.2. Analysis of LPS samples with silver staining

V. vulnificus b. I LPS sample showed one broadd band with molecular weight ranging from 50 to 99KD approximately.

DISCUSSION

Prior to receiving the isolate under study it had already been identified as a member of the genus Vibrio. Further investigation was carried out to confirm the genus and determine the species.

The isolate was Gram negative, a short rod and motile. Oxidase reaction, fermentation of glucose and sensitivity to the vibriostat 0/129 (150μ) were positive. The isolate was able to grow in TCBS media forming green colonies showing its was inability to utilize sucrose. These selected features are common to the *Vibrio* spp placing the isolate in the genus *Vibrio*, thus confirming the original identification.

Results from API tests gave a tentative identification of *V. vulnificus*, in particular biotype I as it is glucose positive and biotype II is negative.

Doubts were raised about the organism being *V. vulnificus* or *V. harveyi* as they both have very similar biochemical characteristics. In Lightner's personal experience (personal communication) *V. harveyi* is urease positive, although insufficient number of samples had been tested to conclude that this is a definitive distinguishing characteristic. Urease positive *V. vulnificus* may be *V. harveyi*.

Two week old plates showed a "regrowth" of small colonies growing in the edges of the former colonies. Although they had the same appearance in colour and shape it was thought that they might be a second organism, which

would have a slower growth. Subcultures of the former colonies showed that they were dead. These new colonies arise from viable organisms, growing at the edges of dead colonies where they had access to nutrients. It proved possible to utilise these colonies in the API test where they provided identical results to the original rapidly-growing colonies.

No growth was observed when TSA media was either supplemented with 8 or 10% NaCl or not supplemented at all. Colonies grew only at 2, 4 and 6% NaCl concentration. It showed sensitivity to all the antibiotics tested except to amoxycillin. The greatest sensitivity was shown to oxytetracycline, then to co-trimoxazole and furazolidone, and the smallest sensitivity to oxolinic acid and nitrofurantoin. A uniform growth pattern was observed rejecting the possibility of a mixture culture.

Only *V. alginolyticus*, *V. damsela* and *V. charcariaceae* are recognised as urease positive vibrios (Austin and Austin 1988) but it is becoming more common to find *Vibrio* spp with urease activity, perhaps because farmers are using urea to fertilize ponds and bacteria are adapting to use this new nutrient source.

The isolate's urease activity was variable. Even colonies which showed positive activity in urease media were negative in the API test. It was thought that this microorganism had a variable tendency to express its capacity to produce the urease enzyme.

Gelatinase activity was negative when it was expected to be positive as it is characteristic of a *V. vulnificus* organism. Later trials with charcoal gel confirmed its negativity, giving the isolate a final classification of gelatin negative *V. vulnificus* biotype I.

Vibrio spp appear to have a relatively simple outer membrane protein (OMP) profile.

The OMP profile of the *V. vulnificus* b. I isolate showed two major bands of 23 and 25KD approximately, in contrast with *V. vulnificus* which only showed a major band of 27KD. The presence of minor bands in the *V.vulnificus* b. I isolate profile could be due to a larger amount of sample loaded into the gel, as the major bands were heavier and more intensively stained compared with the major bands of the other organisms.

The whole cell extracts of *V. vulnificus* b. I showed an almost continuous succession of bands, which major ones (82, 80, 65, 60, 55 and 30KD) did not coincided with the major OMP (23 and 25KD) showing that these last ones are present in a minor proportion when examining at the whole cell.

In the present study V. anguillarum OMP profile showed two bands of approximately 27-28 and 35kd. Aoki *et al* (1981) described the OMP profile of six different serotypes of *V. anguillarum*. All of them were different but all except for one presented a major protein. Buckley *et al* (1981) described

eight major proteins in *V. anguillarum* in which the predominant protein had an apparent molecular weight of 38KD. Chart and Trust (1984) described the OMP profile of eight strains each of *V. anguillarum* and *V. ordali*. The profiles of both species were dominated by a major protein of an apparent molecular weight of 40KD.

In the present study *V. parahaemolyticus* OMP profile showed two bands of approximately 29 and 28KD. Koga and Kawata (1983) described the OMP profile of *V. parahaemolyticus* as six major proteins with molecular weights ranging from 22 to 40KD depending on the strain. They concluded that the OMP of the *Vibrio* spp varies with the NaCl concentration in the growth medium.

V. fluvialis kept a similar pattern to the other vibrios, showing a single major band of 27-28KD. In contrast, *P. fluorescens* displayed a rich pattern of three major bands (20, 34 and 40KD) and six minor ones (55, 52, 33, 32, 25 and 14KD).

OMP are used to characterise bacterial species. However, discrepancies between profiles of the same bacterial species are commonly found. The different results might be due to the use of different extraction methods and gel electrophoresis system, different growth conditions and strain variability.

The LPS fraction appeared as a continuous band of 50-99KD approximately. This band had a uniform staining, showing no areas more intensively stained.

CHAPTER III

PRODUCTION OF *VIBRIO VULNIFICUS* BIOTYPE I ANTISERUM AND IDENTIFICATION OF THE BACTERIAL ANTIGENS

INTRODUCTION

The purpose of the work described in this chapter was to produce a polyclonal serum against *V. vulnificus* b. I isolate which posteriorly allowed the identification of bacterial antigens after the infectivity trials.

The specificity of the serum was tested with three tests of increasing sensitivity. These tests were bacterial agglutination assay, immunoblot assay and indirect enzyme-linked immunosorbent assay (ELISA).

Finally, a Western blot was used to characterise antigenically *V. vulnificus* b. I isolate. This technique combines electrophoresis with an enzyme-linked immunoassay.

MATERIALS AND METHODS

6.1. POLYCLONAL ANTIBODY SERUM PRODUCTION

Polyclonal antibodies were raised in rabbits against *V. vulnificus* b. I. Two rabbits, 15 weeks old and about 3kg weight, were used for this purpose.

6.1.1. Inoculum preparation:

Bacteria cultured in NaCl supplemented TSB for 24h in exponential growth phase were killed with a 60 minutes bath at 60°C. TSB media was inoculated to confirm bacterial death.

Bacterial concentration required for the inoculum was $2x10^8$ cells/ml. Cells were collected by centrifugation at 2000g for 15 minutes and washed twice with sterile saline. The desired concentration was calculated using the standard curve produced previously (chapter 2). Finally 0.1% of thimerosal was added to prevent bacterial contamination.

6.1.2. Rabbit inoculation:

Prior to inoculation, rabbits were bled from the marginal ear vein. The blood collected was left at room temperature for 4h, the serum was aliquoted and frozen down for later use as negative control.

For the first inoculation, complete Freund's adjuvant was used. Bacterial suspension and adjuvant were emulsified at a 1:1 ratio. 1ml containing approximately 10^8 cells was injected subcutaneously.

The second and third inoculations were performed as described above but using incomplete Freund's adjuvant separated by three weeks on each occasion.

A fourth inoculation was performed 10 days after the third subcutaneous one. In this occasion 1ml of dead bacteria (10^8 cells) resuspended in sterile saline, with no adjuvant or thimerosal was injected intravenously.

The rabbits bleed out was performed 7 days after the last inoculation. Their blood was collected and allowed to coagulate in a beaker at room temperature for 4h. Serum was centrifuged to free it from any residual cells. Specificity and titration studies of immunised and preimmunised sera were then conducted.

7.1. SERUM SPECIFICITY

Serum obtained from the rabbits was tested against the organisms listed in table 5, following three different procedures.

7.1.1. Bacterial agglutination assay

Agglutination reaction is one of the most easily performed immunological tests.

One or more bacterial colonies were resuspended in a small amount of saline until the suspension reached the colour and density of skimmed milk. Serum anti-*V. vulnificus* b. I was used at full strength. One drop of each dilution was added to each bacterial suspension drop on the slides. Incubation was performed at room temperature. The slides were examined macroscopically as well as under low power microscopy (10X) at various intervals for clumping of bacteria. Strong agglutination was expected to occur within 5 minutes (Roberson, 1990).

Setting patterns were examined and scored as:

+++ Strong agglutination

++ Moderate agglutination

+ Slight agglutination

- No agglutination

7.1.2. Immunoblot assay

In the immunoblot assay the antigen was bound by simple adsorption to a

nitrocellulose membrane and detected by an immuno-enzymatic reaction.

1. **Materials**

.Nitrocellulose membrane (Bio-rad)

.Skimmed dry milk

.Rabbit *V. vulnificus* b. I antiserum

.Antirabbit HRP conjugated IgG (SAPU)

.Trizma buffered saline (TBS): 10mM Trizma base

0.15mM NaCl

Stored at 4°C for not longer than 4 weeks.

.Substrate: 6mg 3,3'diamino benzidine (DAB)

10μl $H_2)_2$

10ml TBS

This had to be prepared fresh.

2. **Method**:

Nitrocellulose membrane was wetted with water and air dried in order to avoid the diffusion of the solution added. A pencil was then used to mark where the drops would be placed.

The different bacteria were cultured in TSA supplemented with NaCl if required. A few colonies of each culture were resuspended in sterile saline

to a similar turbidity. Six drops of 1μl each from each organism were placed on the membrane. The drops were allowed to dry.

The whole membrane was immersed in a blocking solution of 2.5% skimmed dry milk in TBS for 20 minutes and rinsed with 0.5% of skimmed dry milk. The membrane was allowed to dry. *V. vulnificus* b. I antiserum diluted 1:2, 1:10, 1:30, 1:60, 1:100 and 1:200 was placed onto the bacterial drops and incubated for 30 minutes at room temperature.

The membrane was then washed in 0.5% skimmed dry milk for 15 minutes in a shaker and dried. HRP-antirabbit antiserum diluted 1:100 in TBS was placed on the membrane and incubated for 30 minutes. Finally, the membrane was soaked in the substrate for 10 minutes and then washed with 0.5% skimmed dry milk.

Because reactive antigens appears as a vivid coloured dot on the membrane, the immunoblot results can be interpreted by visual inspection.

7.1.3. Enzyme linked inmunoabsorbent assay (ELISA):

The ELISA is a sensitivity system which allows for the quantification of serum antibodies. Some of the advantages of this assay are its high sensitivity compared to other tests, the small amounts of serum required and the ability

to determine the serum titre.

The ELISA was performed with serum from immunised rabbits and preimmunised rabbits as control and with the *Vibrio* spp organisms listed in table 5.

1. **Materials**:

1.1. Equipment:

.MSE Mistral 3000 i

.Dynatech MR 5000 ELISA plate reader

.Microtitre plates

1.2. Reagents and solutions:

.2M H_2SO_4

.Foetal calf serum (FCS)

.Blocking solution: 1% bovine serum albumin (BSA) in distilled water

.Coating buffer: 0.5M $NaCO_3$

0.5M $NaHCO_3$

0.01%(w/v) Thiomersal

pH was adjusted to 9.6 and stored at 4°C no longer than 2 weeks.

.1% Poly-L-lysine solution in coating buffer

.Low salt wash buffer: 0.2M Trizma base

0.38M NaCl

0.01%(w/v) Thimerosal

0.05% Polyoxethylene (20) sorbitol monolaurate (Tween-20)

pH was adjusted to 7.4 and stored at 4°C up to 2 months.

.High salt wash buffer: 0.02M Trizma base

0.5M NaCl

0.01%(w/v) Thimerosal

0.01% Tween-20

pH was adjusted to 7.4 and stored at 4°C no longer than 8 weeks.

.Phosphate buffered saline (PBS): 0.02M $NaH_2PO_4H_2O$

0.02M $Na_2HPO_42H_2O$

0.15M NaCl

0.01%(w/v) Thimerosal

pH was adjusted to 7.2 and stored at 4°C no longer than 8 weeks.

.Substrate buffer: 0.1M Citric acid

0.1M Sodium acetate (anhydrous)

pH was adjusted to 5.4 and 33.3 μl of H_2O_2 added to 100ml of the above solution and stored at 4°C.

.0.05% Glutaraldehyde in PBS

.Rabbit *V. vulnificus* b. I antiserum

.Pre-immunised rabbit serum

.Antirabbit HRP conjugated IgG (Scottish antibody production unit, SAPU)

.Chromogen: 42mM of 3,3',5,5'tetramethylbenzidine dihydrochloride (TMB) in 1:2 solution of glacial acetic acid and distilled water.

2. **Method**:

Wells were coated with 50μl of poly-L-lysine solution covered with parafilm and left for one hour at room temperature. The polycationic nature of this molecule allows interaction with the anionic sites of the cells resulting in strong adhesive properties. Plates were washed three times with high salt wash buffer. On the third wash, they were left to soak for 5 minutes. Any excess solution remaining on the plates was removed by tapping the inverted plates onto several paper towels.

Bacteria were grown for 48h in TSB, supplemented with 1.5% (NaCl when required) at 22°. Broth was centrifuged at 1800g for 10 minutes. Supernatants were removed and cells resuspended in PBS. This wash was repeated twice. Bacterial concentration was diluted 10^8-10^9 cells by adjusting the optical density to 1.0 at 610nm. 50μl of bacteria solution was added to each well and left for 1h at room temperature. Plates were shaken to ensure that antigen solution was evenly distributed over the bottom of each well. Without removing the supernatant from the wells , fixation of the bacteria was encouraged by the addition of 0.05% of glutaraldehyde at 50μl/well for 20 minutes at room temperature.

Plates were washed three times with high salt wash buffer as described before and then dried.

To prevent nonspecific adsorption of the serum or conjugates onto the plate surface, 200μl of blocking solution was added and incubated for 1h at room temperature. At this step plates could be wrapped in foil and store in the fridge for a maximum of 48h. Blocking solution was discarded. Plates were washed and dried carefully, as described before, as moisture can dilute any reagent added subsequently to the wells.

Doubling dilutions of the sera (control and anti-*V. vulnificus* b. I) were prepared in low salt wash buffer from 1:100 to 1:3,676,800. 100μl of the serum dilutions were added to each well in duplicate, maintaining a negative control with only low salt wash buffer. Incubation lasted 30 minutes at room temperature. Plates were washed again. 100μl of antirabbit HRP-conjugated IgG were diluted 1:1000 in low salt wash buffer with 10% foetal calf serum (FCS). 100μl of the suspension were placed in each well and incubated for 30 minutes. Plates were washed as described previously.

Chromogen was mixed at 1% with substrate buffer and 100μl/well dispensed. Incubation lasted 2 minutes at room temperature. Reaction was stopped by adding 100μl/well of 2M H_2SO_4 and results were read in an ELISA reader at 450nm.

8.1. WESTERN BLOT

1. **Material**:

1.1. Equipment:

.Trans-blot SD semi-dry transfer cell Bio-rad

.Mini protean II multiscreen Bio-rad

.Nitrocellulose paper (Bio-rad)

.Whatman filter paper number 1

1.2. Reagents and solutions:

.Transfer buffer: 0.025M Trizma base

0.192M Glycine

3.75ml 10% SDS

200ml Methanol

800ml Distilled water

.10% n-Lauryl sulphate sodium salt (SDS)

.Phosphate buffer solution (PBS): 0.02M $NaH_2PO_4H_2O$

0.02M $Na_2HPO_42H_20$

0.15M NaCl

Stored at 4°C for not longer than 4 weeks.

.Trizma buffered saline (TBS): 10mM Trizma base

0.15mM NaCl

pH was adjusted to 7.6 and stored at 4°C no longer than 4 weeks.

.Foetal calf serum (FCS)

.Staining solution: 0.1% Coomassie Blue R250 CI 42660
16.9% Glacial acetic acid
41.5% Methanol
41.5% Distilled water

Dye was dissolved in water and solvents added, then filtered through Whatman paper number 1 and stored no longer than 8 weeks.

.Tween-20

.Blocking solution: 0.5% casein in PBS. Prepared fresh.

.Rabbit *V. vulnificus* b. I antiserum

.Antirabbit HRP conjugated IgG (SAPU)

.Substrates: a. 6mg 3,3'diamino benzidine (DAB)
10μl H_2O_2
10ml TBS

It had to be prepared fresh

b. TMB membrane peroxidase substrate (1-component) (Kirkegaard & Perry Laboratories, Inc).

2. **Method**:

Following electrophoresis, gel was equilibrated in transfer buffer for 20-30 minutes for removing electrophoresis buffer salt and detergents. If salts were not removed, they would increase the conductivity of the transfer buffer and the amount of heat generated during the transfer. In addition equilibration allowed the gel to adjust to its final size prior to electrophoretic transfer.

Nitrocellulose membrane was cut to the gel size as well as 6 pieces of Whatman filter paper number 1 and soak carefully in transfer buffer for 20-30 minutes. Air bubbles were avoided in the membrane as they can block transfer of molecules. It was handled with gloves or forceps in order to prevent protein contamination.

Three layers of filter paper were laid one on top of the other inside the transfer cell. The membrane was placed on top of them and then the gel. Another 3 sheets of filter paper were placed on top. Air bubbles were removed rolling a pasteur pippette as they would interfere with the electrophoretic transfer. Gloves were worn for the handling of the filter papers, nitrocellulose membrane and gel. Electrodes were connected and turn on at 15 volts for 15-20 minutes. The transfer efficiency was monitored by the later staining of the gel in electrophoresis staining solution.

The remaining free protein binding sites on the membrane were blocked with casein solution for 2h. After blocking, the membrane was washed 3 times in PBS with 0.05% Tween-20 for 5 minutes each. The membrane was placed in the multiscreen and antibodies against *V. vulnificus* b. I diluted 1:200, 400 and 800 were injected into the multiscreen alternating with blocking solution (600μl). It was then wrapped with parafilm and incubated for 2h at room temperature.

Antibody solution was removed with a pasteur pipette and the membrane washed 3 times in PBS with 0.05% Tween-20 for 5 minutes each. HRP-antirabbit serum was diluted 1:100 in PBS with 1% of FCS and the membrane left to soak in it for 30 minutes. A third wash was given to the membrane as described before. Two substrate were used: DAB and TMB for different occasions. Colour was developed in a few minutes and the reaction stopped by immersing the membrane in tap water.

RESULTS

7.2. SERUM SPECIFICITY

7.2.1. Bacterial agglutination assay

Results were read macroscopically as well as under the microscope at 10X. Agglutination was observed as described in table 9.

Table 9: Results from the bacterial agglutination test.

Organism	Degree of agglutination
V. vulnificus b. I isolate	+++
V. anguillarum	+
V. parahaemolyticus	+
V. vulnificus	++
V. alginolyticus	+
V. fluvialis	+
E. coli	-
P. fluorescens	-
P. shigelloides	-

+++: strong agglutination,

++: moderate agglutination,

+: slight agglutination,

-: no agglutination.

7.2.2. Immunoblot assay

The results from the immunoblot assay are shown in table 10.

Table 10: Results from the immunoblot assay using *V. vulnificus* b. I isolate antiserum.

Organism/Dilution	**full**	**1:2**	**1:10**	**1:20**	**1:50**	**1:100**
V. vulnificus b. I	+	+	+	+	+	+
V. fluvialis	+	+	+	+	-	-
V. anguillarum	+	+	+	+	-	-
V. alginolyticus	+	+	+	-	-	-
P. shigelloides	+	+	-	-	-	-
V. parahaemolyticus	+	+	+	+	-	-
V. vulnificus	+	+	+	+	-	-
E. coli	+	+	-	-	-	-
P. fluorescens	+	+	-	-	-	-

+: positive reaction,

-: negative reaction.

7.2.3. Enzyme linked immunoassay (ELISA)

The results of the assays using rabbit serum prior to inoculation, after the first

two injections and after final boosting against *V. vulnificus* b. I isolate, checking affinity (figure 5) and crossreactivity with other organisms are displayed in the following graphs (figure 6, 7, 8, 9 and 10).

The working dilution chosen later for immunohistochemistry was 1:800 as at that dilution the absorbance of *V. vulnificus* b. I was remarkably larger than the absorbance of the other organisms. The values of absorbance for the different organisms at this dilution are listed in table 11.

Table 11: Absorbance values for the different organisms at 1/800 dilution using anti-*V. vulnificus* b. I serum.

Organism	Absorbance
V. vulnificus b. I	1.449
V. vulnificus	0.691
V. fluvialis	0.737
V. parahaemolyticus	0.453
V. anguillarum	0.360
V. alginolyticus	0.517
E. coli	0.117
P.piscicida	0.351
P. shigelloides	0.075

The absorbance value for *V. vulnificus* b. I isolate using pre-immunized serum at 1:800 dilution was 0.167.

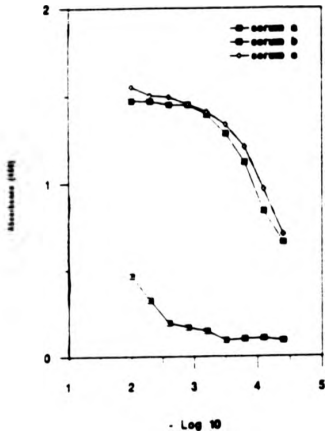


Figure 5. Titration curve for rabbit anti-*V. vulnificus* b. I by ELISA. Serum a: serum prior immunization, serum b: serum after two subcutaneous immunizations and serum c: serum after final boost.

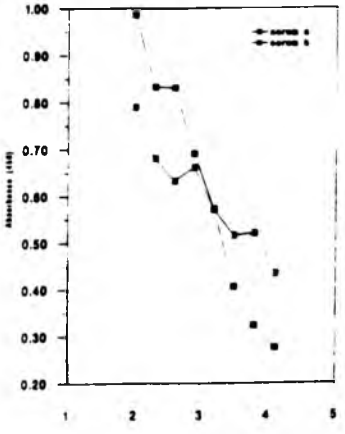


Figure 6. Cross reactivity of rabbit anti-*V. vulnificus* b. I against *V. vulnificus* by ELISA. Serum a: serum prior immunization. Serum b: serum after final boost.

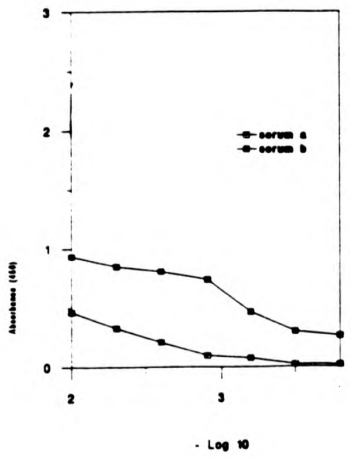


Figure 7. Cross reactivity of rabbit anti-*V. vulnificus* b. I against *V. fluvialis* by ELISA. Serum a: serum prior immunization. Serum b: serum after final boos

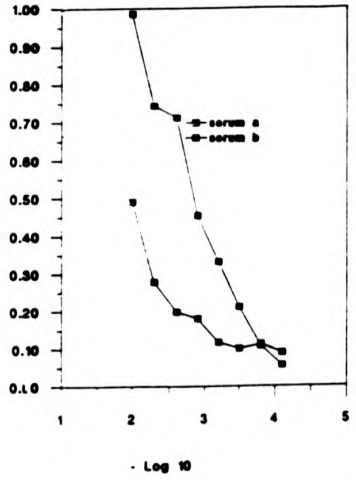


Figure 8. Cross reactivity of rabbit anti-*V. vulnificus* b. I against *V. parahaemolyticus* by ELISA. Serum a: serum prior immunization. Serum b: serum after final boost.

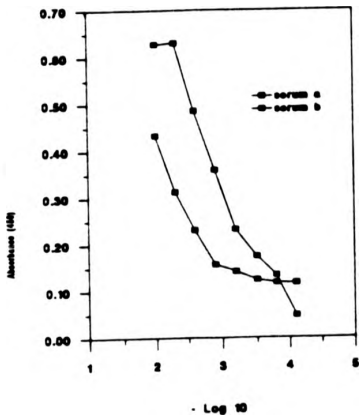


Figure 9. Cross reactivity of rabbit anti-*V. vulnificus* b. I against *V. anguillarum* by ELISA. Serum a: serum prior immunization. Serum b: serum after final boost.

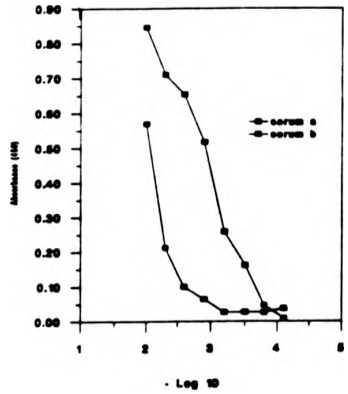


Figure 10. Cross reactivity of rabbit anti-*V. vulnificus* b. I against *V. alginolyticus* by ELISA. Serum a: serum prior immunization. Serum b: serum after final boost.

8.2 WESTERN BLOT

Two components of *V. vulnificus* b. I isolate provoked an antigenic reaction in the rabbit. These components were a 31KD protein and a 90-99KD LPS fraction. They were both found in the OMP sample and whole cell sample.

DISCUSSION

The rabbits used for the production of the serum showed a strong local reaction to the inoculum. Freund's complete adjuvant is a very potent adjuvant consisting of a mixture of mineral oil, emulsifier and killed mycobacteria. The presence of the oil stimulates a local inflammatory response and granulomatous tissue formation around the site of the inoculum, while the antigen is slowly leached from the aqueous phase of the emulsion. The presence of mycobacteria probably serves to enhance interleukin 1 production thus promoting effective presentation of antigen to T and B cells. Freund's incomplete adjuvant is a simple mixture of emulsifier and mineral oil. Although it is not as potent adjuvant, a second use of the complete one could lead the rabbit to an autoimmune disease affecting the kidneys.

As soon as the serum was collected, its specificity was tested with an agglutination test.

V. vulnificus b. I isolate provoked a strong agglutination, followed by *V. vulnificus* which caused a moderate agglutination reaction. All the other *Vibrio* organisms tested showed at least a slight agglutination in contrast with *E. coli*, *P. fluorescens* and *P. shigelloides* which did not show any reaction. These results suggest that the organisms of the genus *Vibrio* may share common antigens.

The immunoblot assay produced quick information not only about the specificity of the serum but also about its strength. Undiluted and 1:2 serum

caused nonspecific binding with every organism tested due to its high concentration of protein. Serum diluted 1:10 and 1:20 showed common antigens between the spp of the genus *Vibrio*, in contrast with *E. coli*, *P. shigelloides* and *P. fluorescens*. Serum diluted 1:50 and 1:100 gave only positive results with *V. vulnificus* b. I.

Finally, an ELISA test was used. This test is more sensitive than the previous ones and allows to titre the serum.

There was a large difference in the titration of the sera prior to immunization and post-immunization when testing *V. vulnificus* b. I. This difference was not so remarkable when testing the other *Vibrio* spp as the reaction of the post-immunization serum was not so strong.

The study of the results suggested the use of the serum at a dilution 1:800 which gave an absorbance for *V. vulnificus* b. I at least double than for the rest of the organisms.

Outer membrane proteins and LPS play a relevant role as antigens. In this study a 31KD protein and a 90-99KD LPS fraction were identified as antigens of the *V. vulnificus* b. I isolate. The results were the same for OMP and whole cell samples which suggests that molecules which act as antigens are located in the outside of the bacterial cell in contact with the host.

CHAPTER IV

PATHOGENESIS OF VIBRIOSIS

The objective of this study was to examine the pathogenesis of vibrio infections in shrimps. The preceding work provided the background information and necessary techniques to undertake such a study.

This chapter describes the central component of the project which involved the detailed study of the pathogenicity of *Vibrio* spp bacteria administered to shrimps, through three different routes: immersion, injection and oral.

The pathogenesis of vibrio infection is not clearly understood. The routes for bacterial entry have been reported to be through the gills (Smith and Ratcliffe, 1976), orally and percuticularly (Jiravanichpaisal *et al*, 1994)

It is essential to understand the factors which affect the initiation and progression of the disease and hopefully provide information which allow more effective preventive and control measures to be developed.

Vibrio spp are considered part of the normal microflora in the digestive tract of crustacea and can also be found associated with the exoskeleton (Austin and Austin, 1989). The difficulty which has been encountered when attempting to challenge healthy shrimps with the putative causative bacteria (Johnson, 1983) suggests that host defence mechanisms may play a very important role in the development of the disease. The shrimp has to be in an unsuitable environment before the bacteria can overcome the shrimp defence mechanisms.

It is generally accepted that stress may render aquatic animals more susceptible to diseases (Snieszco, 1974) by compromising the defense mechanisms and increasing the chance of invasion by pathogens (Walters and Plumb, 1980). Overcrowding, handling and poor water quality are some of the most common causes of stress in shrimps.

In the attempt to reproduce a vibrio infection the animals underwent a period of stress prior to challenge with the bacteria, ammonia was selected as the stressor.

Ammonia is a relevant parameter in water quality, which frequently causes problems. It is the principal end-product of protein catabolism in crustaceans (Kinne, 1976) and accounts for up to 90% of the nitrogenous excretion by the shrimp (Parry, 1960). When proteins are metabolised for energetic requirements the amino group is cleaved and ammonia is excreted directly. Bacterial decomposition of uneaten proteins from food utilises a similar process (Chamberlain, 1988).

The major clinical signs described for acute ammonia intoxication in fish are hyperventilation, hyperexcitability, coma and convulsions. The primary effect is on the nervous system (Visek, 1968). In chronically toxic concentrations, ammonia causes gill damage characterized by a large number of aneurysms in the lamellae, in addition to hyperplasia of the gill epithelium (Roberts, 1989). In contrast to the effect of ammonia in fish, Donnelly (1992) reported

that the toxic effect of ammonia in *P. monodon* produced no damage in the gill epithelium, but scanning electron microscopy studies showed alterations in the surface of the gill cuticle.

The results from the infectivity trials were studied by means of the indirect peroxidase-labelled antibody method. For this technique the serum anti-*V. vulnificus* b. I previously produced in rabbits was utilized. These antibodies react with the antigen present in the shrimp tissue. The combination of the bacterial antigen with the rabbit immunoglobulin is confronted with a donkey anti-rabbit serum that has been labelled with the enzyme horse-radish peroxidase. Finally, this is exposed to the substrate diamino benzidine tetra hydrochloride (DAB) producing a brown end-product at the site of the bacterial antigen which can be examined by ordinary light microscopy.

MATERIALS AND METHODS

9.1. SHRIMP FACILITIES

9.1.1. Holding system

A basic system was constructed for challenging the shrimps. The main water holding tank with a covering lid was kept outside the laboratory, from there the water was moved into a smaller inside tank and allowed to come up to the temperature of the challenge facilities. The holding facilities consisted of 8 perspex aquaria (36 x 19.5 x 28cm), covered with a lid to prevent evaporation, each with a capacity of 20 litres. Water was heated and maintained at a constant temperature by means of electric heater plates in contact with the bottom of the aquaria. Drainage water was collected in another tank where it received a buffered iodophor treatment of 200ppm as recommended by Bell and Lightner (1992) before entering the main drainage.

Each tank was aerated through a small airstone connected to an airline from a high volume blower (Crompton Gryphon, Class E, Huddersfield, England). The dissolved oxygen was near saturation (5mg/l).

The room holding the system was kept in dim lighting conditions (approximately 20 lux) on a 12:12 hours light:dark cycle. The temperature of the room was maintained at 25°C by a fan-heater to reduce the heat loss from the tanks.

The cleaning of the tanks and water exchanges required were performed with a siphon.

9.1.2. Feeding of the shrimps

Shrimps were fed on fresh mussel flesh every 24h.

9.1.3. Water quality parameters

Four water quality parameters: temperature, salinity, ammonia and nitrite concentrations were monitored.

Water temperature was maintained at 29°C +/-1°C.

The salinity was read with a hand refractometer (CSP) as 27ppt with an accuracy of +/-1ppt.

Ammonia concentration was checked with a Tetra Ammonia test kit and kept below 0.25mg/l. If higher levels of ammonia were detected it was corrected with water exchanges, since a reduction in the food supply was undesirable due to a possible increase in cannibalism.

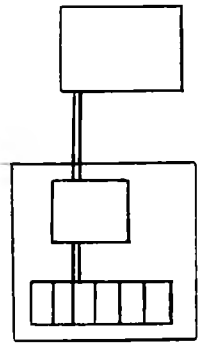
Nitrite concentration was checked with a Tetra NO_2-N test kit and kept below 0.25mg NO_2-N/l. If higher levels of nitrite were detected, it was corrected with

Nitrite concentration was checked with a Tetra NO_2-N test kit and kept below 0.25mg NO_2-N/l. If higher levels of nitrite were detected, it was corrected with water exchanges for the same reason as for high ammonia level.

10.1. BACTERIAL COUNTS

Water from the shrimp culture facilities was collected, in sterile containers from three different areas of the system (figure 11).

Figure 11: Water flow system of the shrimp holding facilities.



1. Sea water brought in refrigerated tanks and kept outside.
2. Water before entering the shrimp tank.
3. Shrimp holding tanks.

Bacterial counts were performed using the spread plate technique. Serial dilutions (10^{-1} -10^{-7}) of water samples from tanks 1, 2, and 3 were prepared in sterile saline and inoculated in duplicate plates each of TSA (2% NaCl) and TCBS. Plates were incubated for 48h at 22°C and reading performed at 24 and 48h.

11.1. INFECTIVITY CHALLENGES

Three different infectivity challenges were carried out with *V. vulnificus* b. I using a bath challenge developed by Roque *et al* (1993), an injection challenge and an oral challenge.

11.1.1. *V. vulnificus* b. I isolate bath challenge

Animals used for the study.

A total number of 224 *P. monodon* with an approximate weight of 0.8g and 2.5 cm in length were used for the challenge.

Bacterial suspension.

Challenge bacteria were grown on TSA (2% NaCl) plates for 24h at 22°C. Bacterial colonies were used to inoculate a 500ml TSB (2% NaCl) flask which was incubated for 24h at 22°C. Bacteria were harvested and washed twice with sterile saline by centrifuging at 2000g for 15 min each. Finally bacteria were resuspended at an approximate concentration of 10^8 CFU/ml.

Challenge procedure.

Six bath challenges were performed. Six to eight tanks with 8 to 10 shrimps in each, were used for each challenge. Approximately two thirds of the animals were subjected to stress with ammonia under the following

procedure. NH_4Cl was used to achieve a final concentration of 20mg NH_4^+/l in the tanks where the animals were kept in a static bath for 48h prior to the bacterial challenge. After that period 50ml of 10^8 CFU/ml bacterial suspension was added to the 10 litres of marine water held in each tank, making a final concentration of $5x10^5$ CFU/ml of *V. vulnificus* b. I isolate. It was run as a static water system and no water was exchanged for the length of the trial. No bacteria or ammonia was added to the water in which the control animals were kept. Sampling time started after 10 min until 15 days after challenge (10min, 20min, 30min, 40min, 50min, 1h, 1h20min, 1h40min, 2h, 2h30min, 3h, 3h30min, 4h, 4h40min, 5h, 5h30min, 6h, 7h, 8h, 9h, 10h, 12h, 14h, 15h, 23h, 24h, 28h, 48h, 53h, 3d, 3.5d, 4d, 4.5d, 5d, 5.5d, 6d, 6.5d, 7d, 7.5d, 8d, 8.5d 9d, 9.5d, 10d, 10.5d, 11d, 11.5d, 12d, 12.5d, 13d, 13.5d, 14d, 14.5d, 15d). At each sampling time two or three animals were collected from each tank.

11.1.2. *V. vulnificus* b. I isolate injection challenge

Animals used for the study.

A total number of 44 juvenile *P. monodon* with an approximate weight of 1g and in length of 3cm were used for this challenge.

Bacterial suspension.

Challenge bacteria were grown on TSA (2% NaCl) plates for 24h at 22°C.

Bacterial colonies were used to inoculate a 200ml TSB (2% NaCl) flask which was incubated for 24h at 22°C. Bacteria were harvested and washed twice with sterile saline by centrifuging at 2000g for 15 min each. Finally bacteria were resuspended at the approximate concentration of 10^5 and 10^7 CFU/ml. The high dose was used in the first trial and the low dose in the two following ones.

Challenge procedure.

Three injection challenges were performed. Shrimps were injected intramuscularly between the 3th and 4th abdominal segment with 0.05ml of 10^5 or 10^7CFU/ml of the bacterial suspension. Control shrimps were inoculated at the same site with 0.05ml of sterile saline. Sampling started after 10min until 96h (10min, 20min, 40min, 1h, 1h30min, 2h, 3h, 4h, 5h, 6h, 7h, 8h, 24h, 48h, 72h, 96h). One or two animals were collected at every sampling.

11.1.3. *V. anguillarum* isolate injection challenge.

The purpose of this experiment was to observe the *in vivo* specificity of the antiserum raised against *V. vulnificus* b. I isolate.

Animals used for the study.

Eight juveniles of *P. monodon* with an approximate weight of 1g and 3cm length were used for the challenge.

Bacterial suspension.

V. anguillarum isolate was grown on TSA (2% NaCl) plates for 24h at 22°C. Bacterial colonies were used to inoculate a 50ml TSB (2% NaCl) flask which was incubated for 24h at 22°C. Bacteria were harvested and washed twice with sterile saline by centrifuging at 2000g for 15min each. Finally bacteria were resuspended to match a concentration of 10^7 CFU/ml as estimated by the *V. vulnificus* b. I isolate growth curve.

Challenge procedure.

Shrimps were injected intramuscularly between the 3th and 4th abdominal segment with 0.05ml of 10^7 CFU/ml of the bacterial suspension. Sampling was performed 4h after the injection.

11.1.4. Sonicated *V. vulnificus* b. I isolate injection challenge.

Animals used for the study.

Twenty juvenile *P. monodon* with an approximate weight of 1.5g and 3.5cm in length were used for this challenge.

Bacterial suspension.

Challenge bacteria were grown on TSA (2% NaCl) for 24h at 22°C. Bacterial colonies were used to inoculate a 200ml TSB (2% NaCl) flask. Bacteria were

harvested, washed twice with sterile saline by centrifuging at 2000g for 15 min each and resuspended again in sterile saline at an approximate concentration of 10^7 CFU/ml. Bacteria were sonicated following the procedure described in section 6.1. The sample was then centrifuged at 2000g for 15 min and the supernatant collected. The OD of the supernatant was read in order to estimate the amount of protein/ml. As a general rule 1mg protein/ml has an OD of 1.4 at 280 nm wave length (Adams, 1994, personal communication). The minimum amount of protein injected into a mouse able to be detected is known to be 0.050 mg (Adams, 1994, personal communication). With this information a final suspension was prepared in order to inject approximately 0.045mg protein/animal.

Challenge procedure.

Shrimps were injected intramuscularly between the 3th and 4th abdominal segment with 0.05ml of the sonicated bacteria suspension. Sampling started 10 min after the challenge until 32h (10min, 20min, 40min, 1h, 2h, 3h, 4h, 5h, 6h, 7h, 24h, 32h). Two animals were collected at every sampling time.

11.1.5. *V. vulnificus* b. I isolate oral challenge

Animals used for the study.

A total number of 37 juvenile *P. monodon* with an approximate weight of 5g and 10cm in length were used for the challenge.

Bacterial suspension.

Challenge bacteria were grown on TSA (2% NaCl) plates for 24h at 22°C. Bacterial colonies were harvested and resuspended in sterile saline at an approximate concentration of 10^8 CFU/ml.

Challenge procedure.

Two oral challenges were performed. Half of the animals were subjected to stress with ammonia for 48h prior to the challenge as described for the bath challenge. One ml of the bacterial suspension was inoculated by mouth with a blunted needle and animals returned to their original tanks. The animals were sampled after 30 minutes until 72h post-inoculation (30min, 1h, 2h30min, 6h, 9h, 24h, 48h, 72h). Two animals were collected at each sampling time.

11.1.6. Bacteria fixation trial

As described later, bacteria inoculated orally were difficult to observe microscopically in haematoxylin and eosin preparations. This experiment was performed to eliminate the possibility that the fixative injected into the shrimp, Davidson's fixative (section 15), was responsible for the disruption of the bacterial cells.

A colony of *V. vulnificus* b. I isolate grown in TSA (2% NaCl) was resuspended in 1ml of sterile saline and smeared onto glass slides. The slides were heat-fixed with a flame and each covered with a different fixative, Davidson's and formalin. The latter is a widely used fixative and was included in the trial for comparison. After 5 minutes incubation, fixatives were washed off and the smears stained with crystal violet for 5 minutes.

11.1.7. Bactericidal activity of the hepatopancreatic extracts.

This experiment was conducted in order to determine the effect of the hepatopancreatic enzymes on the bacterial cells.

Method

The hepatopancreas and stomachs from 5 adult *P. monodon* were removed in sterile conditions and ground with sterile sand, mixed with a volume of sterile saline twice the weight of the removed tissue. Once the tissue was disrupted, the mixture was centrifuged at 1800g for 10 minutes and the supernatant filtered through 0.22 μm membrane. Several dilutions of the extract were prepared, 1:10, 1:100, 1:1000.

A bacterial suspension of 10^6 CFU/ml of *V. vulnificus* b. I isolate was prepared as described for the oral challenge and 100 μl of the suspension

placed in each well.

Another 100 µl of undiluted, 1:10, 1:100 and 1:1000 hepatopancreatic extract were added to each well, except for the controls which had the same volume of saline added.

Plates were incubated at 30°C. 20µl from each well was collected after 10min, 30min, 1h and 2h and spread with a sterile glass rod onto TSA (2% NaCl) plates for CFU counting.

This experiment was repeated with 10 adult *Penaeus indicus* since no *P monodon* were available.

12.1. SHRIMP SAMPLING AND FIXATION

Shrimps sampled were fixed alive to prevent autolysis following the procedure recommended by Bell and Lightner (1988).

1. **Materials**:
    1. Equipment:
        .1ml syringes
        .27 gauge 5/8" needles

2. Reagents and solutions:

.Davidson's fixative: 330ml of 95% ethyl alcohol 220ml of 100% formalin (saturated aqueous solution of formaldehyde gas, 37-39% solution)

115ml of Glacial acetic acid

335ml of Distilled water

Stored at room temperature

.50% Ethanol in water

2. **Method**

Davidson's fixative was injected by means of needle and syringe into the living shrimp. The sites of injection were the hepatopancreas, the region anterior to the hepatopancreas and the posterior and anterior abdominal regions. The cephalothorax, specifically the hepatopancreas received the larger share of fixative. Approximately 50% of the shrimp's body weight were injected and all signs of life ceased.

The shrimps remained in a volume of fixative approximately 10 times their own volume for 24h and then transferred into 50% ethyl alcohol, where they could be stored for an indefinite period of time. Shrimp were cut for histology (appendix III)

13.1. SAMPLE PROCESSING

The aim of processing is to impregnate the tissue with an embedding medium which will give support to the tissue section during section cutting. For this method standard procedure was used.

Following section cutting, sections were allowed to dry and stained with haematoxylin and eosin (appendix IV) if required. The sections cut for later use with immunoperoxidase were not stained and poly-L-lysine coated glass slides (Sigma) used for a better attachment of the tissue.

14.1. INDIRECT PEROXIDASE-LABELLED ANTIBODY METHOD

The protocol used is an adaptation of the method described by Adams *et al* (1992)

1. **Materials**:

    1. Equipment:

        .PAP pen (DAIDO Sangyo Co, ltd. Japan)

        .Poly-L-lysine coated slides (Sigma)

    2. Reagents and solutions:

.Tris buffered saline (TBS): 0.05M Tris

0.15M NaCl

Adjust ph at 7.6 and store at 4°C not longer than 4 weeks.

.Xylene

.100% and 70% ethanol

.10% H_2O_2 in methanol

.Pertex

.Substrate: 0.5mg of 3,3 Diaminobenzidine tetrahydrochloride (DAB)

100μl of 1% H_2O_2 in TBS

5ml of TBS

.Normal donkey serum (SAPU)

.Donkey antirabbit HRP conjugated IgG (SAPU)

.Rabbit antiserum against *V. vulnificus* b. I isolate

.Haematoxylin

2. **Method**:

Paraffin-embedded sections from control and challenged shrimps were dewaxed in xylene for 5 minutes, then placed in 100% ethanol for another 5 minutes and finally in 70% ethanol for 3 minutes. Slides were rinsed in distilled water. Rings were made around tissue sections using the PAP pen, providing a waxy circle around the sample enabling small volumes of serum

to be used. Endogenous peroxidase activity was blocked by incubating slides for 10 minutes in 10% H_2O_2 in methanol and then washing 3 times with TBS. The non-specific binding sites were blocked with normal donkey serum diluted 1:10 in TBS for 10 minutes. The donkey serum was poured off and slides dried by tapping them edge up on paper towel.

Rabbit antiserum against *V. vulnificus* b. I diluted 1:800 in TBS was found to give the best specific staining. Slides were incubated for 1h at room temperature in a moist chamber. Control slides had no incubation with antisera. Slides were washed 3 times with TBS. Normal donkey serum diluted 1:10 was used for a 10 minute incubation again.

Presence of antibody bound to *Vibrio* cells was detected by adding donkey antirabbit HRP conjugate diluted 1:50 in TBS for 30 minutes at room temperature in a moist chamber.

Excess conjugate was removed by washing the slides as described previously. Finally, antigens of *V. vulnificus* b. I isolate were visualised after 10 minutes of incubation with the substrate. The reaction was stopped by immersing slides in tap water. Sections were counterstained for 3-4 minutes with haematoxylin, placed in tap water for 10 minutes, dehydrated for 3 minutes in 70% ethanol and 5 minutes in 100% ethanol and rinsed twice for 5 minutes in clean xylene.

Slides were mounted using pertex and coverslip. Brown staining was visualised under the microscope.

RESULTS

10.2. Bacterial counts

Water held in tank number 1 situated outside gave $13x10^4$ CFU/ml in TSA (1.5% NaCl) plates. No growth was observed in TCBS plates.

Water held in tank number 2, kept inside gave $2x10^2$ CFU/ml in TSA (1.5% NaCl) plates and 4 CFU/ml in TCBS.

Water held in tank number 3, where shrimps were kept, gave $33x10^5$ CFU/ml in TSA (1.5% NaCl) and 12 CFU/ml in TCBS.

11.2. *V. vulnificus* b.I isolate bath challenge.

Ten minutes after commencing the challenge, positive staining corresponding to an accumulation of bacterial cells were found in gill lamellae (figure 12).

After twenty minutes, positive stained bacterial cells were found in the content of the gut (figure 13). Areas of melanization were observed underneath the cuticle, at the insertion of a cuticular setae site, with the presence of an intense haemocytic reaction (figure 14).

After forty minutes, lesions of the cuticle were frequently observed, commonly

associated with cellular reaction (figure 15) and some of them melanized (figure 16). Cuticle setae appeared in close association with positive staining (figure 17).

After eighty minutes, haemocytic nodules were found in the muscle (figure 18). In some of them melanin or positive staining could be detected inside the nodule. At this stage, necrosis of connective tissue underneath the cuticle associated with cuticular damage was observed in every animal. In some cases the lesion was melanized (figure 19) whereas some other lesions presented positive staining (figure 20). Positive staining reaction was found associated with a few phagocytic haemocytes containing bacteria found in the hepatopancreas capsule. The positive staining of the gut content became more intense.

After two hours, melanized haemocytic nodules were found distributed along the subcuticular connective tissue (figure 21). Positive stained material was found inside the hepatopancreatic tubules (figure 22). Positive stained affected gill lamellae were also commonly observed.

After three hours, positive staining had disappeared from the hepatopancreas tubules resulting in positive staining in the surrounding haemolymph (figure 23). Melanisation of lesions in cuticle became generalised (figure 24) and extensive affected areas were found (figure 25). Some of the animals presented irregular profile of the midgut epithelium with individual cells

associated with cellular reaction (figure 15) and some of them melanized (figure 16). Cuticle setae appeared in close association with positive staining (figure 17).

After eighty minutes, haemocytic nodules were found in the muscle (figure 18). In some of them melanin or positive staining could be detected inside the nodule. At this stage, necrosis of connective tissue underneath the cuticle associated with cuticular damage was observed in every animal. In some cases the lesion was melanized (figure 19) whereas some other lesions presented positive staining (figure 20). Positive staining reaction was found associated with a few phagocytic haemocytes containing bacteria found in the hepatopancreas capsule. The positive staining of the gut content became more intense.

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After three hours, positive staining had disappeared from the hepatopancreas tubules resulting in positive staining in the surrounding haemolymph (figure 23). Melanisation of lesions in cuticle became generalised (figure 24) and extensive affected areas were found (figure 25). Some of the animals presented irregular profile of the midgut epithelium with individual cells

demonstrating reduction in the volume of the cytoplasm and apical vacuolation of the midgut epithelium (figure 26 and 27).

After four hours, nuclei of hepatopancreatic epithelium had a positive staining in some of the animals (figure 28). Nodules in connective tissue and muscle were melanized (figure 29).

After six hours, the R-cell vacuoles of the hepatopancreatic tubular epithelium were seen to retain the positive staining in two animals (figure 30).

After two days, one of the animals sampled, presented a haemocytic enteritis with no positive staining associated with it (figure 31).

After three days, positive staining was found within large cells in the gills with no surrounding haemocytic reaction (figure 32).

After four days, large cells containing a material whose colour ranged from eosinophilic to brown started to appear in the connective tissue just underneath the cuticle with no surrounding haemocytic reaction (figure 33).

After five days, only one animal was found with a melanized necrotic hepatopancreatic tubule (figure 34).

After eight days, positive staining was found concentrated inside the vacuoles

of the B-cells of the hepatopancreatic epithelium (figure 35). The staining was located in the luminal side of the vacuoles (figure 36).

Sampling lasted for 15 days, melanized cuticle and gill lamellae and modified midgut epithelium were common findings. As the time passed melanized nodules in connective tissue increased together with the subcuticular large cells described previously.

After detailed examination of the samples, it seemed that the animals which underwent stress prior to the bath challenge had a tendency to show earlier and more frequent pathological changes, however, there were insufficient differences between both groups of animals to demostrate such trends conclusively.

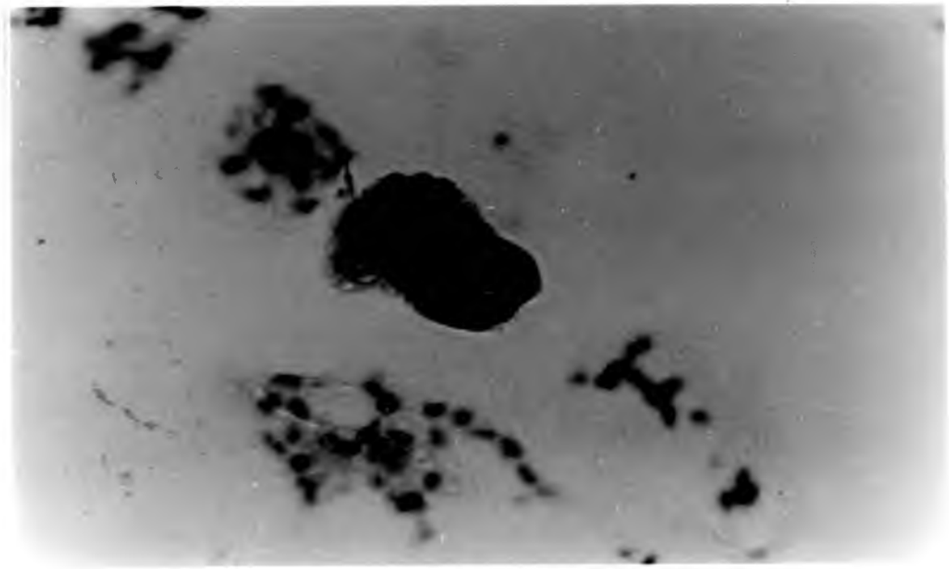


Figure 12. *V. vulnificus* b. I isolate bath challenge, 10min post- introduction. Positive staining associated with an accumulation of bacterial cells on gill lamellae. Immunoperoxidase staining (IP staining). 570X.

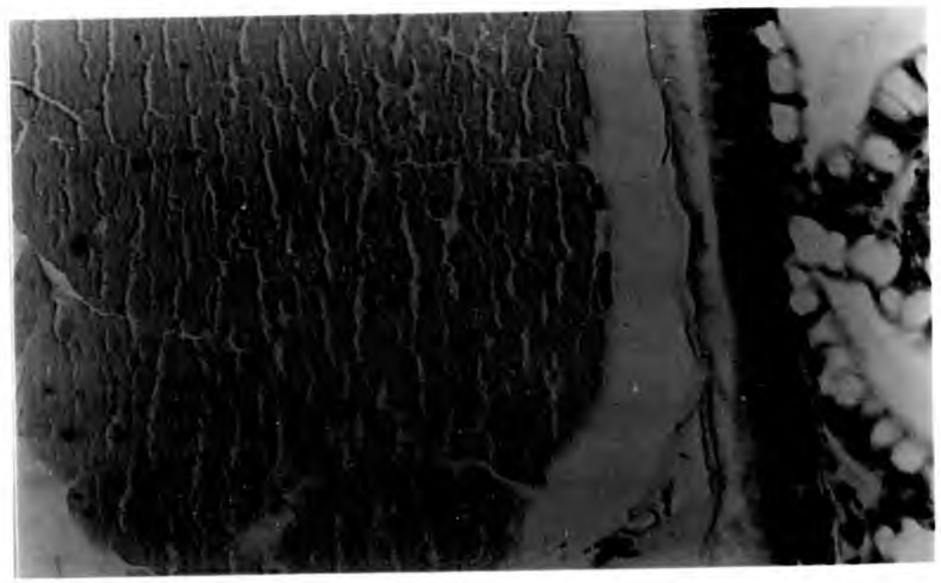


Figure 13. *V. vulnificus* b. I isolate bath challenge, 20min post- introduction. Positive staining in gut content corresponding to bacterial cells. IP staining. 290X.

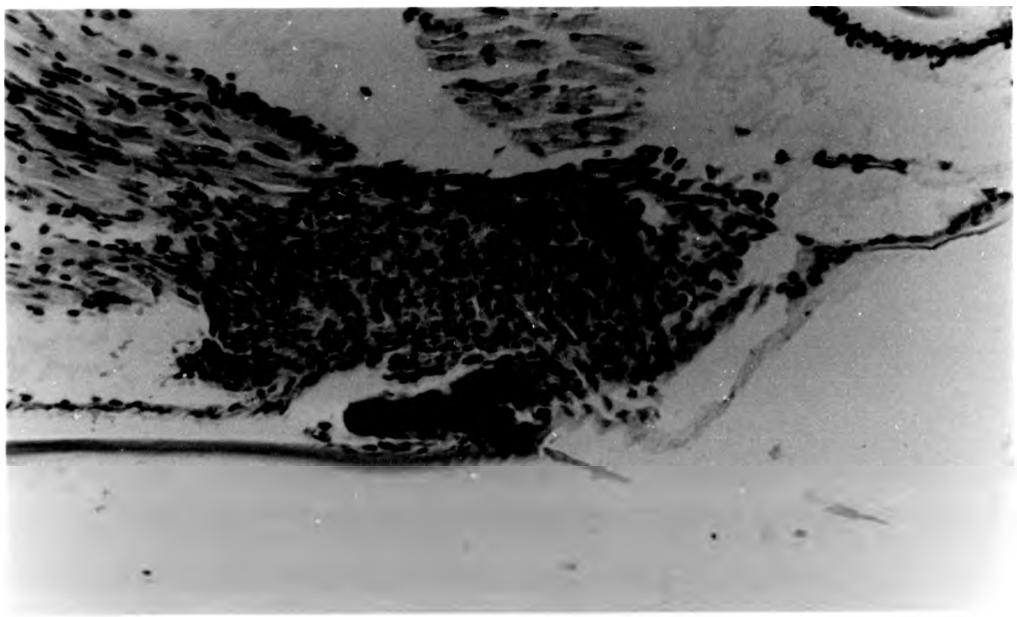


Figure 14. *V. vulnificus* b. I isolate bath challenge, 20min post- introduction. Lesion with melanized central material underneath the cuticle associated to the setae insertion and presence of an intense haemocytic response. IP staining. 290X.

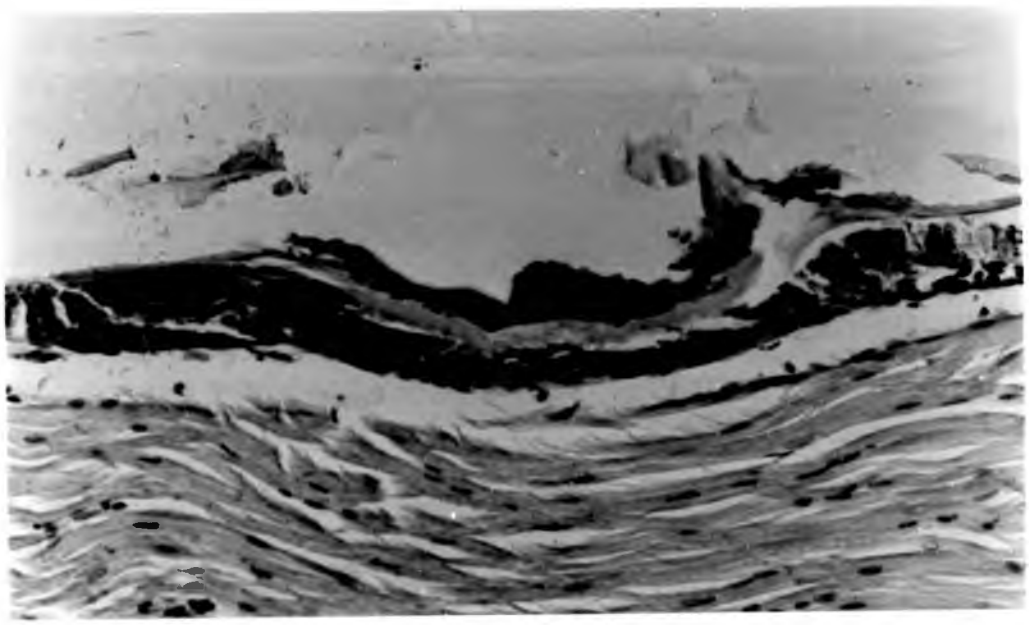


Figure 15. *V. vulnificus* b. I isolate bath challenge, 40min post- introduction. Positive staining associated with cuticle. IP staining. There is a cellular reaction associated with the lesion. 290X.

Figure 16. *V. vulnificus* b. I isolate bath challenge, 40min post- introduction. Melanized area associated with the cuticle. IP staining. 570X.

Figure 17. *V. vulnificus* b. I isolate bath challenge, 40min post-introduction. Positive staining associated with the cuticle setae. IP staining. 290X.

Figure 18. *V. vulnificus* b. I isolate bath challenge, 80min post-introduction. Haemocytic nodule in muscular tissue. Brown staining can be observed inside the nodule. IP staining. 570X.

Figure 19. *V. vulnificus* b. I isolate bath challenge, 80min post-introduction. Melanized area corresponding to a necrosis of the subcuticular connective tissue surrounded by haemocytic reaction. IP staining. 290X.

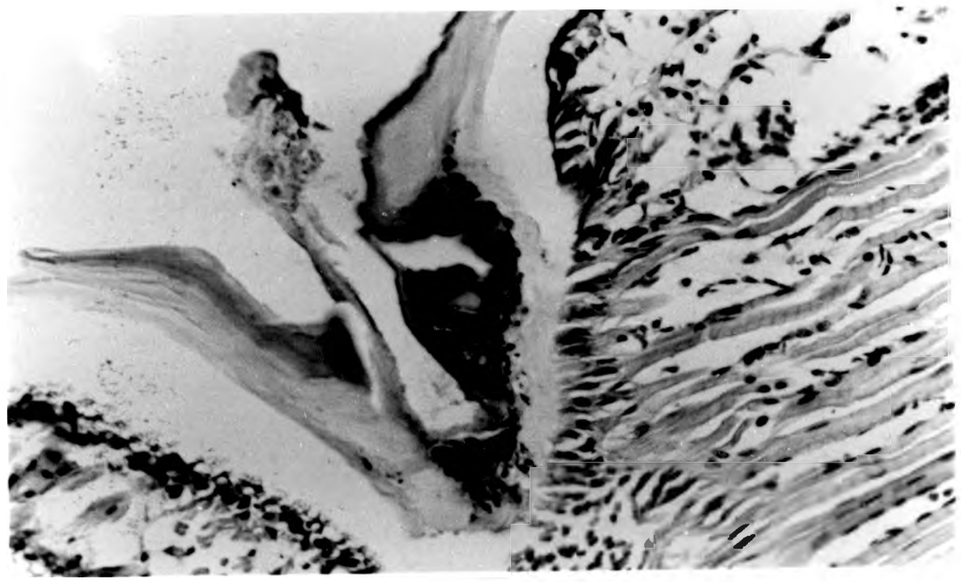
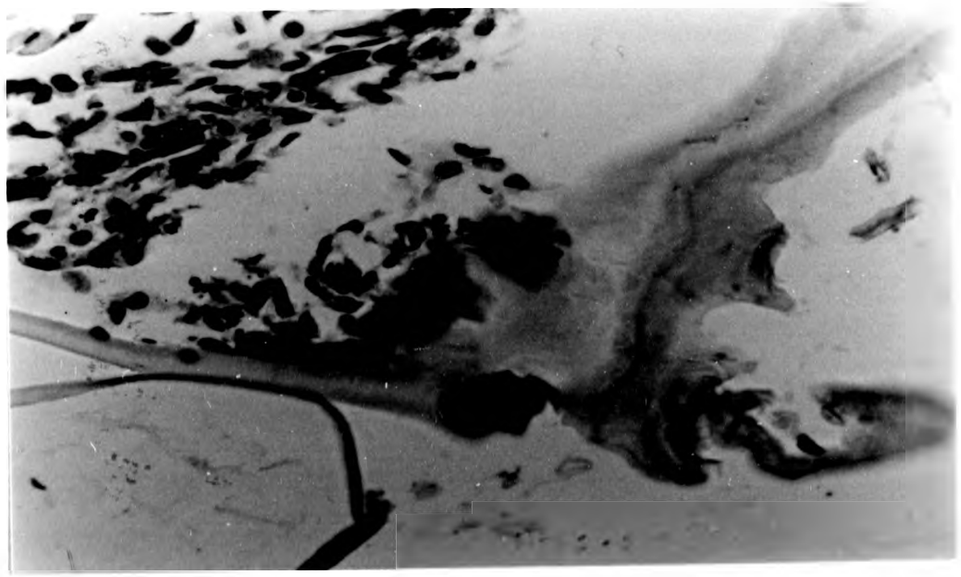


Figure 20. *V. vulnificus* b. I isolate bath challenge, 80min post-introduction. Positive staining material associated with the insertion of a cuticular setae and the connective tissue underneath. IP staining. 570X.



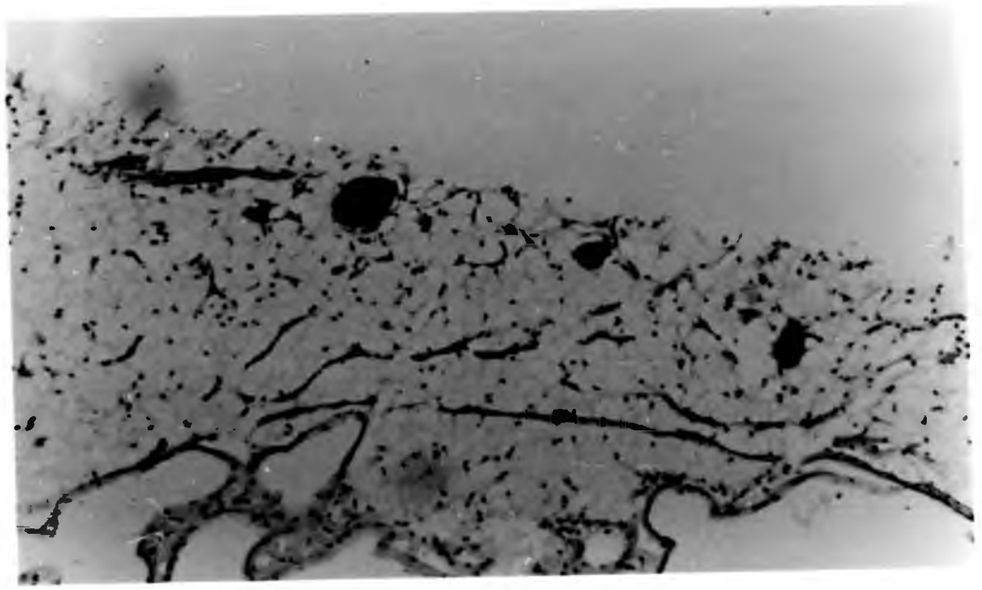


Figure 21. *V. vulnificus* b. I isolate bath challenge, 2h post-introduction. Melanized haemocytic nodules in subcuticular connective tissue. IP staining. 140X.

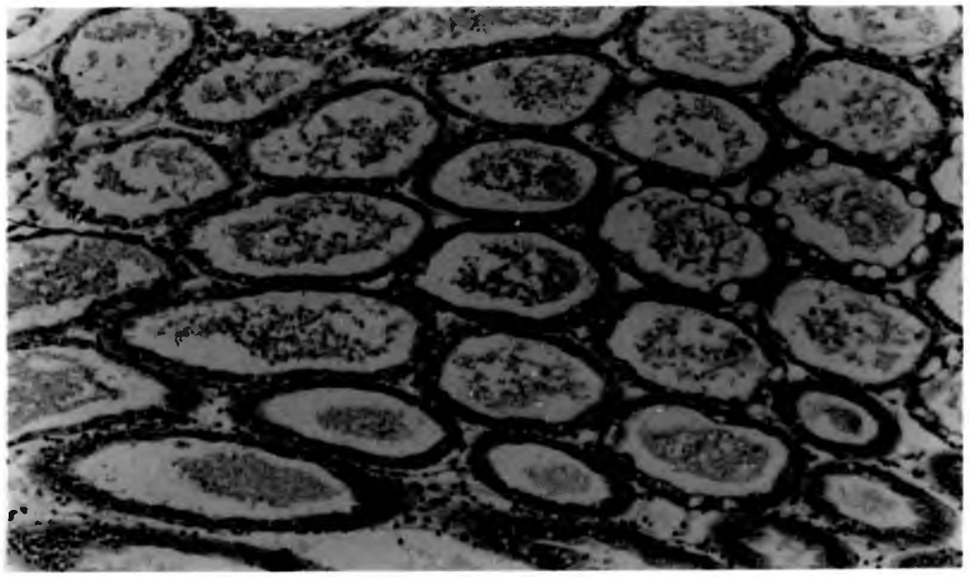


Figure 22. *V. vulnificus* b. I isolate bath challenge, 2h post-introduction. Positive stained material inside the hepatopancreatic tubular content. IP staining. 60X.

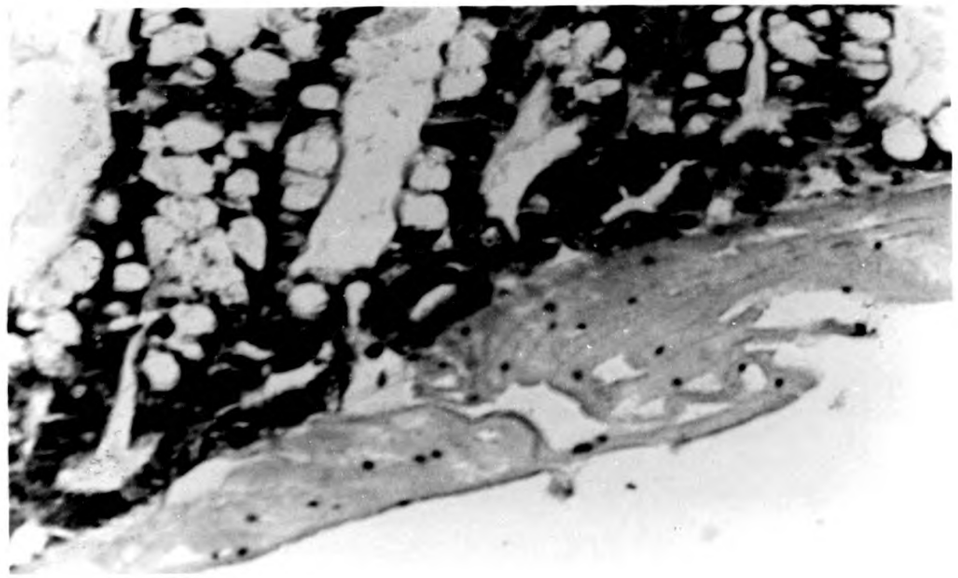


Figure 23. *V. vulnificus* b. I isolate bath challenge, 3h post-introduction. Positive stained haemolymph surrounding the hepatopancreas. IP staining. 290X.

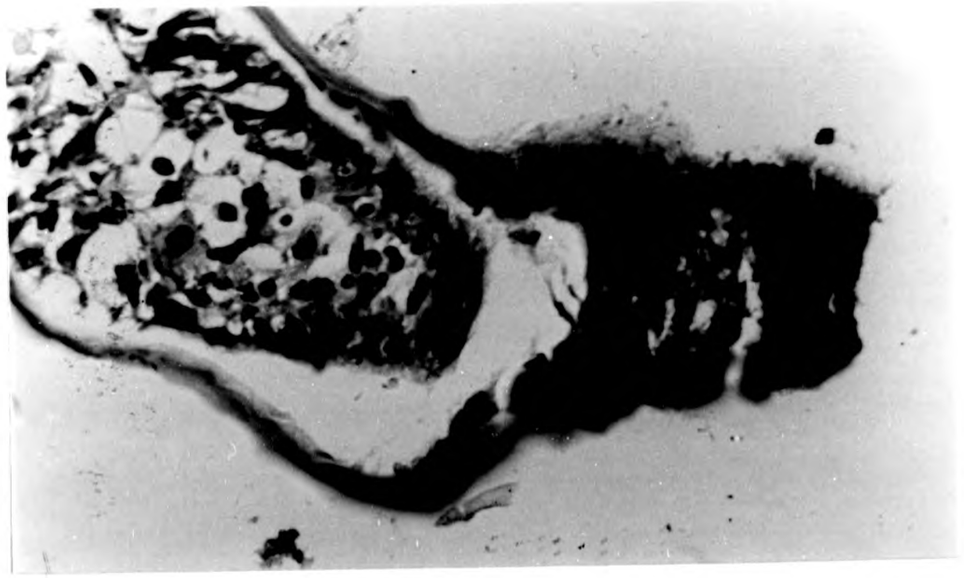


Figure 24. *V. vulnificus* b. I isolate bath challenge, 3h post-introduction. Melanized lesion affecting the cuticle in a pleopod. IP staining. 570X.

Figure 25. *V. vulnificus* b. I isolate bath challenge, 3h post-introduction. Melanised necrotic area affecting muscular and connective tissue of a pleopod. IP staining. 140X.

Figure 26. *V. vulnificus* b. I isolate bath challenge, 3h post-introduction. Positive staining associated with the content of the midgut (C). Modified gut epithelium (E). IP staining. 290X.

Figure 27. *V. vulnificus* b. I isolate bath challenge, 3h post-introduction. Modified epithelium with reduction in the cytoplasm of the epithelial cells and apical vacuolation. IP staining. 570X.

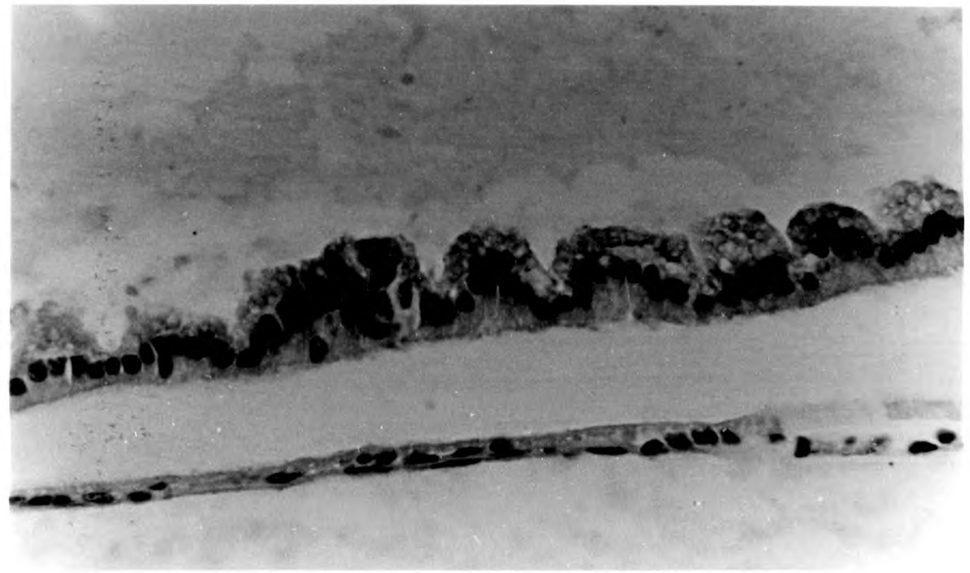
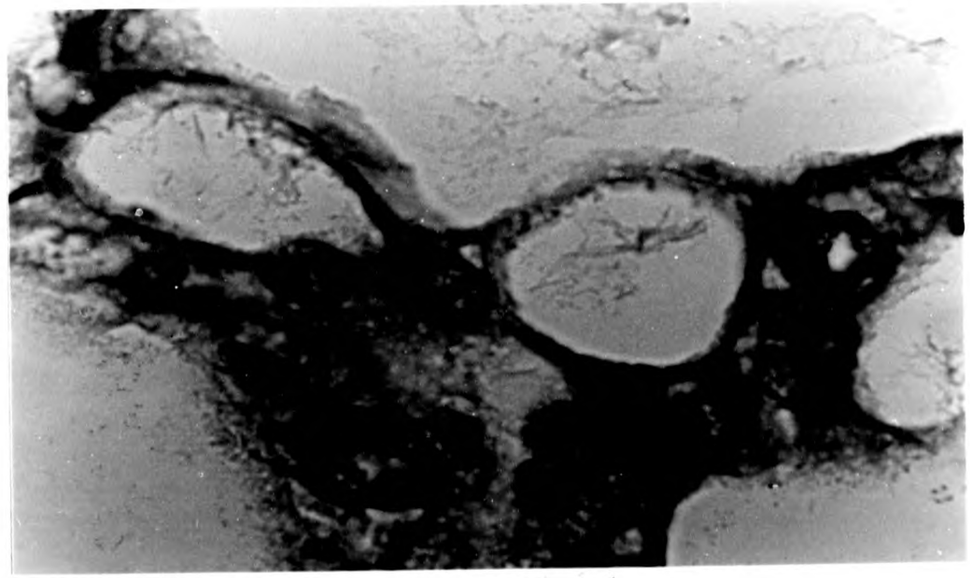


Figure 28. *V. vulnificus* b. I isolate bath challenge, 4h post-introduction. Positive staining associated with the hepatopancreatic epithelial nuclei. IP staining. 1430X.



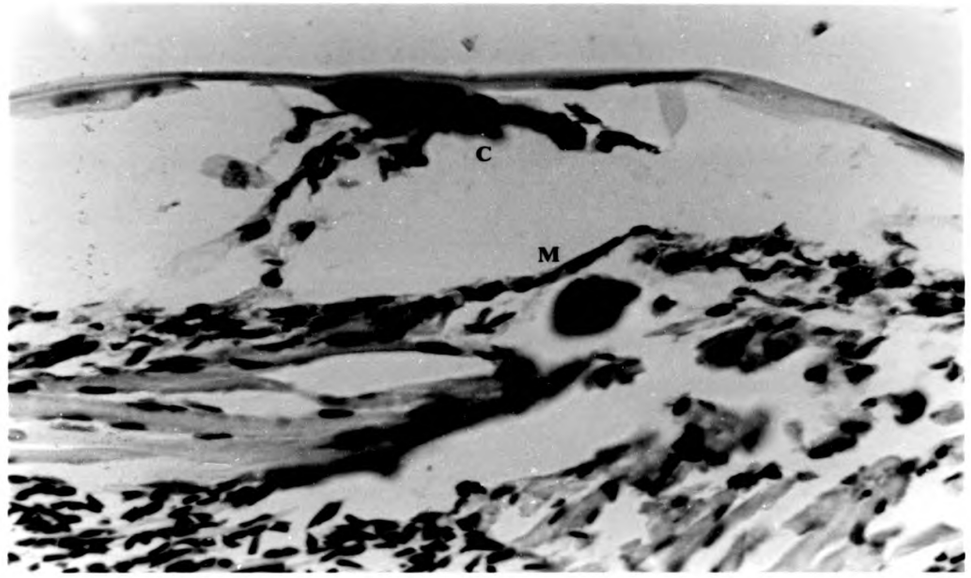


Figure 29. *V. vulnificus* b. I isolate bath challenge, 4h post-introduction. Melanized nodules in muscular tissue (M) and underneath the cuticle (C). IP staining. 570X.

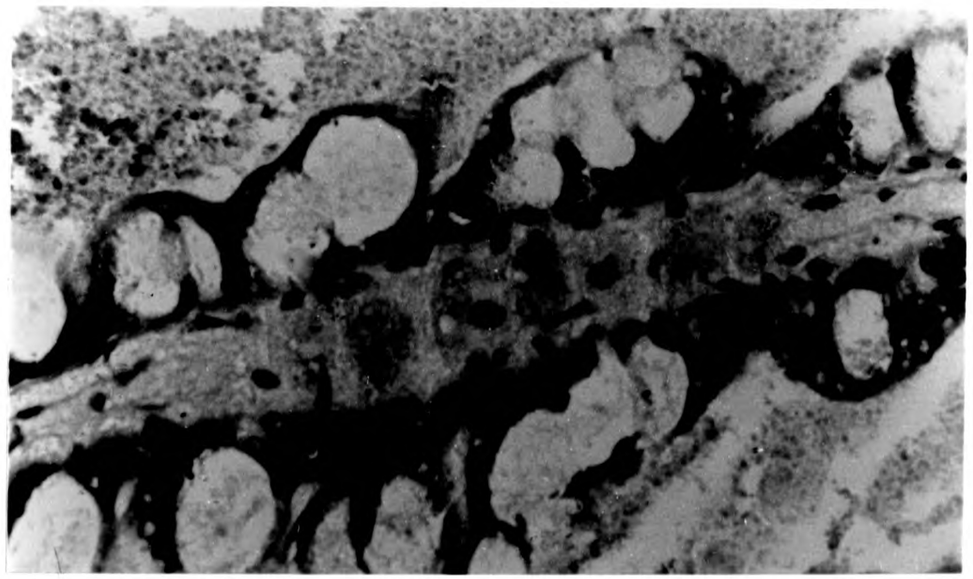
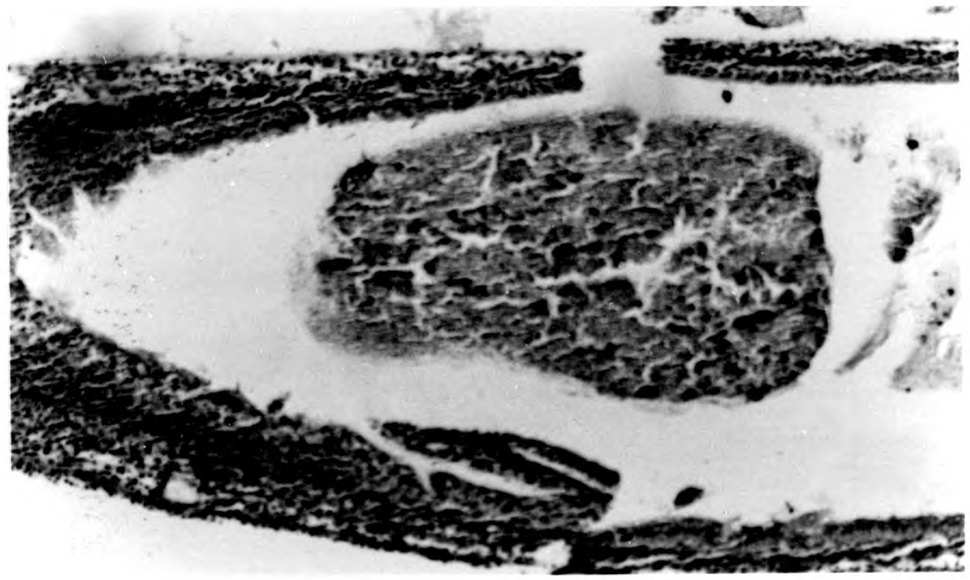


Figure 30. *V. vulnificus* b. I isolate bath challenge, 6h post-introduction. Positive staining associated with the vacuoles of the R-cells. IP staining. 570X.

Figure 31. *V. vulnificus* b. I isolate bath challenge, 2 days post-introduction. Haemocytic enteritis with no positive staining associated with it. IP staining. 140X.



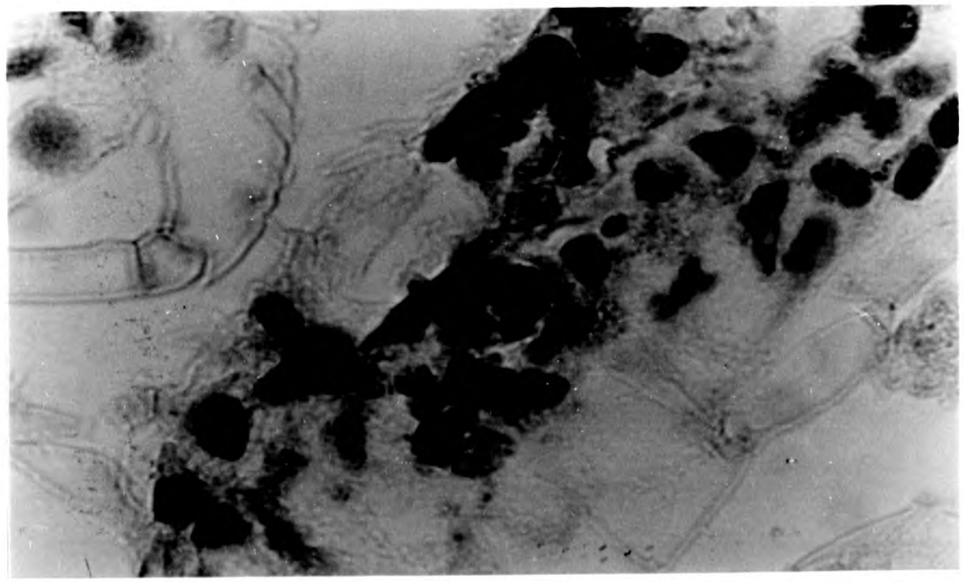


Figure 32. *V. vulnificus* b. I isolate bath challenge, 3 days post-introduction. Positive stained material contained within a cell in gill lamellae with no haemocytic reaction. IP staining. 1430X.

Figure 33. *V. vulnificus* b. I isolate bath challenge, 4 days post-introduction. Positive stained material contained within large cell underneath the cuticle. Two of them appear to overlap. IP staining. 1430X.

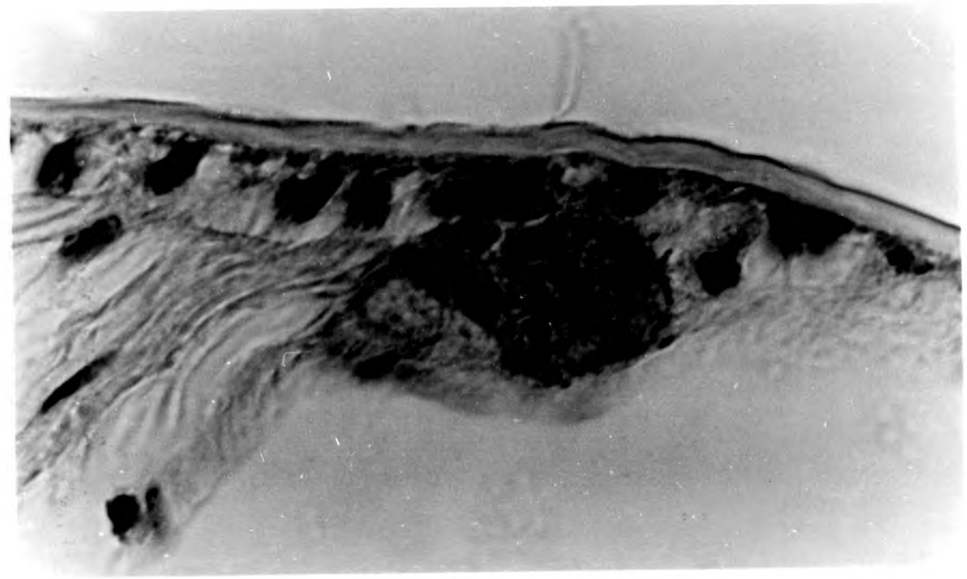


Figure 34. *V. vulnificus* b. I isolate bath challenge, 5 days post-introduction. Melanized material within an encapsulated necrotic hepatopancreatic tubule. IP staining. 290X.

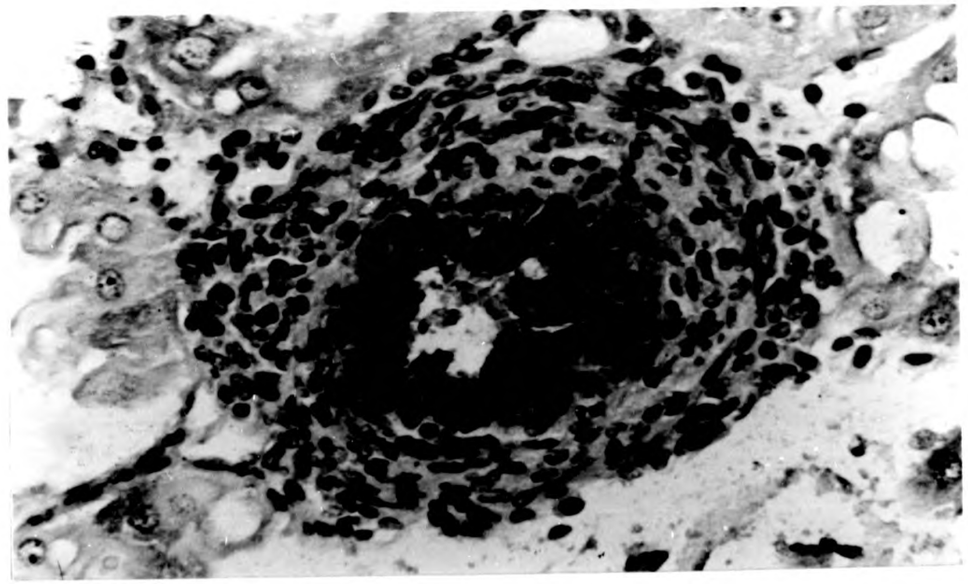


Figure 35. *V. vulnificus* b. I isolate bath challenge, 8 days post-introduction. Positive stained material within the vacuoles of the hepatopancreatic B-cells. IP staining. 290X.

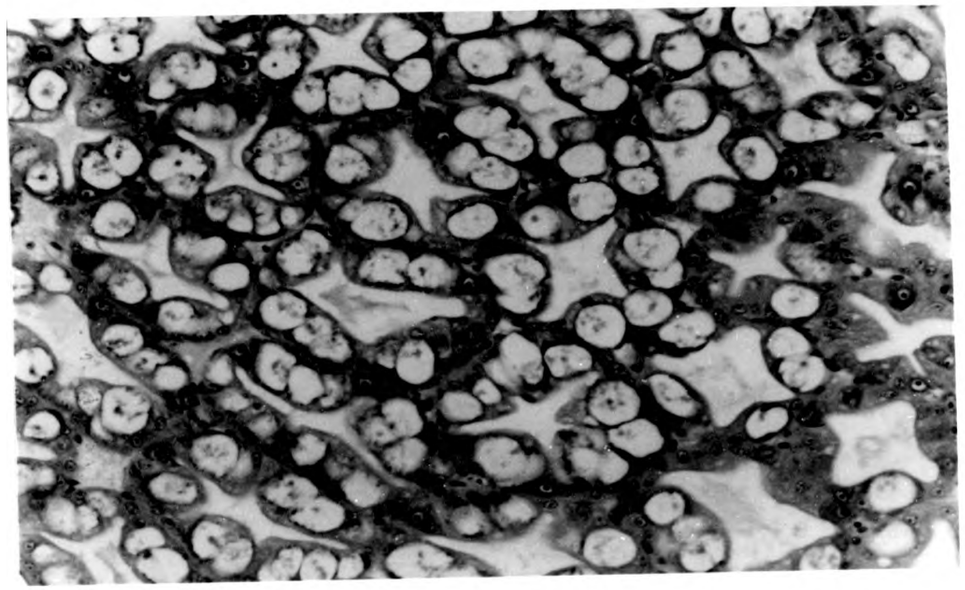
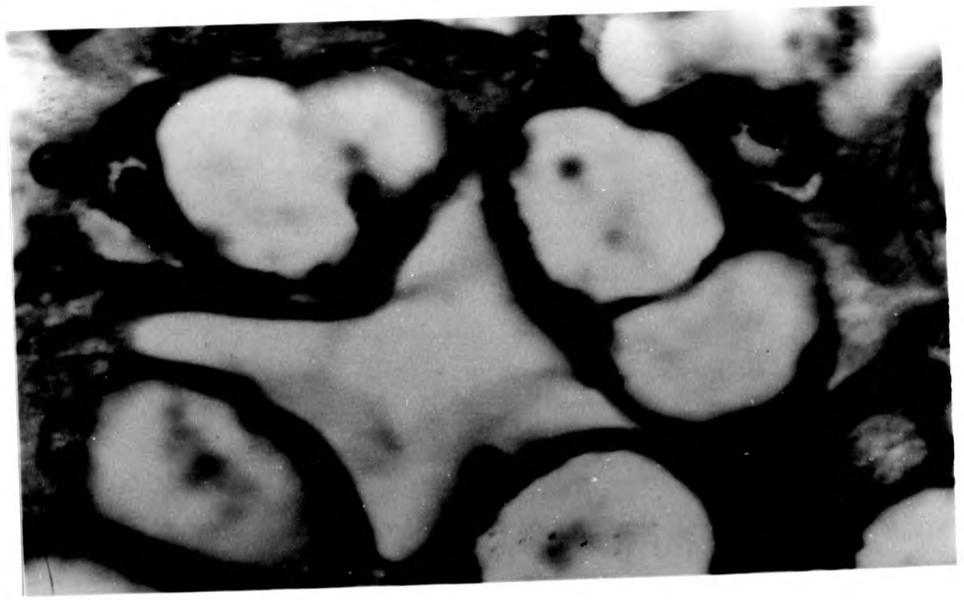


Figure 36. *V. vulnificus* b. I isolate bath challenge, 8 days post-introduction. Positive stained material within the luminal side of the B-cell vacuoles. IP staining. 1430X.



11.2.2. *V. vulnificus* b. I isolate injection challenge.

Ten shrimps of the thirty six shrimps injected with the low bacterial dose were found dead after the challenge, five after 1h, three after 12h and two after 24h. Six of the eight shrimps injected with the high bacterial dose were found dead after 4h and the remaining two shrimps were sampled at 4h.

Twenty minutes post-injection, haemocytes had already migrated to the injection site and phagocytosed bacterial cells. These haemocytes containing bacteria (HCB) and free bacterial cells were transported via haemolymph (figure 37) to the gills, midgut caecum (figure 38), midgut intestine and to the hepatopancreas capsule (figure 39) were they accumulated. Haemolymph had already acquired a positive staining and was found in the ventral sinus and bathing the organs. The muscle tissue of periopods, pleopods and mouth area had more intense positive reaction than the other areas of muscle (figure 40).

HCB were found distributed along the subcuticular connective tissue. Although there was a greater concentration of HCB in the tail region, they were easily found in cephalothorax or periopods (figure 41).

One hour post-injection, more haemocytes had collected at the injection site (figure 42). Many of them had pyknotic nuclei. Haemolymph containing HCB was present in hepatopancreatic vessels (figure 43) and lymphoid organ

vessels (figure 44). HCB nodules were present in the heart and reached the gills through haemolymph sinuses (figures 45 and 46).

Two hours post-injection, HCB were still present in the gills and accumulated in the heart. Haemocytic nodules were found in the antennal gland (figure 47). HCB accumulated in lymphoid organ where it was possible to distinguish the active normal lymphoid organ from the inert spheroids (figure 48). However, some positive reaction could be observed in spheroids (figure 49). HCB which had gathered in the hepatopancreas capsule appeared to penetrate between the tubules (figure 50).

Four hours post-injection, legs and mouth muscle still showed more positive reaction than the rest of the muscle tissue. The injection site retained a strong positive staining that was confined to the injected muscle segment (figures 51 and 52). Gills were rich in free positive stained material but there were not so many HCB at this stage (figure 53). Positive stained material accumulated attached to the heart muscle fibres (figure 54). A similar accumulation of positive material occurred in normal lymphoid organ tissue (figure 55), however, it did not correspond to bacterial cells when the sample was stained with H&E (figure 56).

The two shrimps injected with the high dose of bacteria were sampled after 4h and showed a positively stained generalised necrosis of the connective tissue associated with posterior midgut caecum, midgut and hepatopancreas

together with loss of midgut epithelium (figures 57 and 58).

Six hours post-injection, the injection site started to accumulate fibrous tissue, but HCB were still present in great numbers (figure 59). The heart and lymphoid organ still retained substantial amounts of positive material. Large numbers of HCB were associated with the connective tissue surrounding the posterior midgut caecum, midgut and hepatopancreas tubules.

Eight hours post-injection, there was a reduction in the amount of positive material in the heart and lymphoid organ (figure 60). Gills were still rich in positive material content and a few HCB were still found. The injection site was massively infiltrated with large numbers of new haemocytes with no positive staining associated with them.

As the time advanced, positive material and HCB decreased in the tissues previously described, however, traces of positive material could still be found 96h post-injection.

The degree of response to the inoculum over the time varied substantially between individuals.

Control animals injected with sterile saline, showed a limited haemocytic infiltration and necrosis of muscle tissue. The fibrous regeneration of muscular tissue was very advanced by 72h.

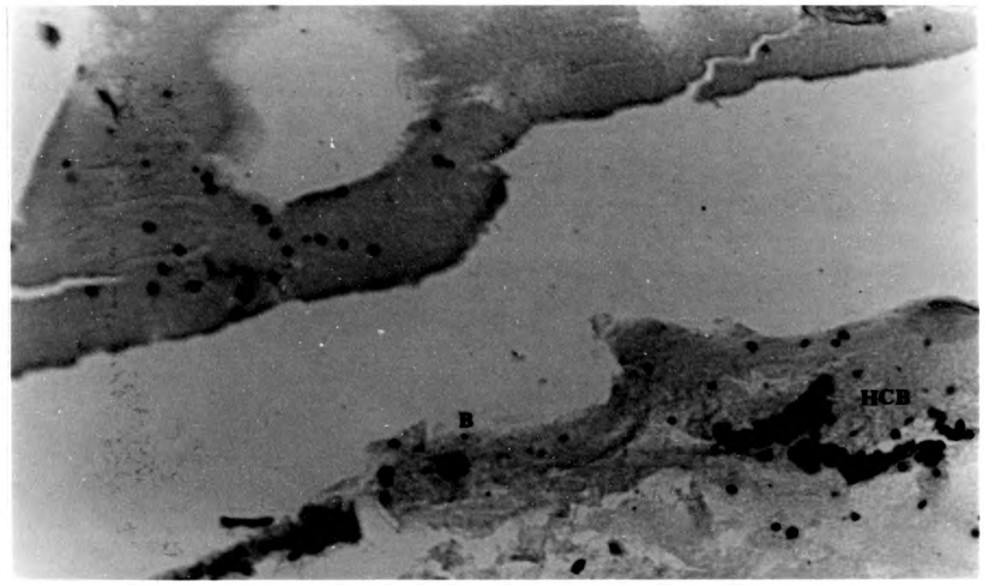


Figure 37. *V. vulnificus* b. I isolate injection challenge, 20min post-injection. Haemolymph with positive staining associated with haemocytes containing bacteria (HCB) and bacterial cells (B). IP staining. 290X.

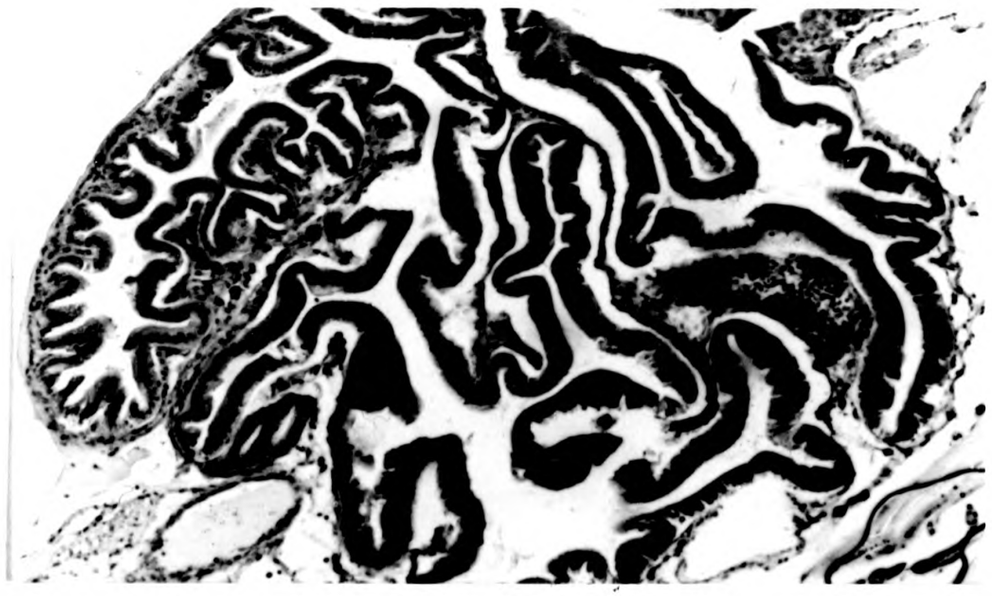


Figure 38. *V. vulnificus* b. I isolate injection challenge, 20min post-injection. Positive staining associated with HCB surrounding the posterior midgut caecum. IP staining. 140X.

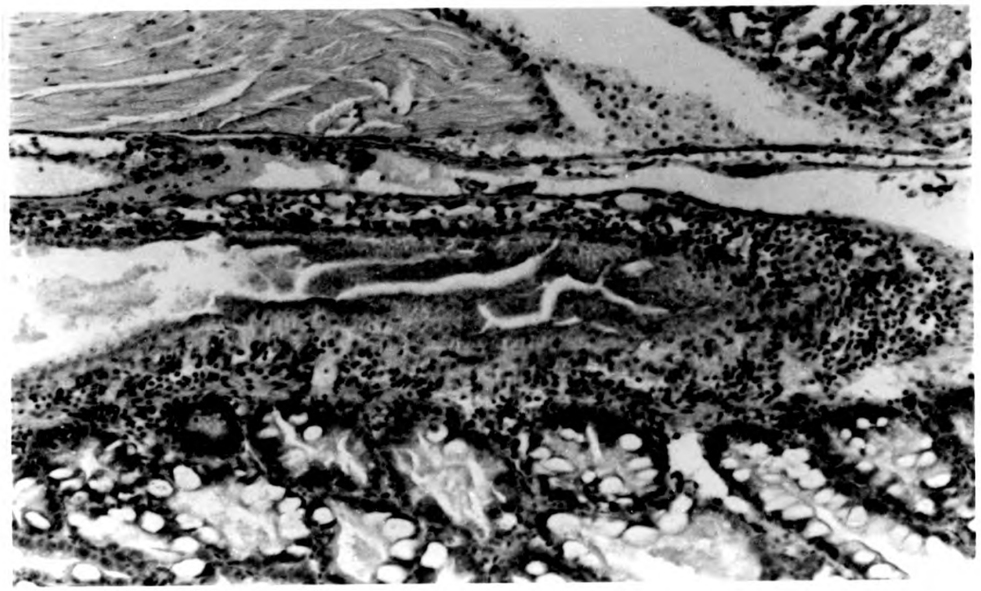


Figure 39. *V. vulnificus* b. I isolate injection challenge, 20min post-injection. Positive staining associated with HCB surrounding midgut and hepatopancreas. IP staining. 140X.

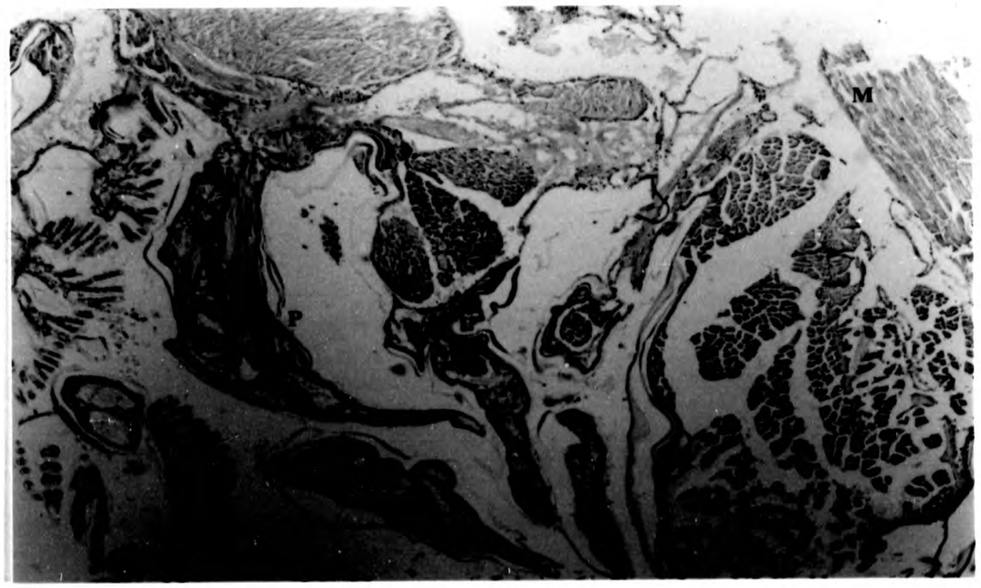


Figure 40. *V. vulnificus* b. I isolate injection challenge, 20min post-injection. More intense positive staining associated with pereiopod muscle (P) than with the other muscle tissue (M). IP staining. 60X.

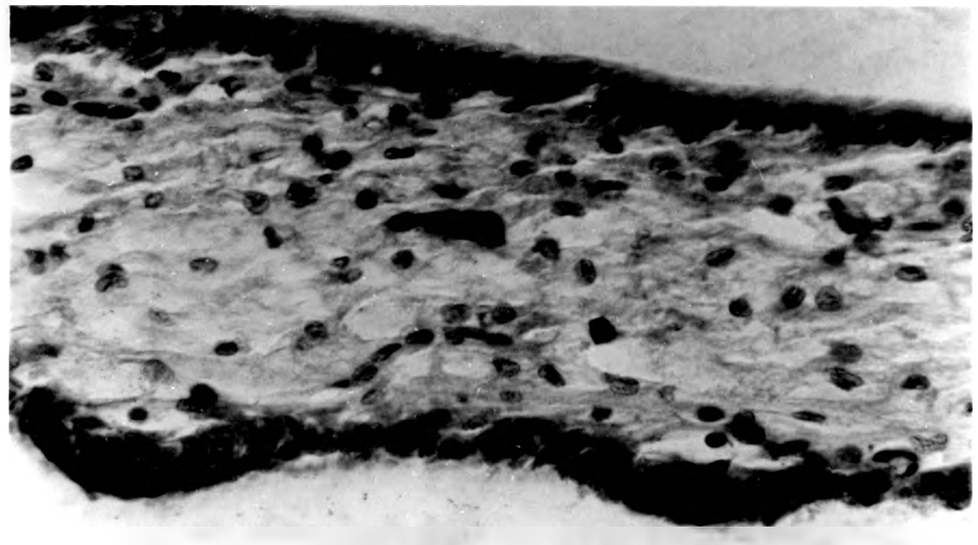


Figure 41. *V. vulnificus* b. I isolate injection challenge, 20min post-injection. Positive staining associated with HCB in subcuticular connective tissue. IP staining. 570X.

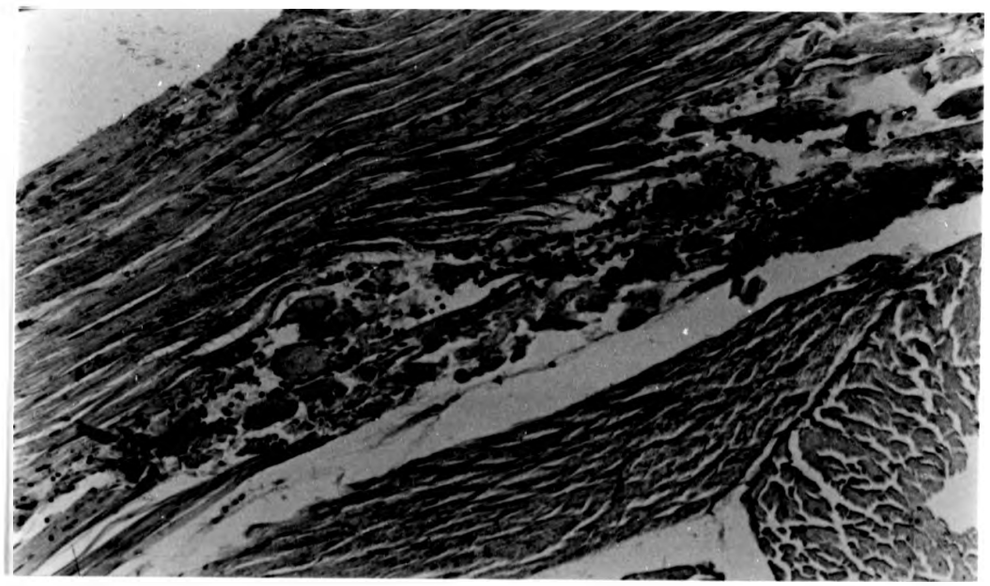


Figure 42. *V. vulnificus* b. I isolate injection challenge, 1h post-injection. Positive staining at the injection site with accumulation of haemocytes. IP staining. 140X.

Figure 43. *V. vulnificus* b. I isolate injection challenge, 1h post-injection. Positive staining associated with HCB contained in a hepatopancreatic vessel. IP staining. 290X.

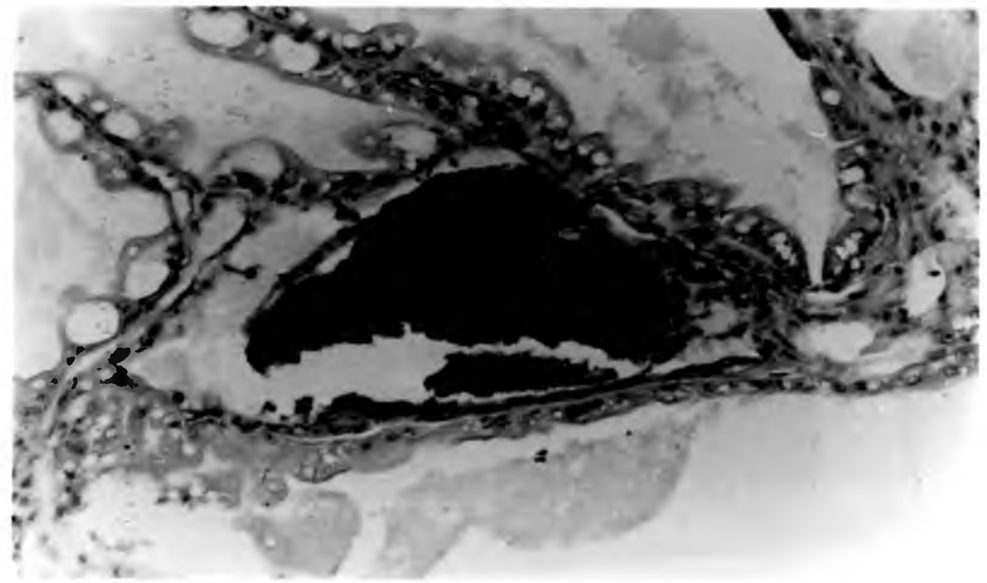


Figure 44. *V. vulnificus* b. I isolate injection challenge, 1h post-injection. Positive staining associated with HCB contained in a lymphoid organ vessel. IP staining. 290X.

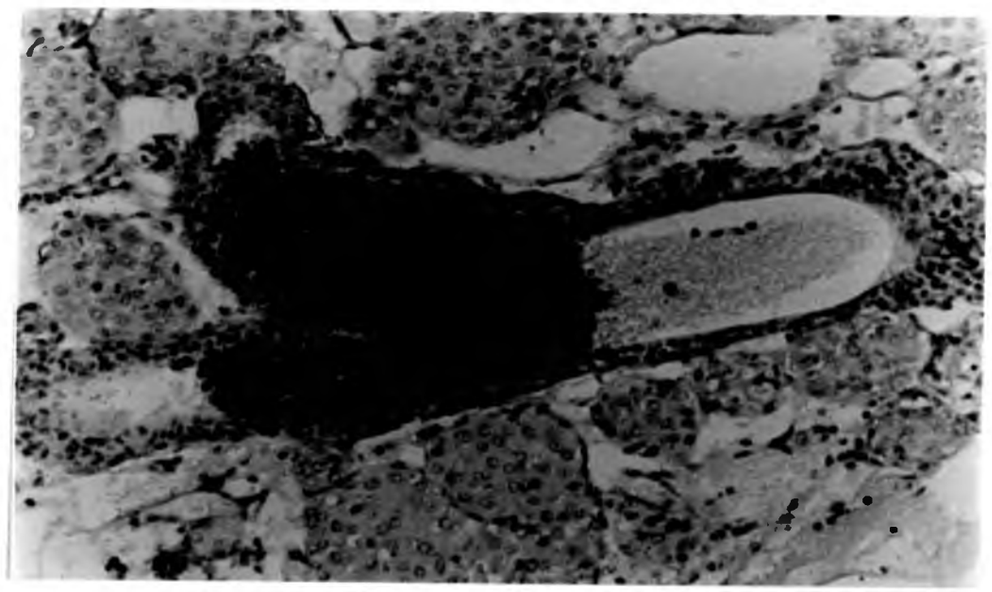


Figure 45. *V. vulnificus* b. I isolate injection challenge, 1h post-injection. Positive staining associated with the haemolymph and haemocytic aggregations in gills. IP staining. 60X.

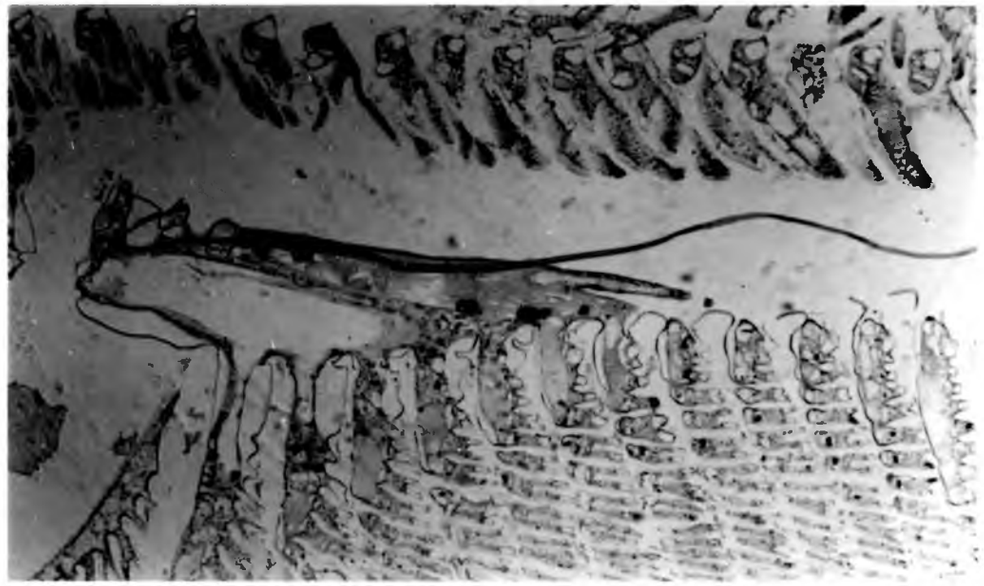


Figure 46. *V. vulnificus* b. I isolate injection challenge, 1h post-injection. Presence of HCB and positive material in the gill lamellae. IP staining. 1430X.

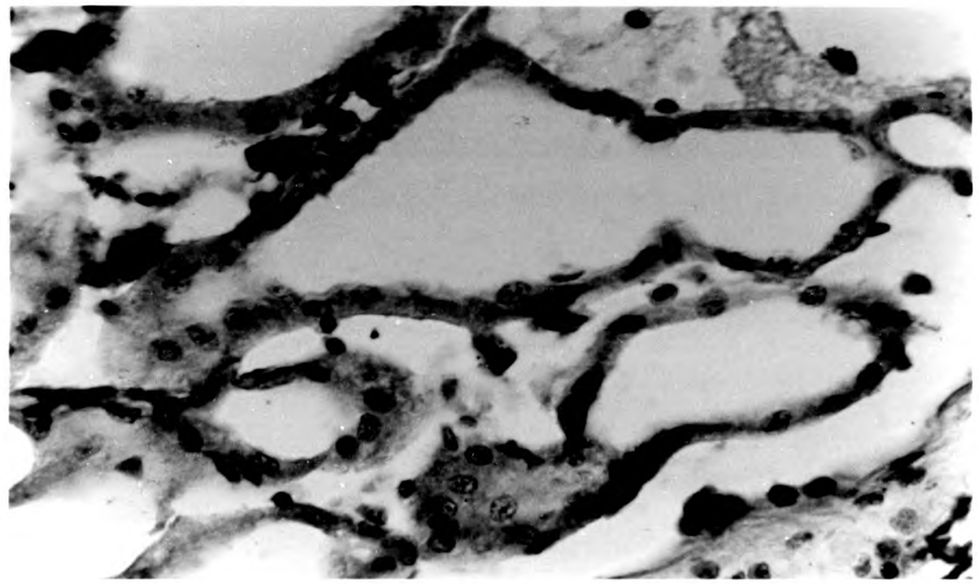


Figure 47. *V. vulnificus* b. I isolate injection challenge, 2h post-injection. Positive staining associated with HCB in antennal gland. IP staining. 570X.

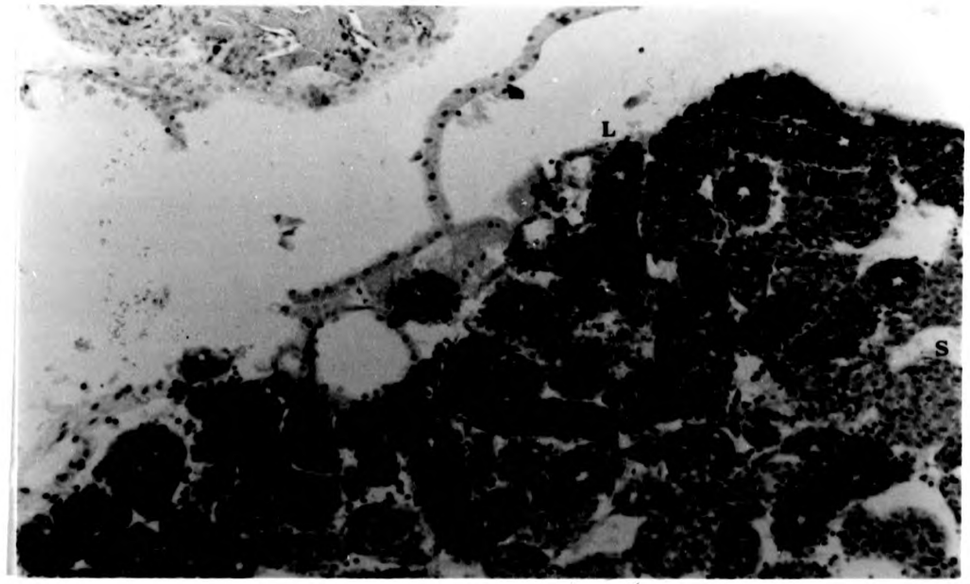


Figure 48. *V. vulnificus* b. I isolate injection challenge, 2h post-injection. Strong positive staining associated with normal lymphoid organ (L) and HCB in contrast with the spheroids (S). IP staining. 290X.

Figure 49. *V. vulnificus* b. I isolate injection challenge, 2h post-injection. Some positive staining associated with the spheroids can be observed. IP staining. 570X.

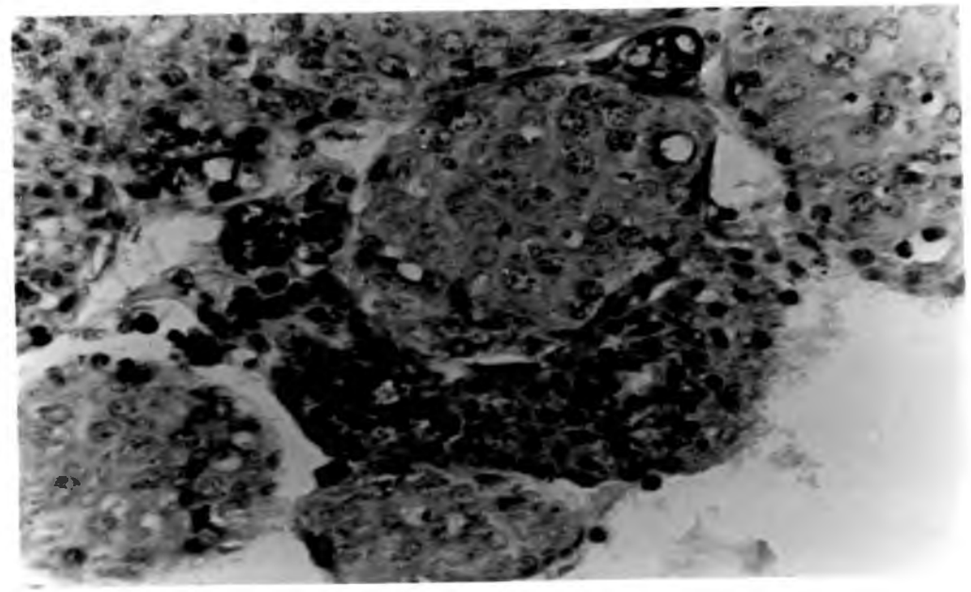
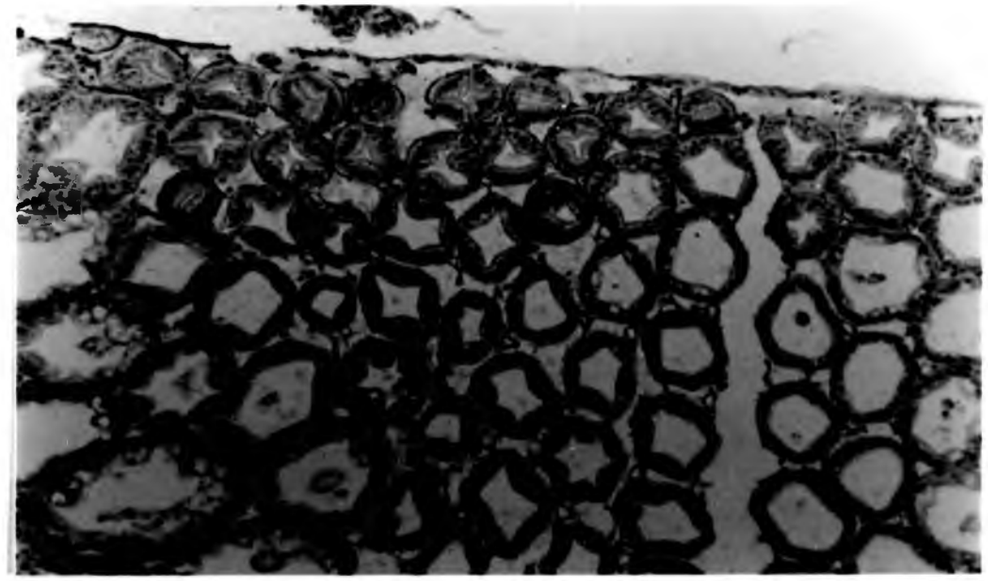
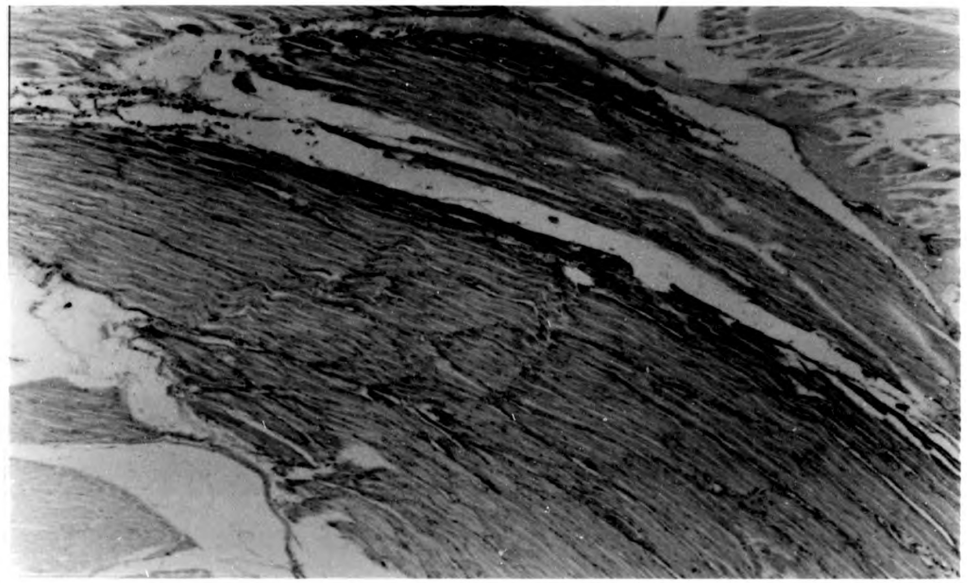


Figure 50. *V. vulnificus* b. I isolate injection challenge, 2h post-injection. Positive staining associated with HCB which are penetrating between the hepatopancreatic tubules. IP staining. 290X.





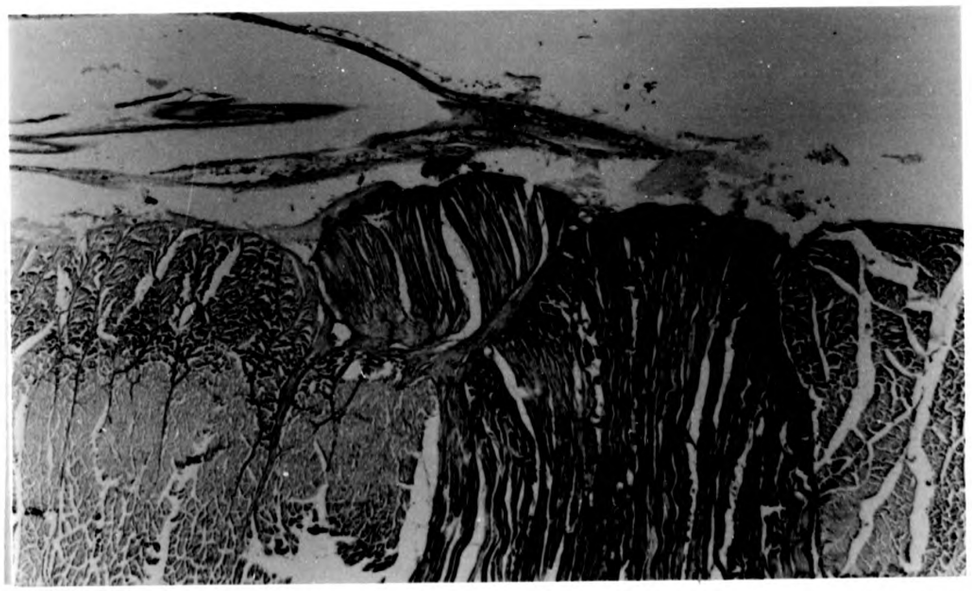


Figure 51 and 52. *V. vulnificus* b. I isolate injection challenge, 4h post-injection. Positive staining associated with muscular tissue at the site of injection, limited to the injected muscular segment. Haemocytes have concentrated in the area. HCB can be observed in connective tissue. IP staining. 140X.

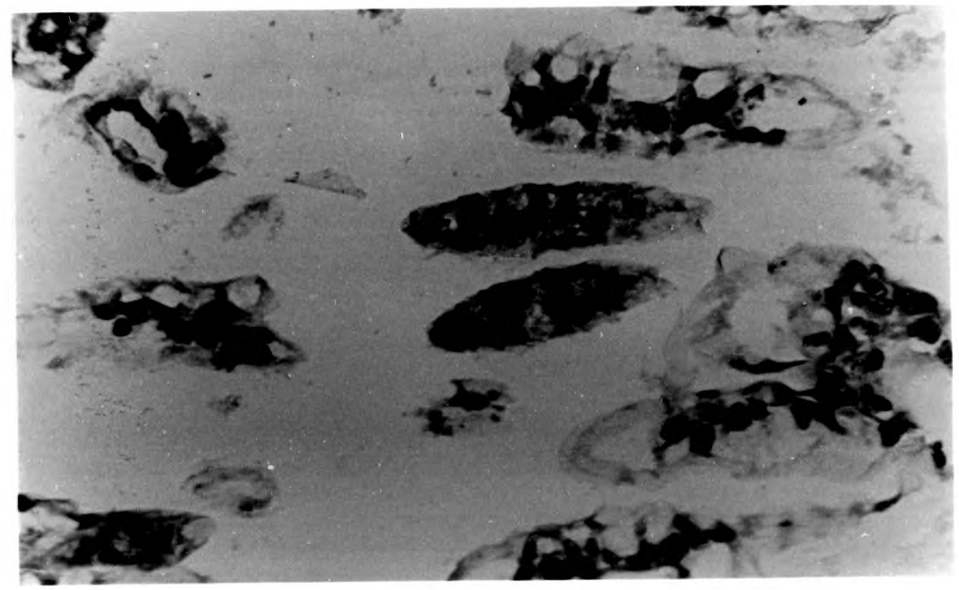


Figure 53. *V. vulnificus* b. I isolate injection challenge, 4h post-injection. Positive staining material associated with gill lamellae tissue. IP staining. 570X.

Figure 54. *V. vulnificus* b. I isolate injection challenge, 4h post-injection. Positive staining material associated with the heart muscular fibres. HCB nodules can be observed (N). IP staining. 290X.

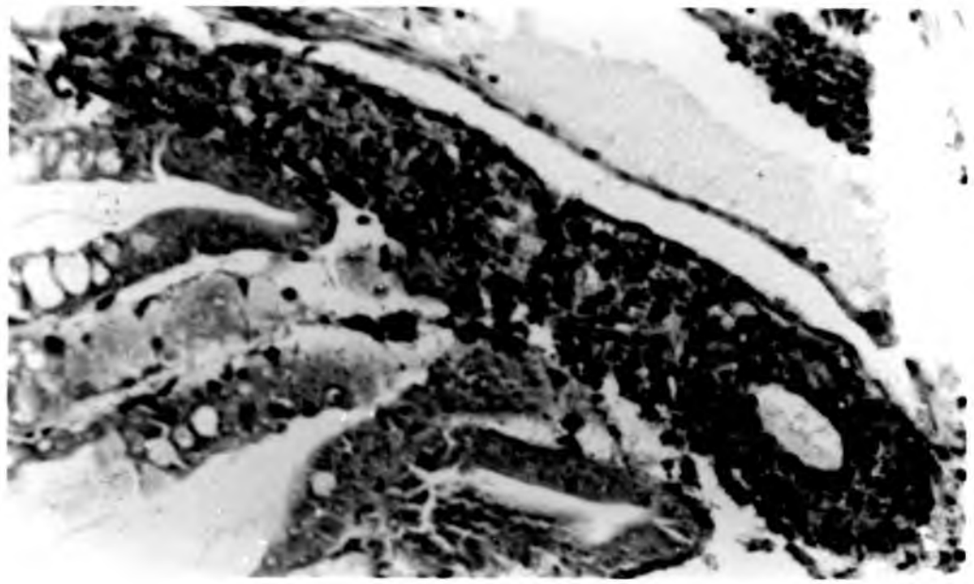


Figure 55. *V. vulnificus* b. I isolate injection challenge, 4h post-injection. Positive staining associated with the lymphoid organ and presence of HCB. IP staining. 290X.

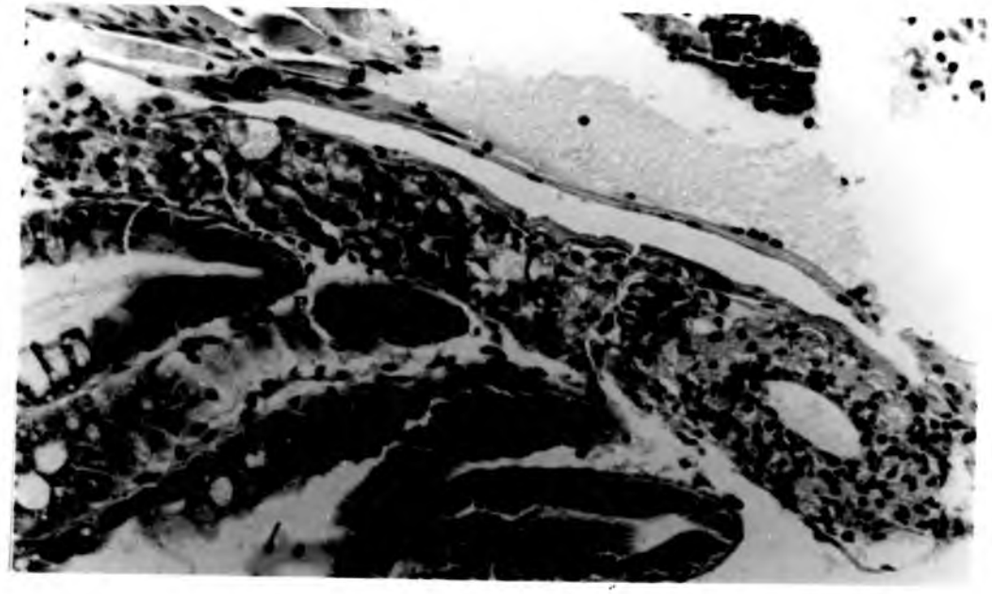


Figure 56. *V. vulnificus* b. I isolate injection challenge, 4h post-injection. Same sample as figure 55 showing a relatively normal lymphoid organ. Pyknotic nuclei can be observed (P). H&E staining. 290X.

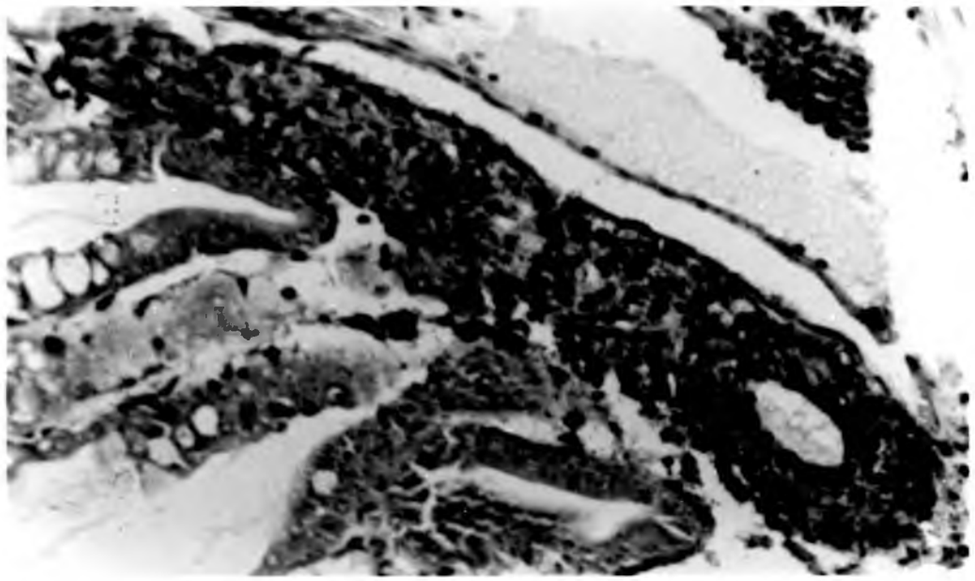


Figure 55. *V. vulnificus* b. I isolate injection challenge, 4h post-injection. Positive staining associated with the lymphoid organ and presence of HCB. IP staining. 290X.

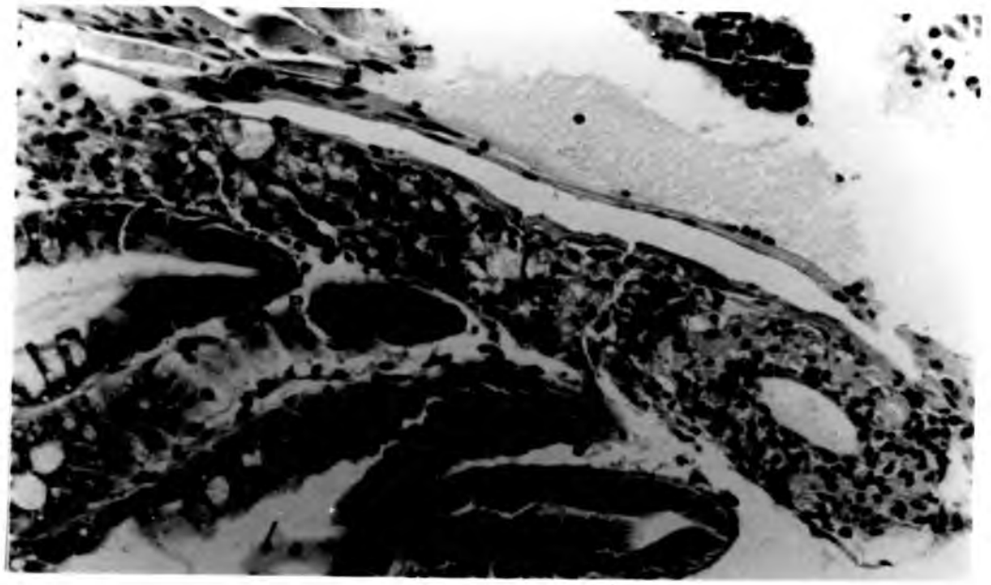


Figure 56. *V. vulnificus* b. I isolate injection challenge, 4h post-injection. Same sample as figure 55 showing a relatively normal lymphoid organ. Pyknotic nuclei can be observed (P). H&E staining. 290X.

Figure 57. *V. vulnificus* b. I isolate injection challenge, 4h post-injection with high bacterial dose. Positive staining associated with the connective tissue surrounding the midgut and dramatic loss of epithelium. IP staining. 570X.

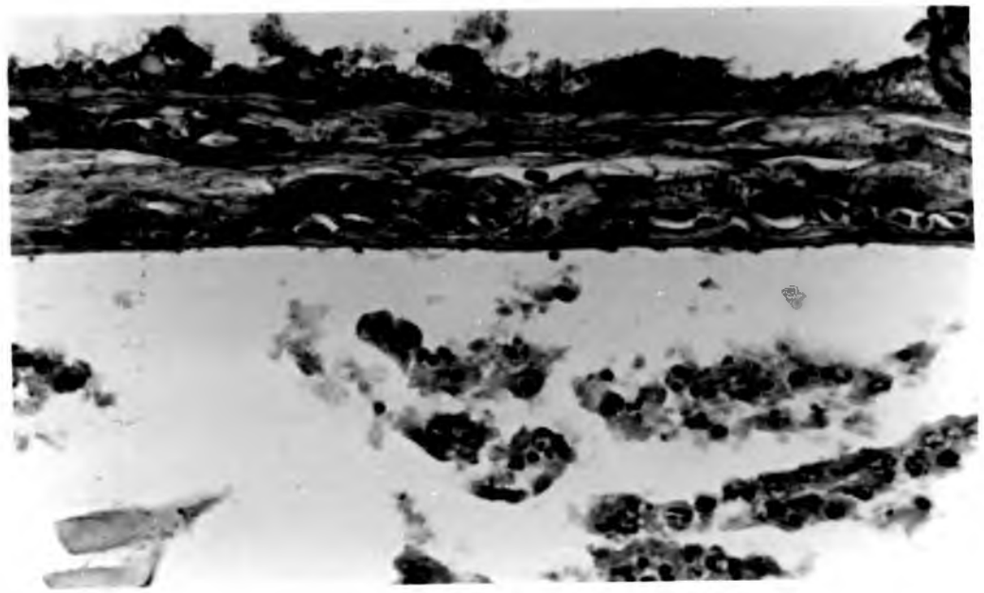


Figure 58. *V. vulnificus* b. I isolate injection challenge, 4h post-injection with high bacterial dose. Positive staining associated with the hepatopancreatic capsule and intertubular tissue. IP staining. 290X.

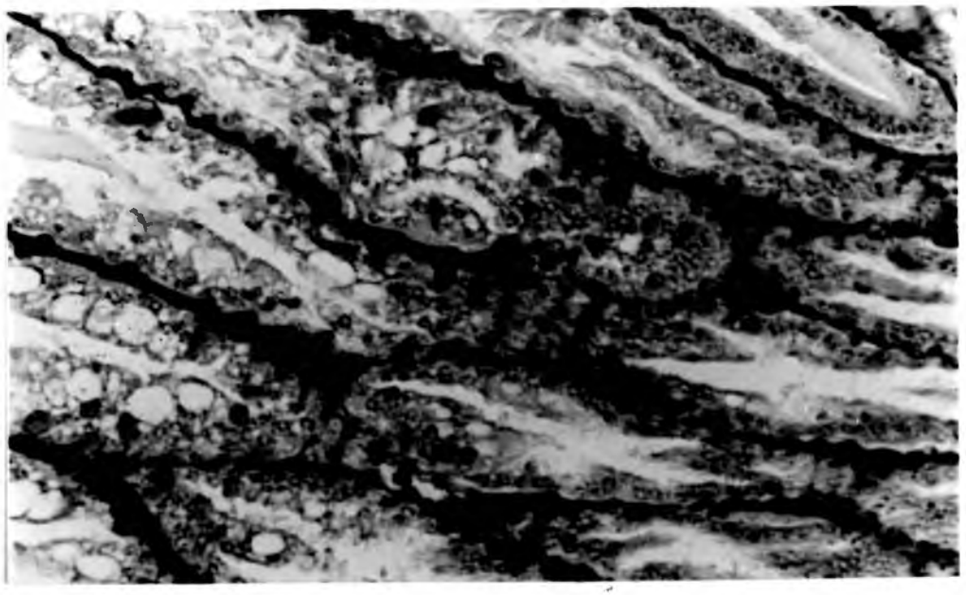


Figure 57. *V. vulnificus* b. I isolate injection challenge, 4h post-injection with high bacterial dose. Positive staining associated with the connective tissue surrounding the midgut and dramatic loss of epithelium. IP staining. 570X.

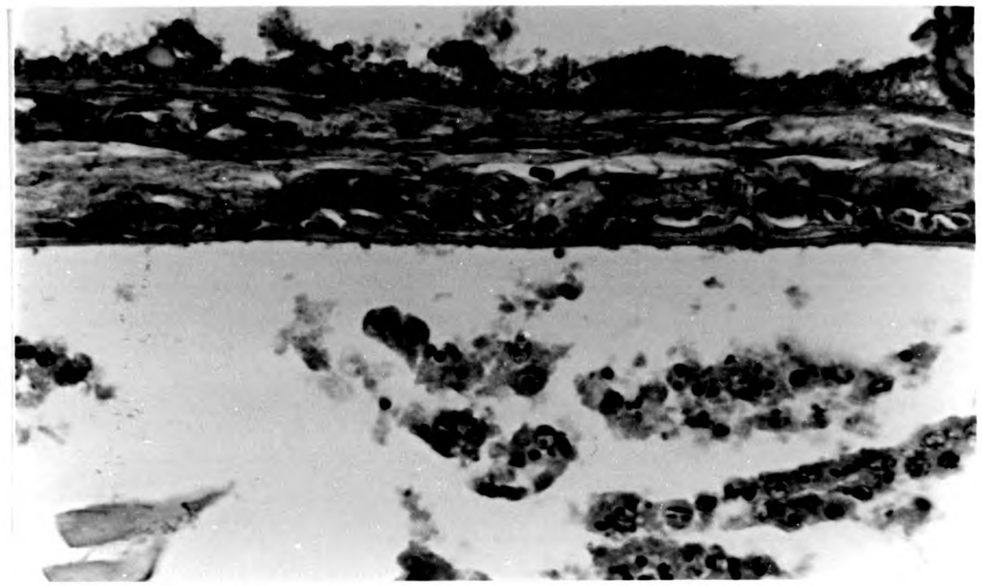


Figure 58. *V. vulnificus* b. I isolate injection challenge, 4h post-injection with high bacterial dose. Positive staining associated with the hepatopancreatic capsule and intertubular tissue. IP staining. 290X.

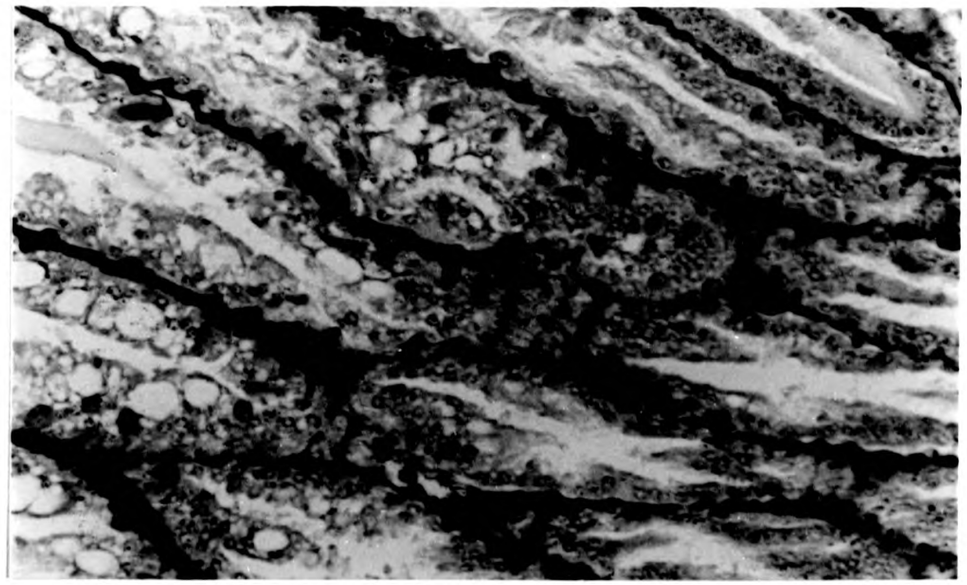


Figure 59. *V. vulnificus* b. I isolate injection challenge, 6h post-injection. HCB and fibrous tissue present in the injection site. IP staining. 60X.

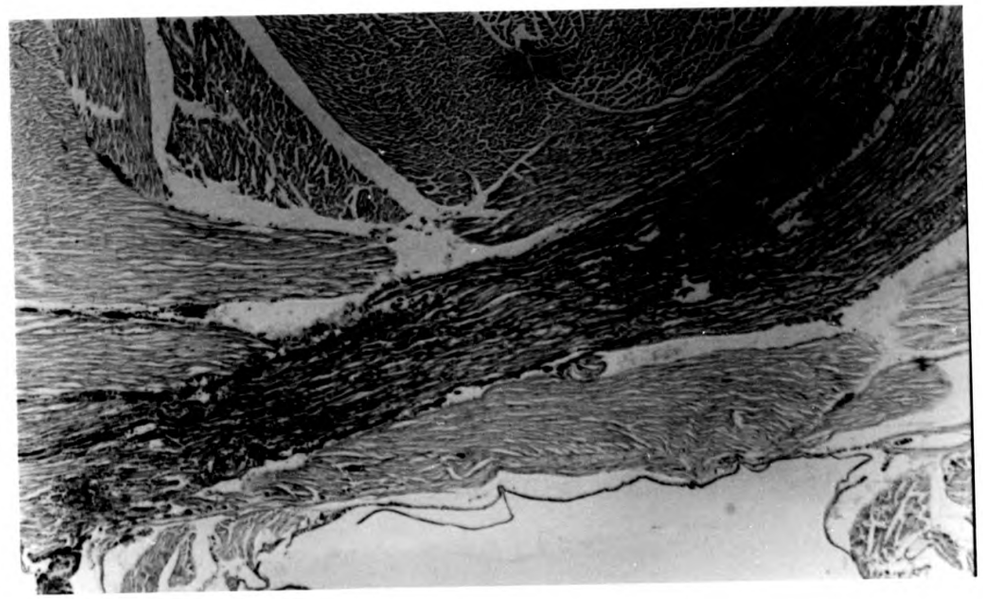
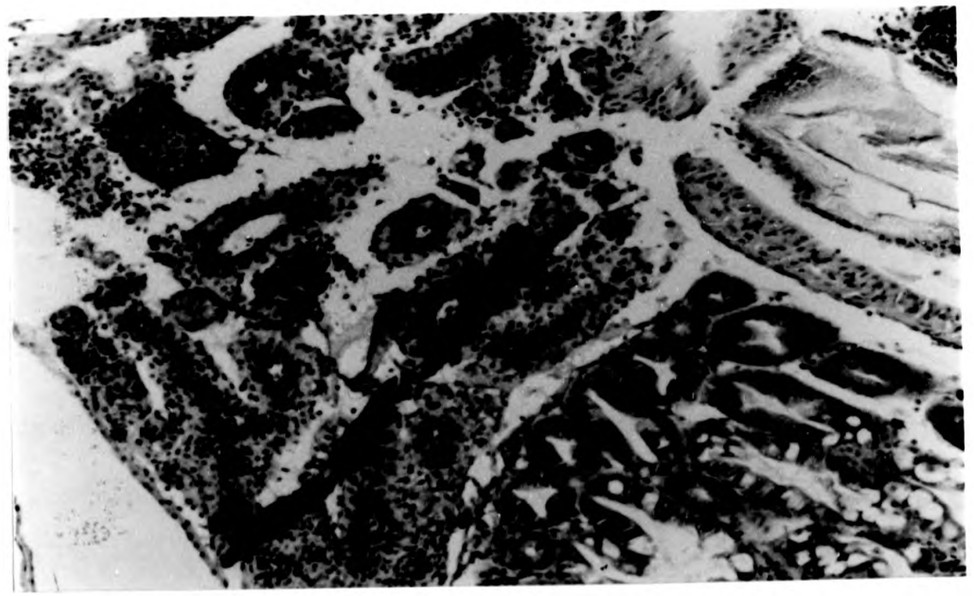


Figure 60. *V. vulnificus* b. I isolate injection challenge, 8h post-injection. Lymphoid organ with a less positive staining. IP staining. 140X.



11.2.3. *V. anguillarum* strain injection challenge.

Two shrimps were sampled 4h post-injection, the other 6 used in the experiment were found dead 24h post-injection.

The pathology caused by *V. anguillarum* was very similar to that caused by *V. vulnificus* b. I isolate, however, its evolution was much faster.

Nodules of haemocytes with pyknotic nuclei were found in heart (figure 61) and lymphoid organ (figure 62). These modified haemocytes were present in the hepatopancreatic capsule and in the distal intertubular spaces (figure 63). Sloughing of epithelial cells of the hepatopancreas and midgut epithelium were observed (figure 64) and were discarded through gut (figure 65) and posterior midgut caecum (figure 66) which was itself surrounded with modified haemocytes.

No positive staining reaction was observed in the samples.

Figure 61. *V. anguillarum* strain injection challenge, 4h post-injection. Aggregation of haemocytes with pyknotic nuclei in heart. IP staining. 290X.

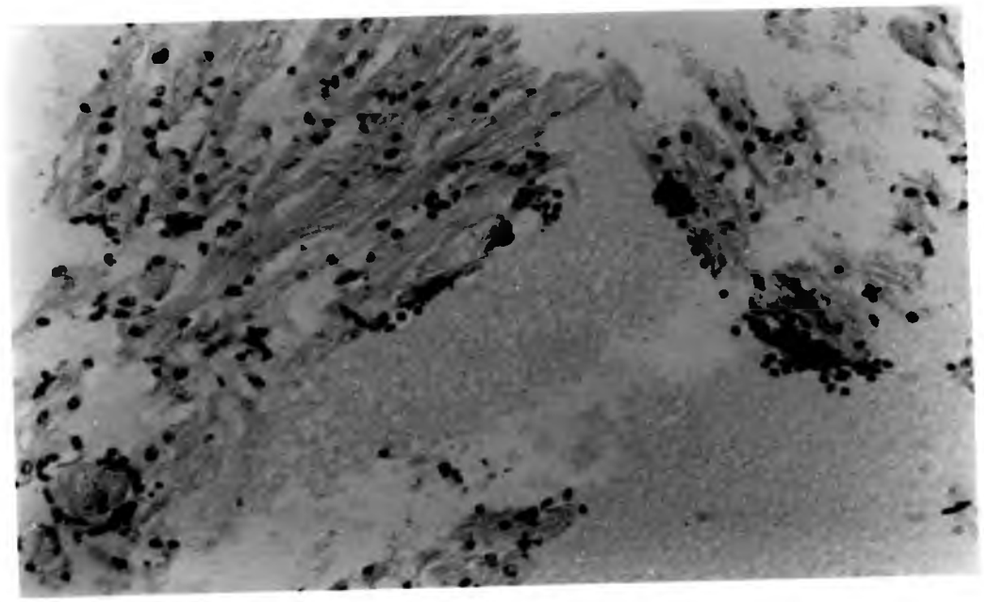
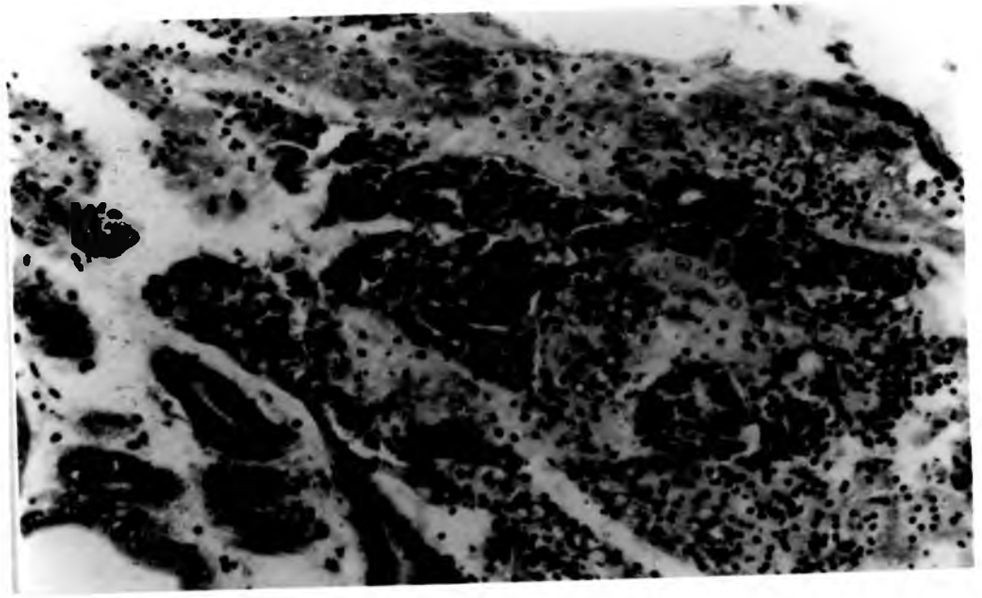


Figure 62. *V. anguillarum* strain injection challenge, 4h post-injection. Accumulation of haemocytes with pyknotic nuclei in lymphoid organ. IP staining. 290X.



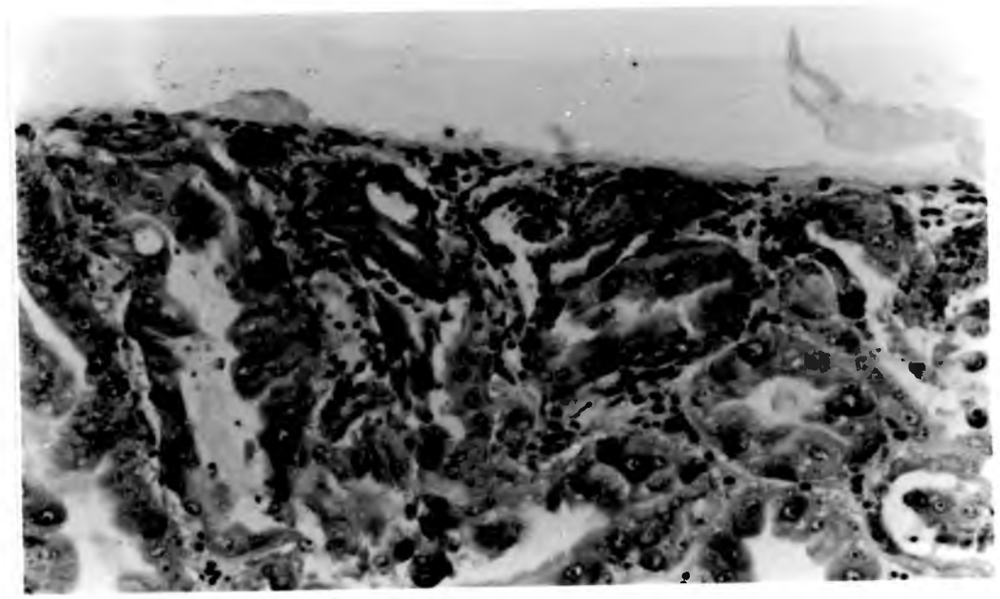


Figure 63. *V. anguillarum* strain injection challenge, 4h post-injection. Haemocytes with pyknotic nuclei in the hepatopancreatic intertubular space. IP staining. 290X.

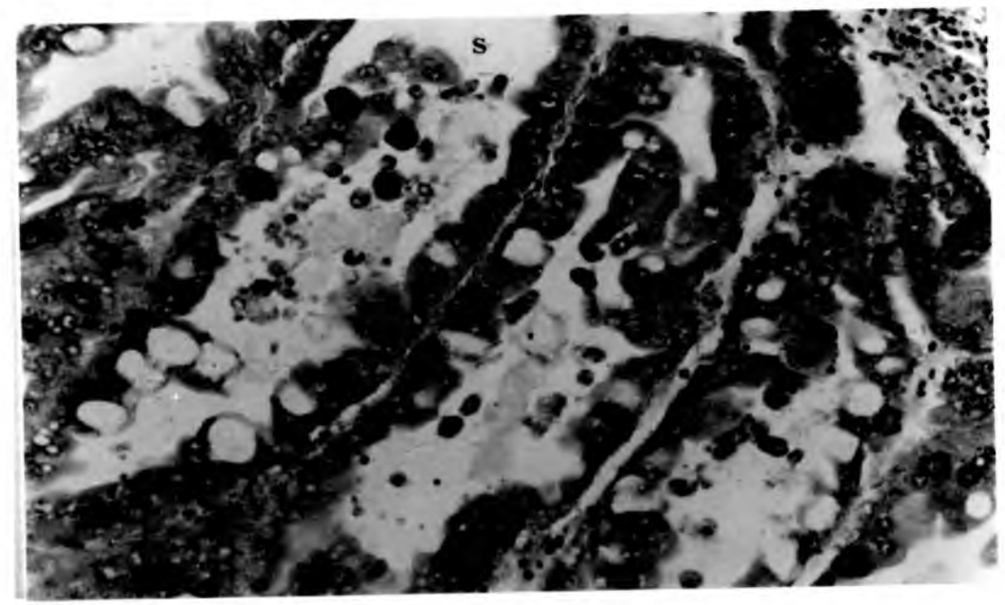


Figure 64. *V. anguillarum* strain injection challenge, 4h post-injection. Haemocytes with pyknotic nuclei between tubules (P) and sloughing hepatopancreatic tubular epithelium (S). IP staining. 290X.

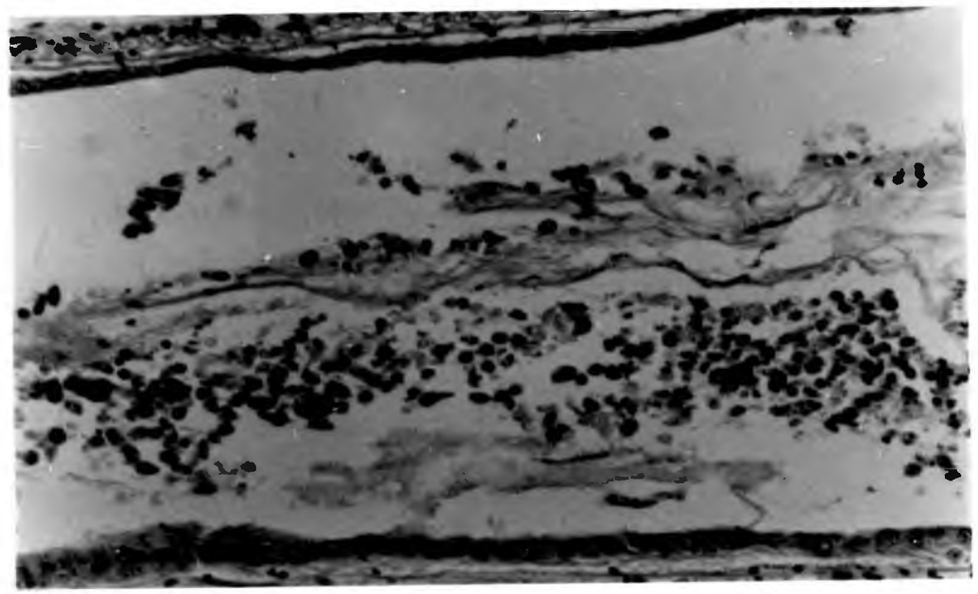


Figure 65. *V. anguillarum* strain injection challenge, 4h post-injection. Sloughed cells from hepatopancreas in midgut. IP staining. 290X.

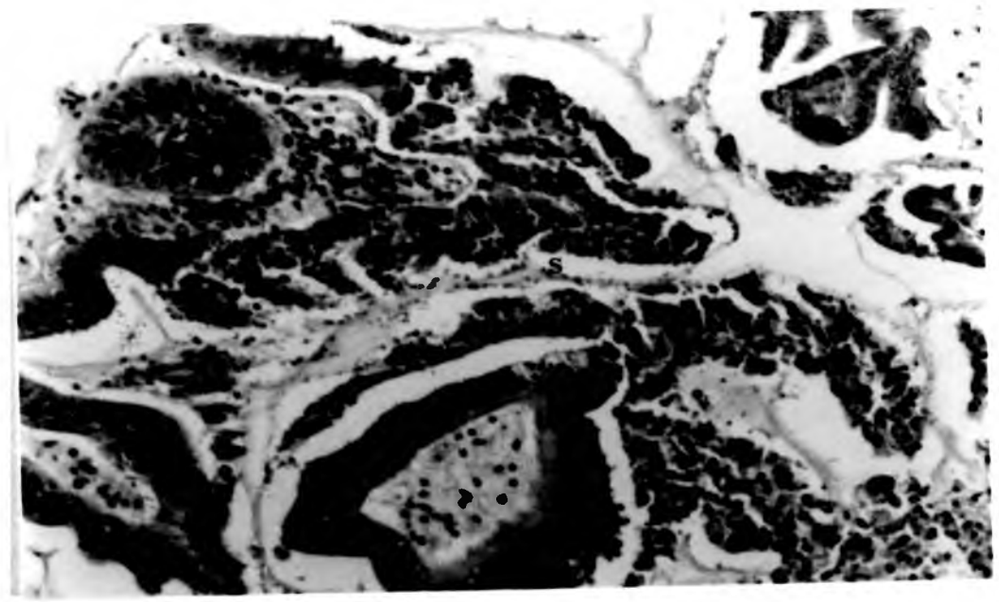


Figure 66. *V. anguillarum* strain injection challenge, 4h post-injection. Sloughed cells from hepatopancreas and midgut accumulated in posterior midgut caecum lumen (S) and haemocytes with pyknotic nuclei surrounding the caecum. IP staining. 290X.

11.2.4. Sonicated *V. vulnificus* b. I isolate injection challenge.

One shrimp out of 21 was found dead after 1h of a total number of 21.

Ten minutes post-injection, positive stained material was carried away with the haemolymph, resulting in haemolymph coloration, and found to accumulate around the gut (figure 67) and hepatopancreas (figure 78). Positive haemolymph was also found between muscle fibres (figure 69). The positive coloration of legs and mouth muscle was more intense than the rest of the tissues similar to the observation following injection of intact cells.

Twenty minutes post-injection, positive material was accumulated in gills (figure 70), heart (figure 71) and lymphoid organ (figure 73). H&E staining of both tissues showed no pathology associated with them (figures 72 and 74).

Four hours post-injection, a small haemocytic response was observed at the site of injection. Positive material had already been cleared from the area.

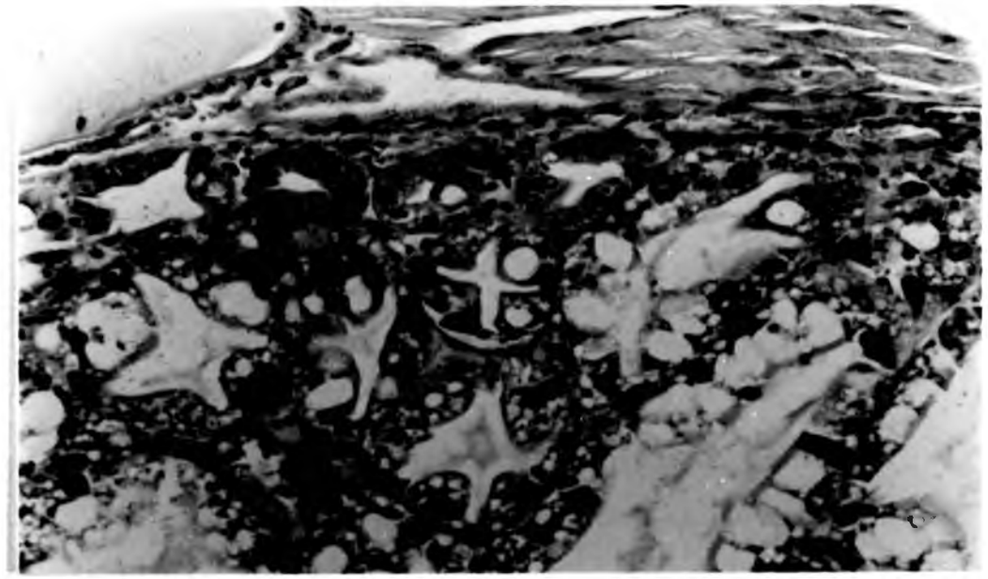
Twenty four hours post-injection, positive staining was fading, although still remained in gill, heart and lymphoid organ.

Although bacteria were sonicated, some cells resisted the treatment and some whole cells were injected into the shrimp provoking a haemocytic reaction. This reaction was rarely observed, only a positive haemocytic

nodule was found in haemolymph (figure 75) and a few HCB could be distinguished in the hepatopancreas capsule and gills (figures 69 and 70).

Figure 67. Sonicated *V. vulnificus* b. I isolated injection challenge, 10min post-injection. Positive staining associated with the haemolymph surrounding the midgut. IP staining. 290X.

Figure 68. Sonicated *V. vulnificus* b. I isolate injection challenge, 10min post-injection. Positive staining associated with the surrounding of the hepatopancreas and presence of HCB around the organ and in the intertubular spaces. IP staining. 290X.



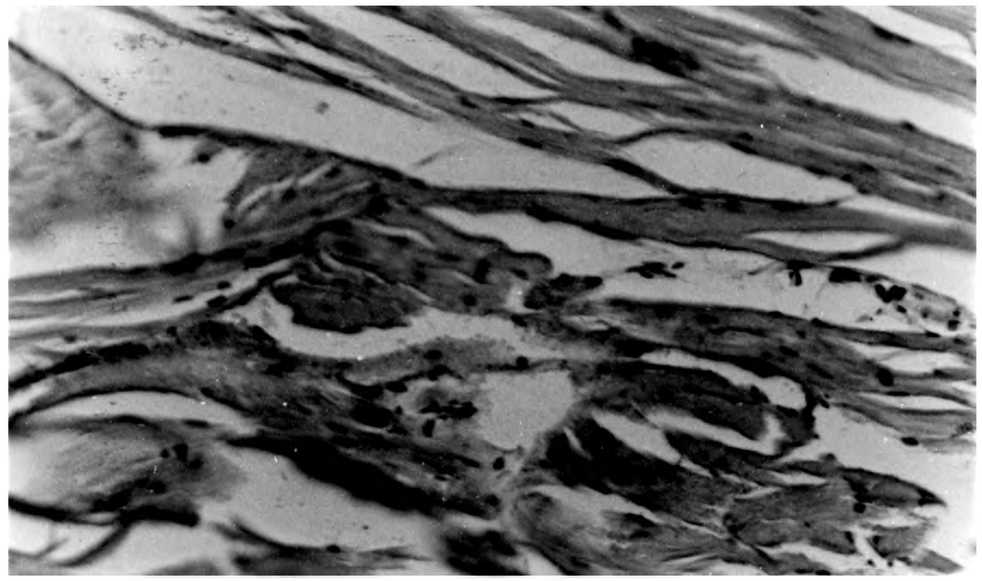


Figure 69. Sonicated *V. vulnificus* b. I isolate injection challenge, 10min post-injection. Positive staining associated with the haemolymph between muscle fibres. IP staining. 290X.

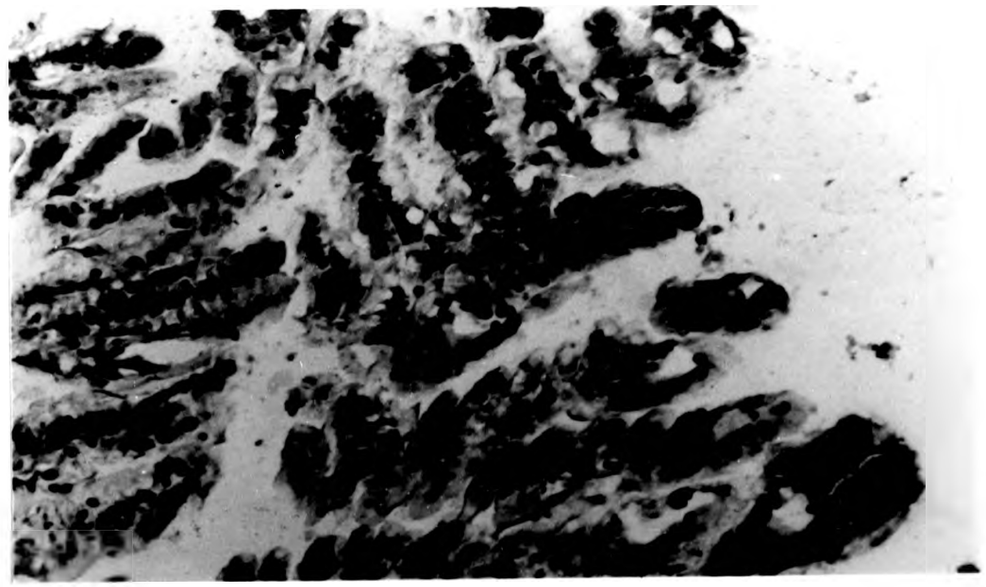


Figure 70. Sonicated *V. vulnificus* b. I isolate injection challenge, 20min post-injection. Positive staining associated with the gill lamellae and presence of HCB. IP staining. 290X.

Figure 71. Sonicated *V. vulnificus* b. I isolate injection challenge, 1h post-injection. Positive staining associated with heart muscle tissue and presence of HCB. IP staining. 140X.

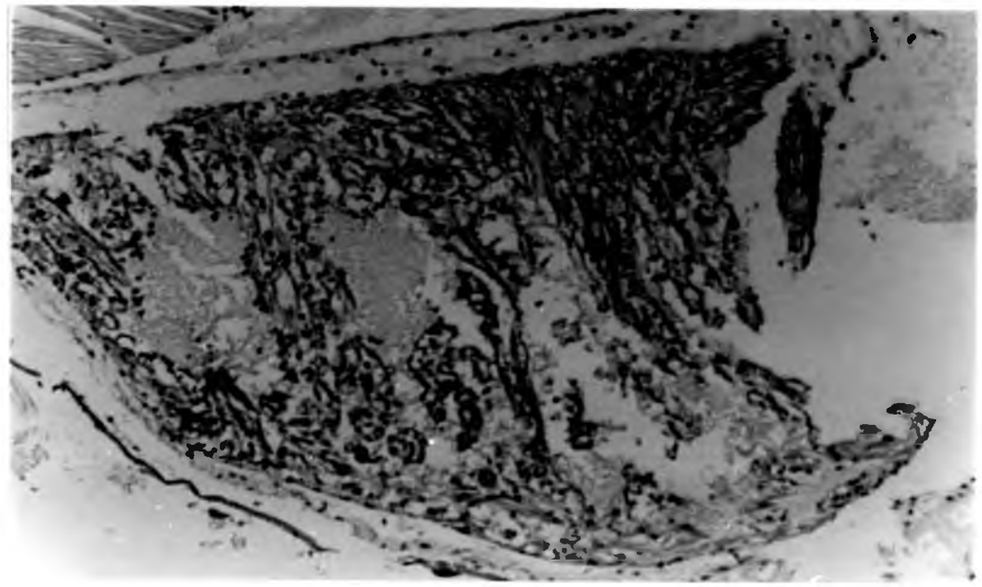


Figure 72. Sonicated *V. vulnificus* b. I isolate injection challenge, 1h post-injection. The same sample as figure 71 showing the heart with haemocytic aggregations. H&E staining. 140X.

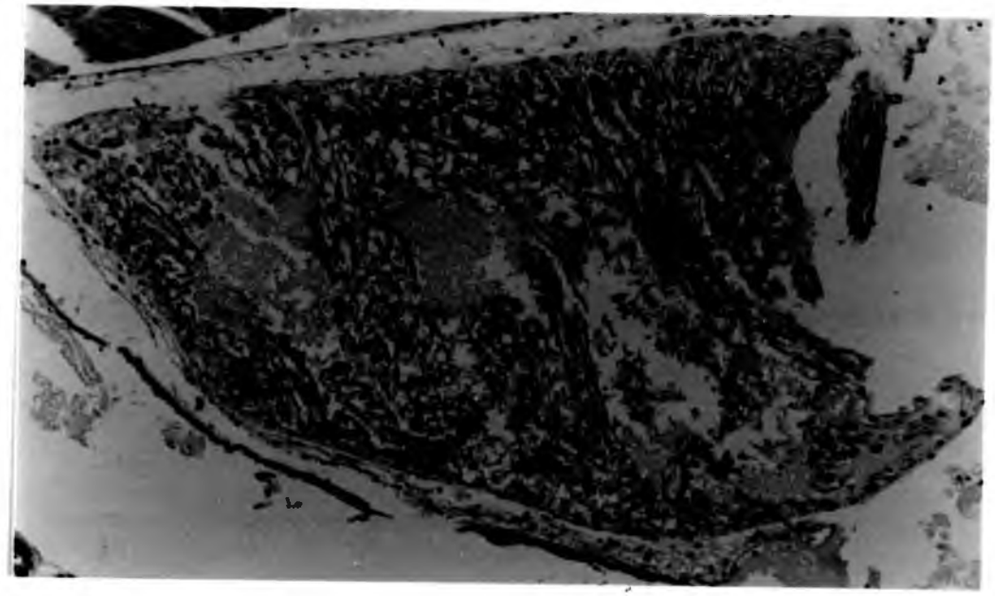


Figure 73. Sonicated *V. vulnificus* b. I isolate injection challenge, 1h post-injection. Positive staining associated with the lymphoid organ. IP staining. 140X.

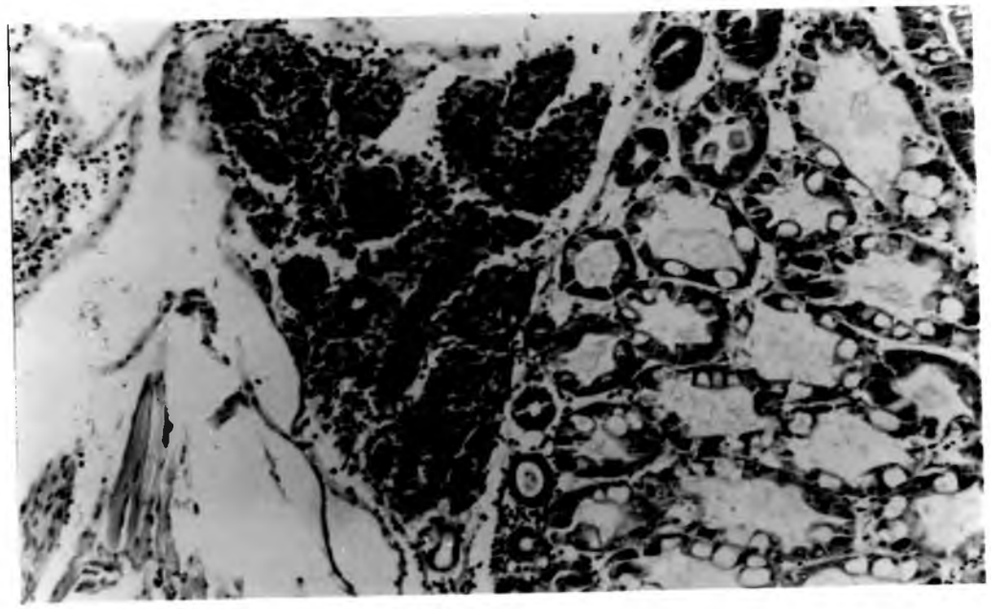
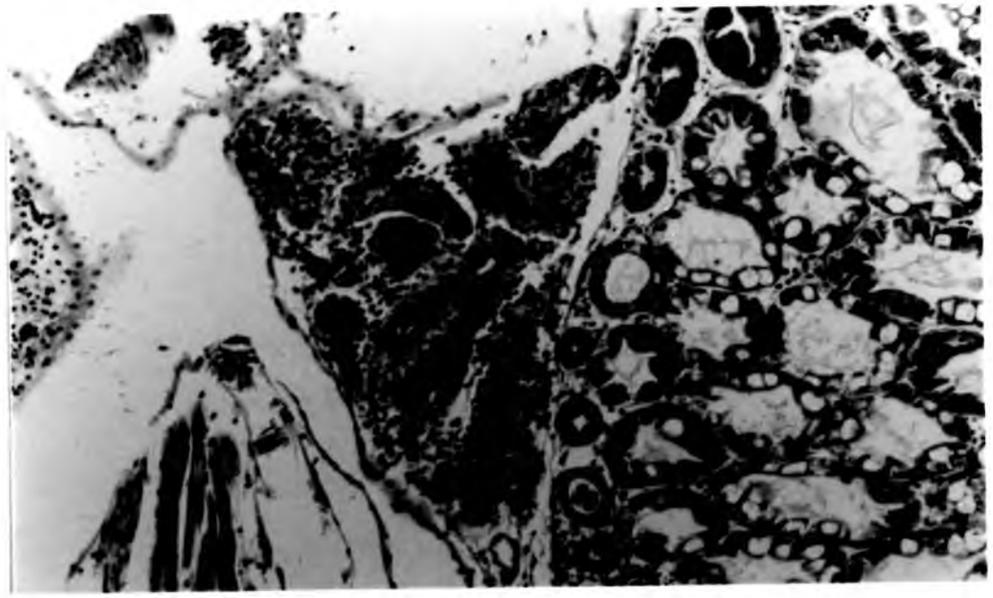


Figure 74. Sonicated *V. vulnificus* b. I isolate injection challenge, 1h post-injection. The same preparation as figure 73 showing a relatively normal lymphoid organ. H&E staining. 140X.



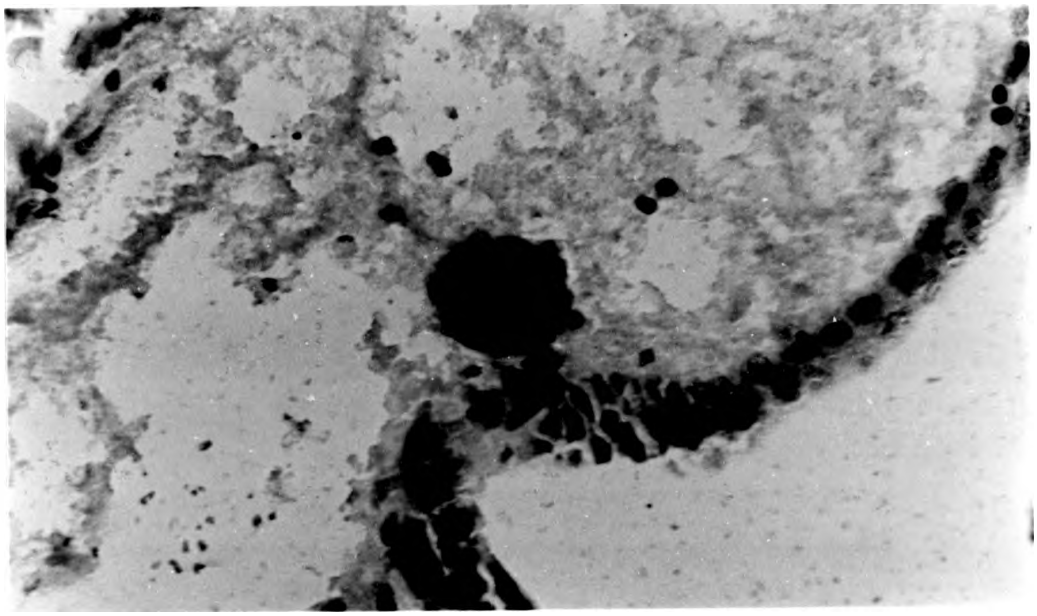


Figure 75. Sonicated *V. vulnificus* b. I isolate injection challenge, 1h post-injection. Positive stained haemocytic nodule in positive stained haemolymph. IP staining. 570X.

11.2.5. *V. vulnificus* b. I isolate oral challenge results

Two of the nineteen shrimps that underwent stress were found dead after 24h.

First sampling was performed 30 min after inoculation. Oesophagus, stomach and gut lumen were full of material stained positive with peroxidase (figure 76). The same sample stained with haematoxylin and eosin (H&E) only showed some patches of basophilic material together with unidentifiable foregut content. The staining reaction and the size of the particles in the patches appeared to be bacteria, however, they appeared to have been somehow modified as cells could not be clearly distinguished (figure 77).

Figure 78 shows the cardiac setal screen filtering into the hepatopancreas the soluble material of the stomach content, this fraction includes the soluble positive stained material and fine particles. The filtered material had a light positive colour in contrast with the darker coarse part which was diverted into the gut. In spite of all this positive bacterial material, no bacterial cells were observed when stained with H&E (figure 79).

The positive material filtered through the sieve started to occupy the hepatopancreatic tubules distributing along their whole length. This material accumulated, packing the tubules (figure 80) but again bacterial cells were not observed in H&E stained preparations (figure 81). A few bacterial cells

were found in two hepatopancreatic tubules in only one animal. These bacterial cells, as the ones found in the foregut, appeared to be somehow modified as the outline of the cells could not be easily distinguished.

One hour post-inoculation, the positive material appeared to diffuse through the walls all along the tubules from the distal to their proximal region (figure 82).

Two hours post-inoculation, positive material could be observed in the haemolymph sinuses between hepatopancreatic tubules and were subsequently observed outside the hepatopancreas (figure 83) until the tubules were empty of positive material, between 4 and 12h post-inoculation (figure 84). Positive material was found associated with the vacuoles of the R-cells as it was observed in the bath challenge (figure 30). Haemolymph with positive material was observed in the sinus along the body before it could be observed in vessels or heart. Haemocytes did not show any reaction to the positive material (figure 85).

Four hours post-inoculation, haemolymph with positive material was observed in the gills, heart, around the antennal gland and lymphoid organ as it happened in the sonicated bacteria challenge, the staining was not accompanied by haemocytic reaction.

Six hours post-inoculation, positive haemolymph was observed in the gill

lamellae (figure 86) and again no bacterial cells were observed when the preparation was stained with H&E (figure 87). A large amount of positive material was retained within the heart. It appeared to be attached to the muscle fibres (figure 88). H&E staining showed no intact bacterial cells (figure 89).

One animal was found to have a positive stained lesion in the midgut epithelium, in the area of the gut not lined with cuticle (figure 90).

Between 6 and 9h all evidence of the inocula had disappeared from gut.

Nine hours post-inoculation, some positive stained material was still found in the gill lamellae and a large amount was found in between the lamellae (figure 91). A necrotic tubule was found in one of the shrimps with a haemocytic reaction encapsulating positively stained or melanized necrotic tissue (as it was observed in figure 34). Another case showed a massive necrosis of hepatopancreatic tubules positively stained and with no hemocytic reaction (figure 92).

Between 9 and 12h, the heart was clear of positive material.

Twenty four hours post-inoculation, positive material was found accumulated inside B-cell vacuoles as it was found in the bath challenge (figure 93). This material was also found inside the lumen of some hepatopancreatic tubules

which had few or no B-cell vacuoles (figure 94). In some cases, positive staining was observed in association with hepatopancreatic epithelial nuclei as it was found in the bath challenge (figure 28). Some animals presented a modified epithelium especially the central area of the hepatopancreas which received the largest amount of filtered inoculum. These modifications ranged from the loss of B-cell vacuoles (figure 95) to a metaplasia into a simple layer of cuboidal epithelium (figure 96).

Animals subjected to stress had a slower clearing of the absorbed material than animals kept in normal conditions. It could be observed a time gap of 12h.

The degree of response to the inoculum over the time varied substantially between individuals.

Figure 76. *V. vulnificus* b. I isolate oral challenge, 30min post-inoculation. Positive stained material associated with the material contained in the foregut. IP staining. 60X.

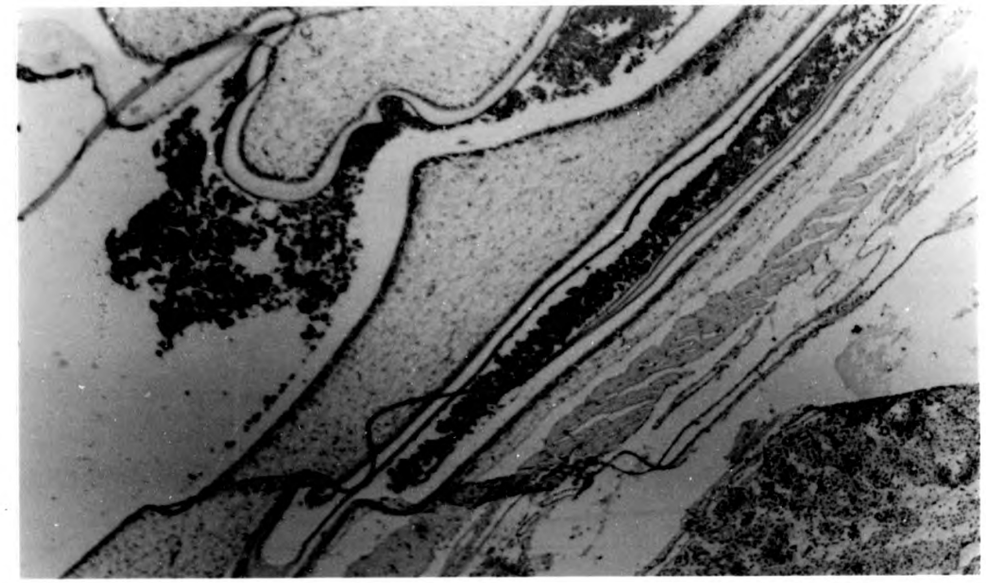
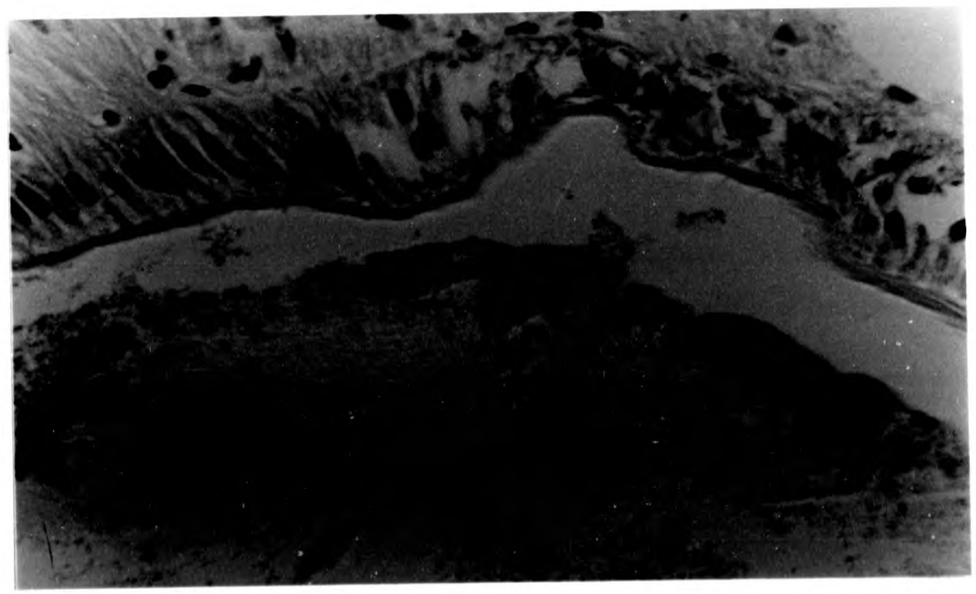


Figure 77. *V. vulnificus* b. I isolate oral challenge, 30min post-inoculation. Bacterial cells within the foregut content. H&E. 1430X.



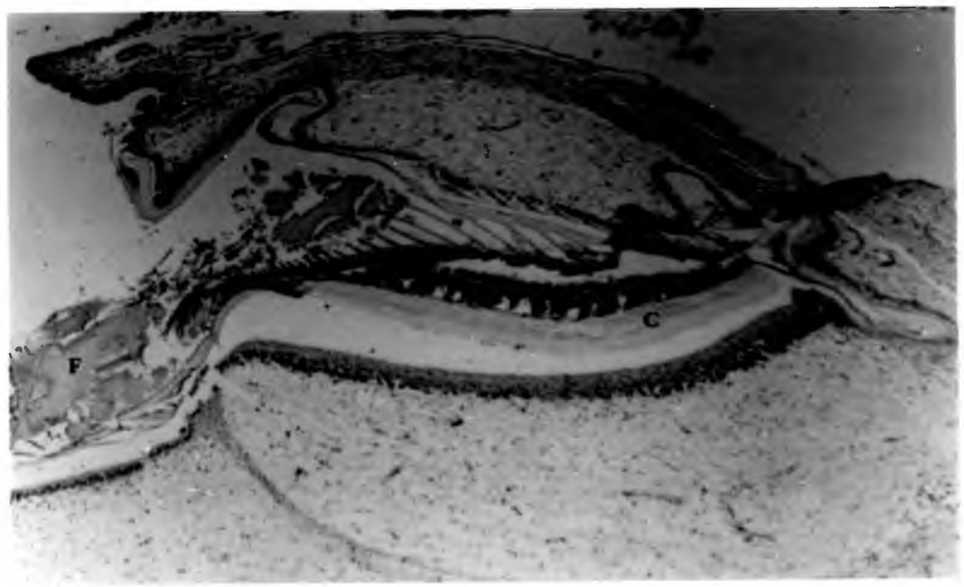


Figure 78. *V. vulnificus* b. I isolate oral challenge, 30min post-inoculation. Setae screen retaining positive stomach content (C). The filtered material has a less intense positive staining (F). IP staining. 60X.

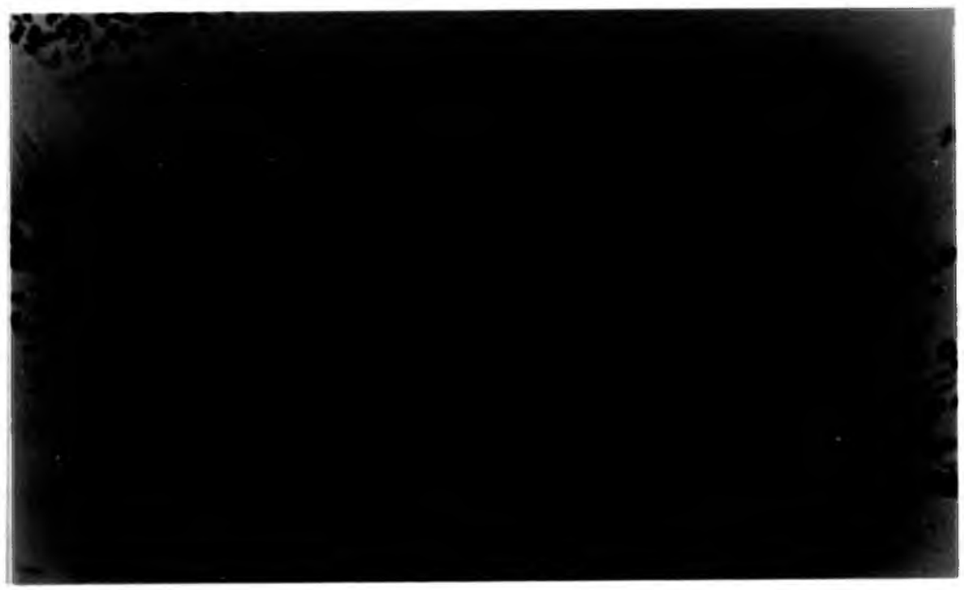


Figure 79. *V. vulnificus* b. I isolate oral challenge, 30min post-inoculation. Detail of figure 78 , no bacterial cells can be observed in the setae screen. H&E staining. 1430X.

Figure 80. *V. vulnificus* b. I isolate oral challenge, 30min post-inoculation. Positive stained material filling the hepatopancreatic tubules. IP staining. 140X.

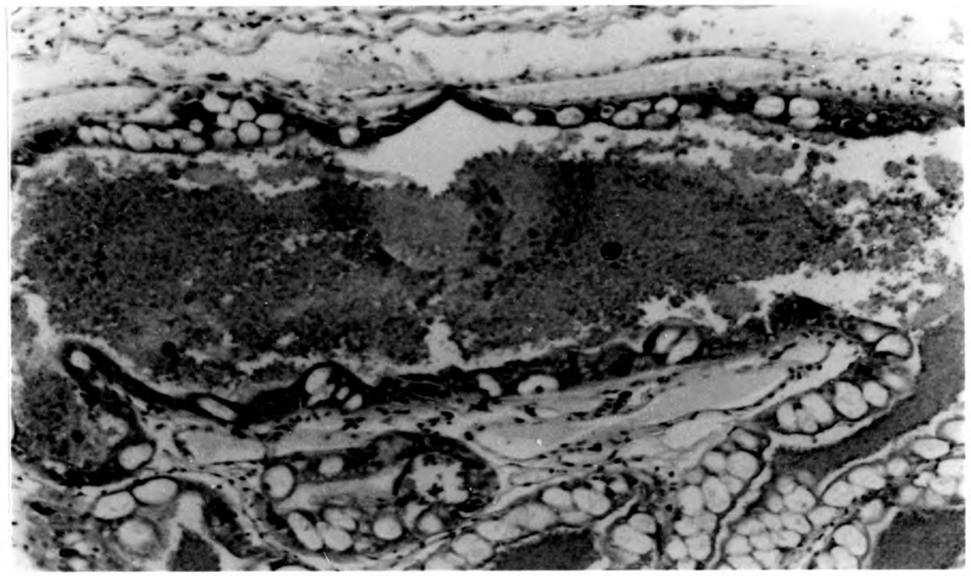
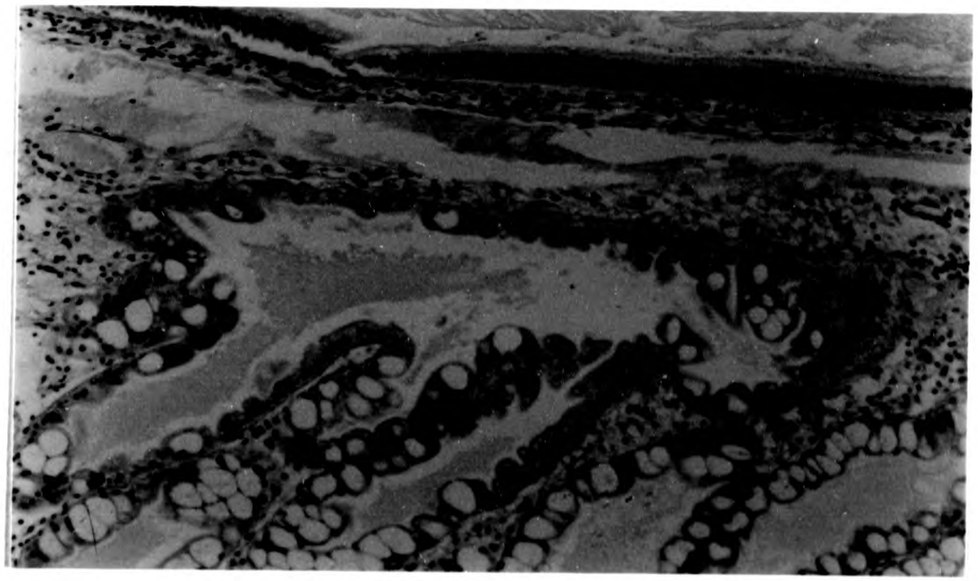


Figure 81. *V. vulnificus* b. I isolate oral challenge, 30min post-inoculation. Same sample as figure 80, no bacterial cells were observed. H&E staining. 140X.



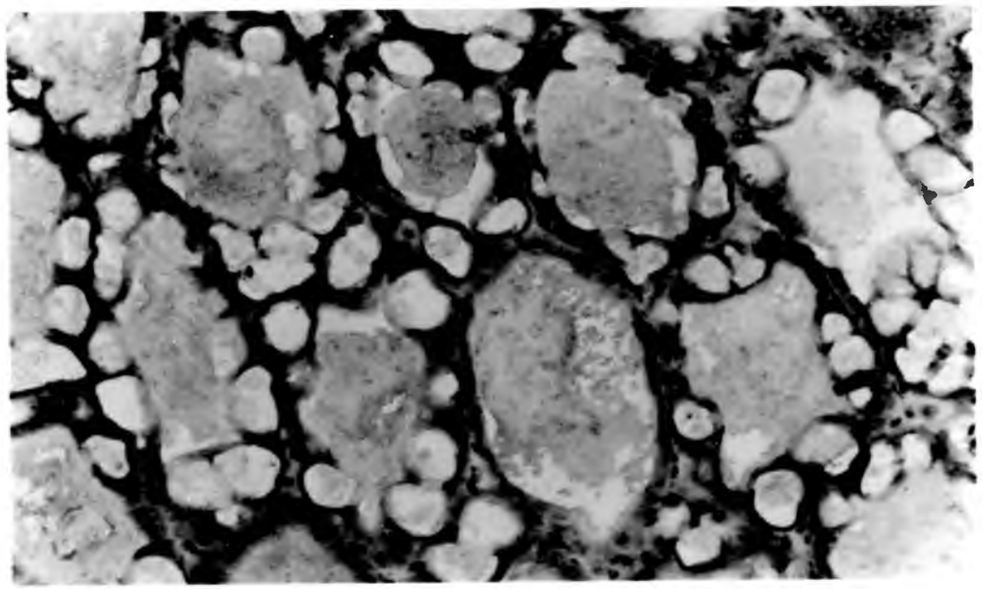


Figure 82. *V. vulnificus* b. I isolate oral challenge, 1h post-inoculation. Positive stained material from the hepatopancreatic tubules diffusing through the epithelial cells. IP staining. 290X.

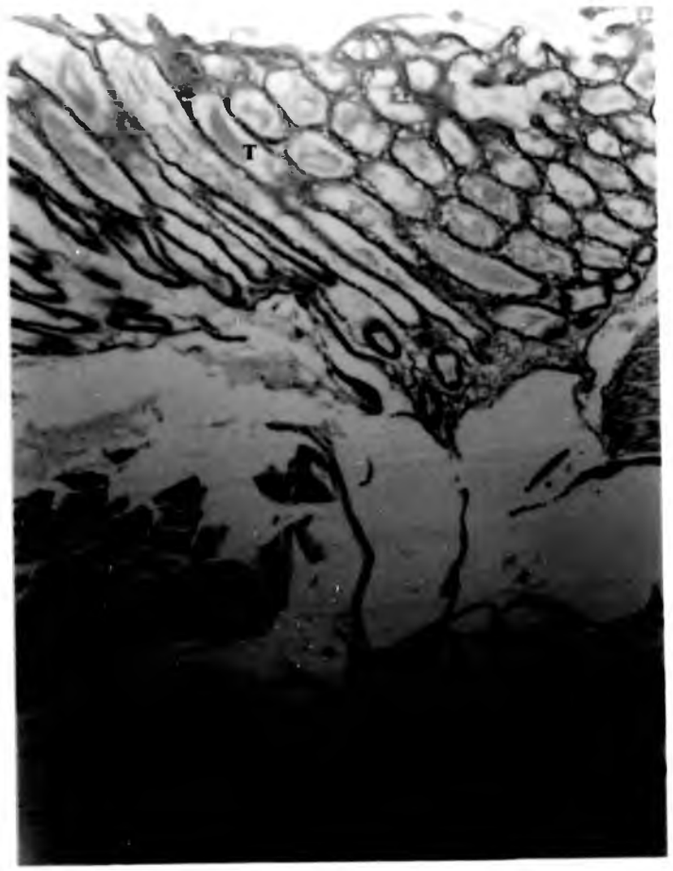


Figure 83. *V. vulnificus* b. I isolate oral challenge, 2h post-inoculation. Positive stained material associated with the hepatopancreatic tubular content (T) and haemolymph (H). IP staining. 60X.

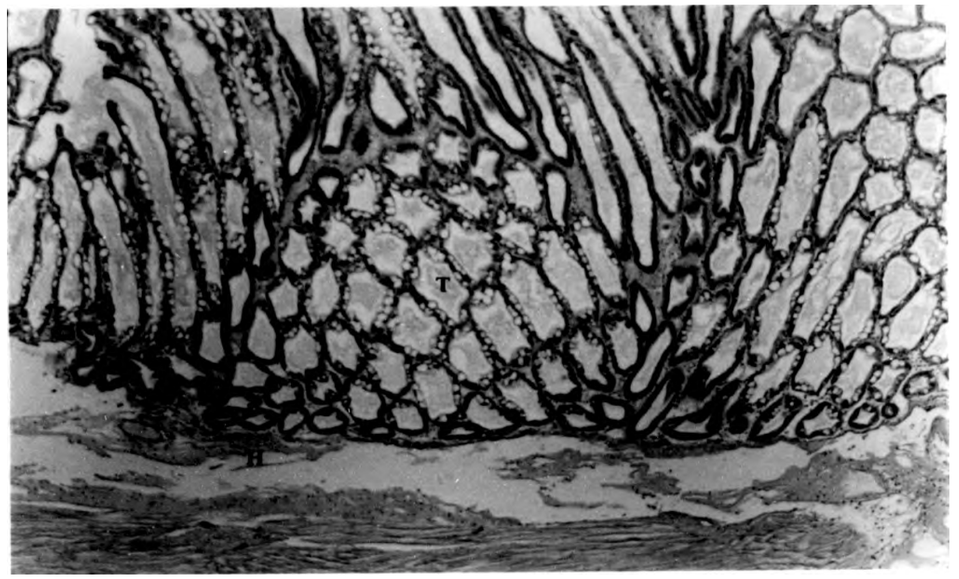


Figure 84. *V. vulnificus* b. I isolate oral challenge, 2h post-inoculation. Hepatopancreatic tubular content free of positive material (T). Positive staining associated with the hepatopancreatic sinuses (S) and the haemolymph surrounding the hepatopancreas (H). IP staining. 60X.

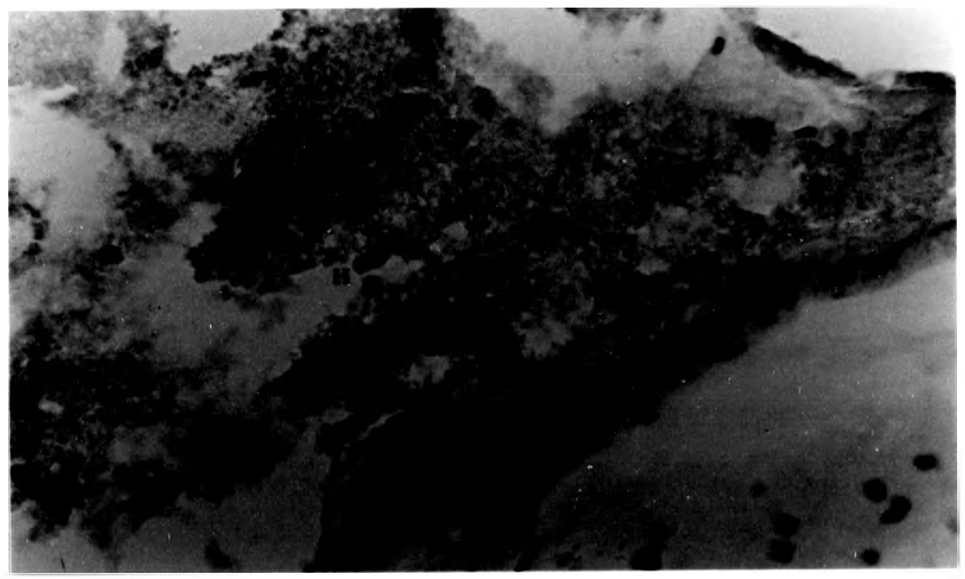


Figure 85. *V. vulnificus* b. I isolate oral challenge, 2h post-inoculation. Haemocytes in positive stained haemolymph (H). No reaction is observed, the haemocyte cytoplasm can be distinguished empty of positive material. IP staining. 570X.

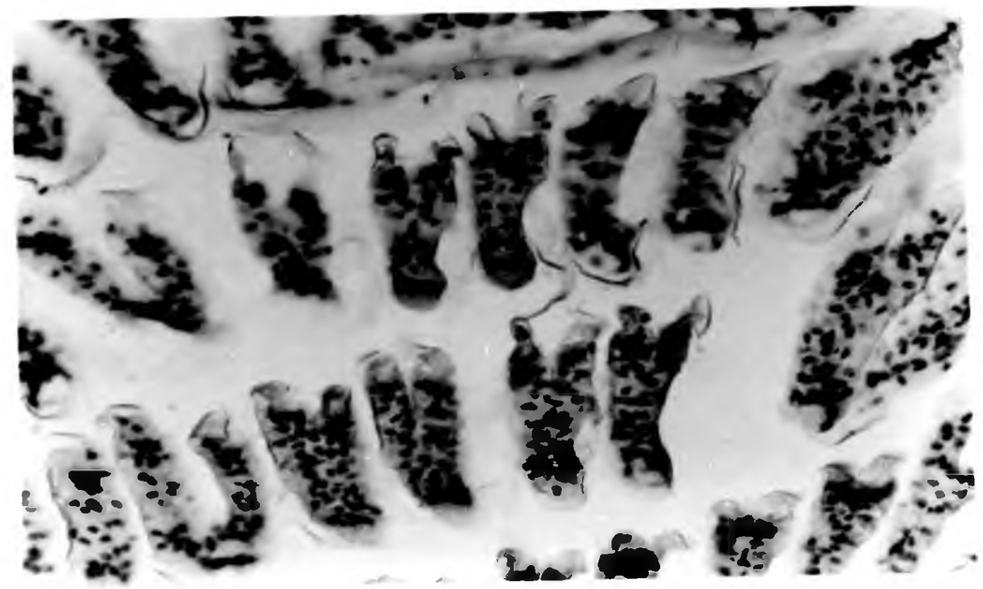


Figure 86. *V. vulnificus* b. I isolate oral challenge, 6h post-inoculation. Positive staining present in gill lamellae. IP staining. 290X.

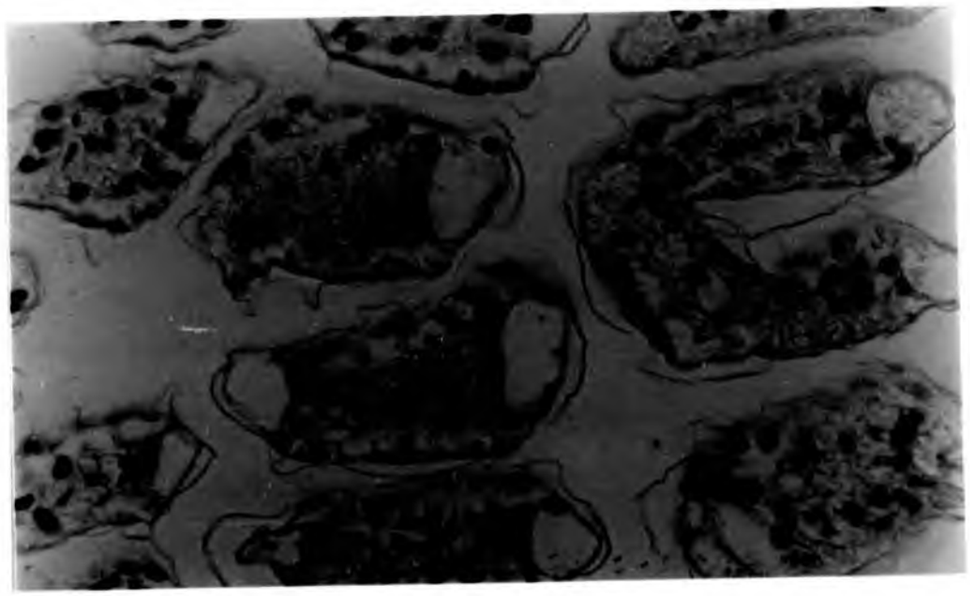


Figure 87. *V. vulnificus* b. I isolate oral challenge, 6h post-inoculation. Detail of figure 86 showing no bacterial cells. H&E staining. 570X.

Figure 88. *V. vulnificus* b. I isolate oral challenge, 6h post-inoculation. Positive stained material associated with the heart muscle fibres. IP staining. 290X.

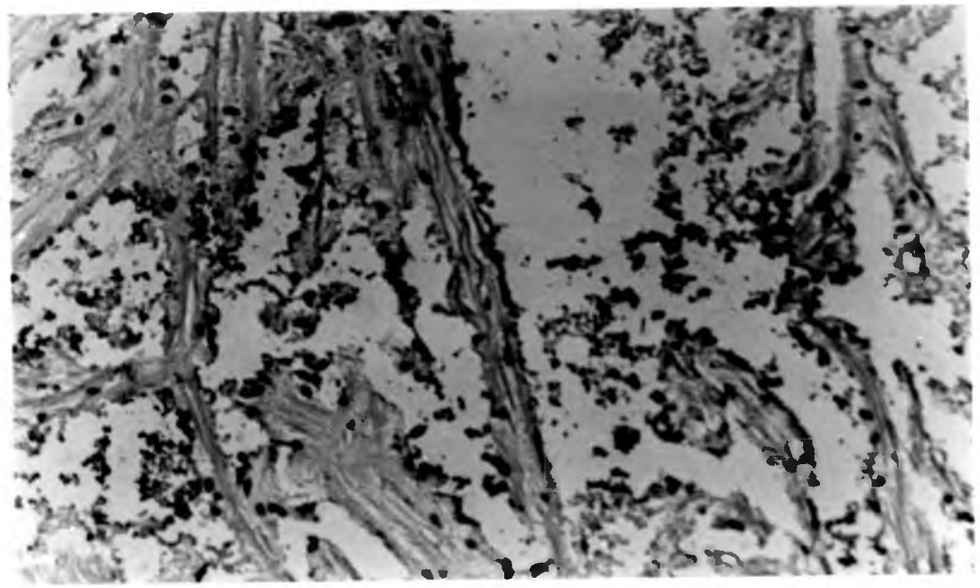


Figure 89. *V. vulnificus* b. I isolate oral challenge, 6h post-inoculation. Detail of figure 88 showing no evidence of bacterial cells associated with the muscle fibres. H&E staining. 570X.

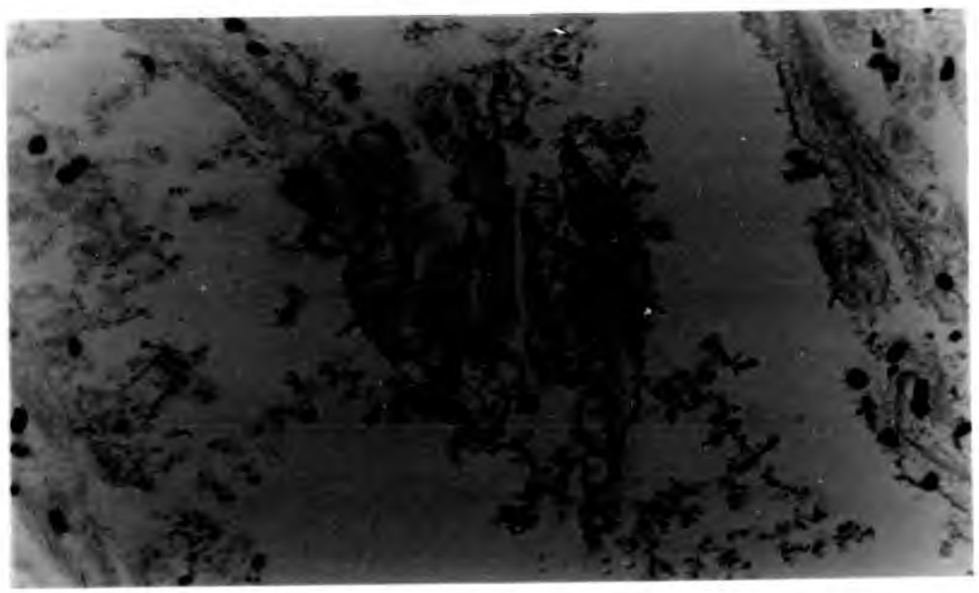


Figure 90. *V. vulnificus* b. I isolate oral challenge, 6h post-inoculation. Positive staining associated with a defect in the midgut epithelium. IP staining. 1430X.

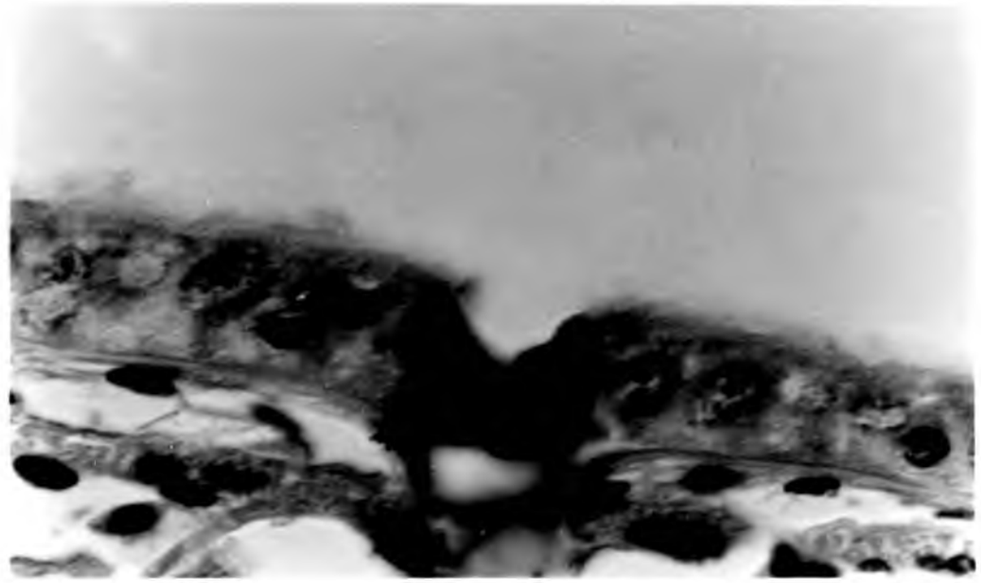
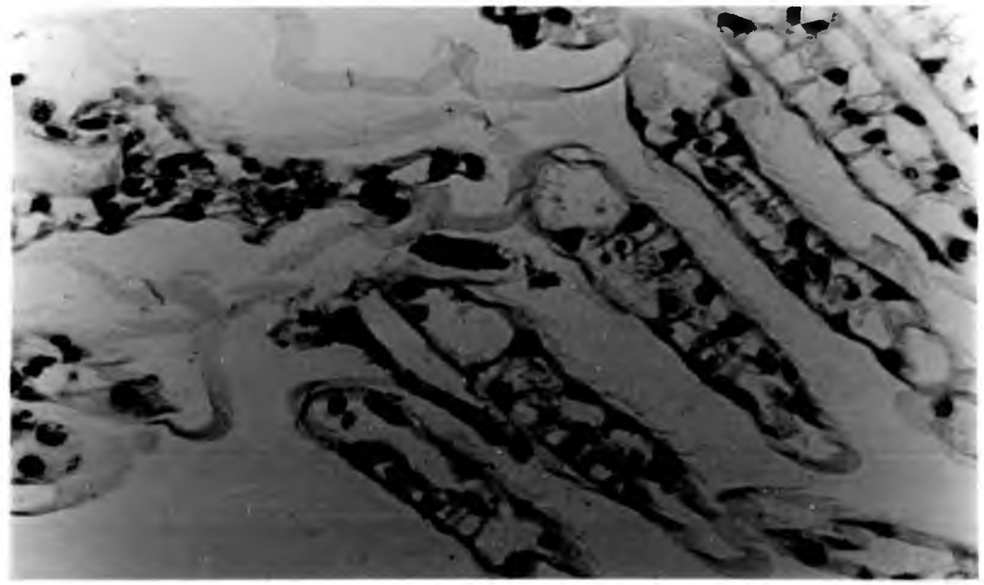


Figure 91. *V. vulnificus* b. I isolate oral challenge, 9h post-inoculation. Positive material present in between the gill lamellae. IP staining. 570X.



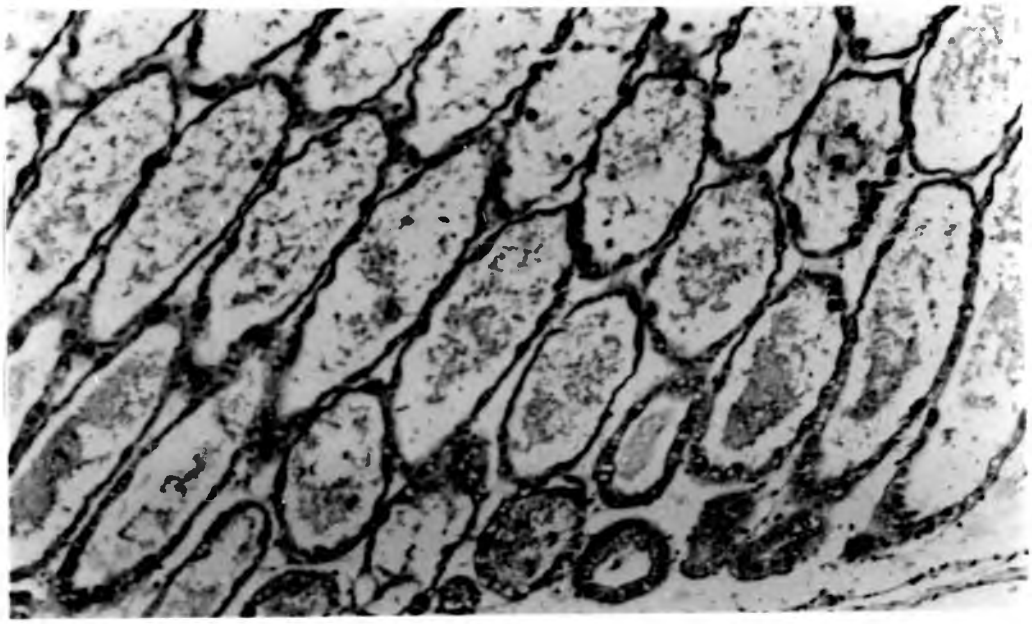


Figure 92. *V. vulnificus* b. I isolate oral challenge, 9h post-inoculation. Positive staining reaction associated with the tubules and cytoplasm within an area of disrupted tubular epithelium. IP staining. 140X.

Figure 93. *V. vulnificus* b. I isolate oral challenge, 24h post-inoculation. Positive staining material contained within the B-cell vacuoles of the hepatopancreatic epithelium. IP staining. 290X.

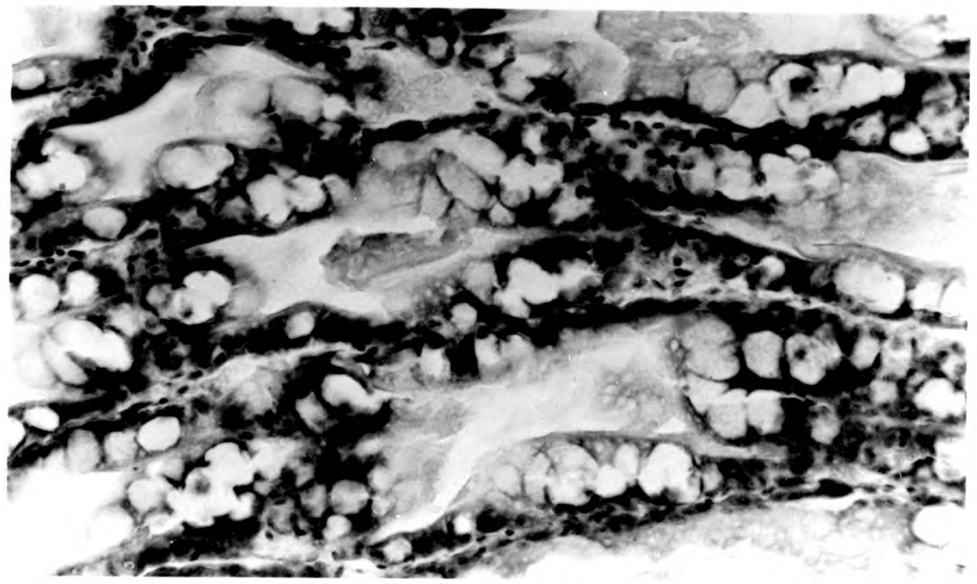
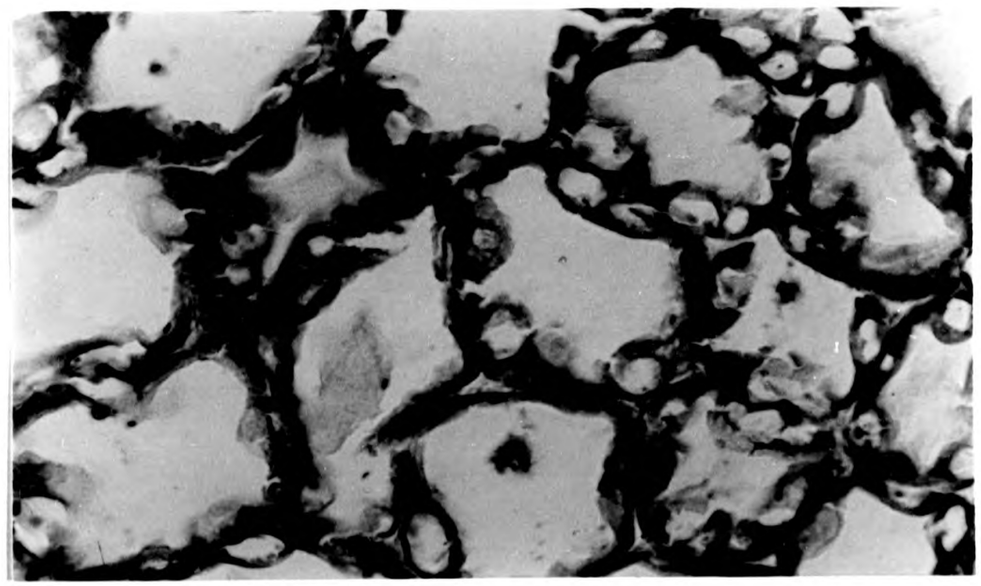


Figure 94. *V. vulnificus* b. I isolate oral challenge, 24h post-inoculation. Positive staining material within the lumen of the hepatopancreatic tubules. IP staining. 290X.



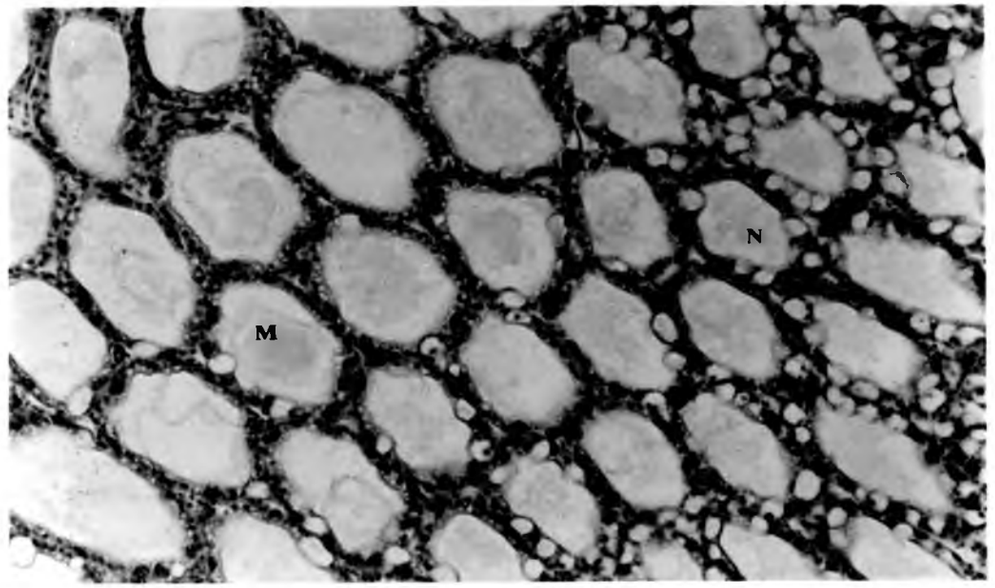


Figure 95. *V. vulnificus* b. I isolate oral challenge, 24h post-inoculation. To the right, there is an area of relatively normal hepatopancreas (N), to the left there is an area of hepatopancreas lacking the characteristic B-cell vacuoles (M). IP staining. 140X.

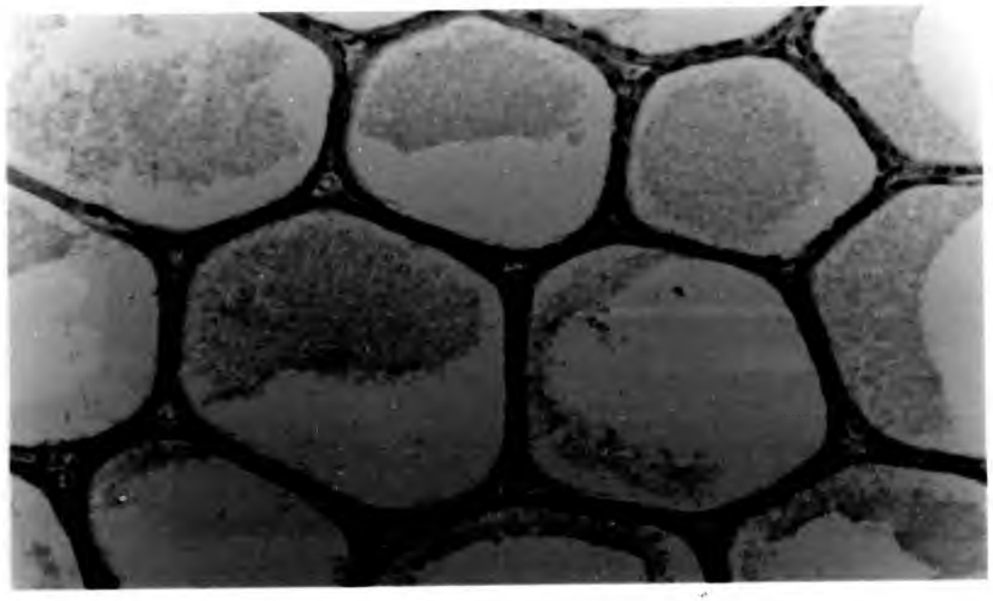


Figure 96. *V. vulnificus* b. I isolate oral challenge, 24h post-inoculation. Metaplasia of the hepatopancreatic tubular epithelium into a layer of simple cuboidal epithelium. IP staining. 290X.

14.3.3.2. Bacterial fixation trial.

The bacterial cells remained intact after being incubated with either Davidson's or formalin fixatives.

14.3.3.3. Bactericidal activity of the hepatopancreatic extract.

No difference was observed in the results of the spread plate count after incubating *V. vulnificus* b. I isolate with hepatopancreatic extract for a range of times (10min-2h) and the negative controls with no hepatopancreatic extract. Same results were obtained for either *P. monodon* or *P. indicus*.

DISCUSSION

The objective of the study was to investigate the aetiology and pathogenesis of *Vibrio* spp infection in shrimp. In order to achieve vibrio infection it was necessary to employ a variety of challenge methods. These methods are not yet reproducible and some aspects may be open to criticism.

Bacterial counts in the water were different within the various compartments of the shrimp facilities. The water held outside, where the temperature did not exceed 25°C, harboured bacteria at a concentration of 10^5 CFU/ml. However, none of the bacterial cells appeared to be *Vibrio* spp or vibrio-like organisms as they were unable to grow in TCBS. The water held inside had a much lower bacterial count (10^2 CFU/ml) nevertheless, *Vibrio* or vibrio-like organisms were present (4 CFU/ml). The water where the shrimps were kept gave the highest count as shrimps and food host bacteria and were probably transferred to the water. *Vibrio* spp and vibrio-like organisms were a small proportion (12 CFU/ml) of the total bacterial count (10^6 CFU/ml).

These levels of bacteria were considered harmless to the shrimp and their presence irrelevant to the infectivity trials performed later. The differences between the work of Lavilla-Pitogo (1990) may be due to a difference in teh pathogenicity of the bacteria. In addition, Lightner (1988) observed that Koch's postulates with *Vibrio* sp isolates from diseased shrimp were fulfilled only if the host was challenged with a relatively massive inoculum.

As already emphasized, *Vibrio* spp are mostly regarded as secondary pathogens, which require the shrimp to be compromised before they can

cause disease. In an attempt to reproduce a vibrio infection as similar as possible to a natural episode, challenging bacteria were administrated as a bath, after subjecting the shrimp to a 48 hour period of stress with ammonia.

In every experiment mortalities were frequently observed after the first 24h of stress probably due to an acute ammonia intoxication. Ammonia has the capacity to diffuse across the cell membrane into the circulation due to its high lipid solubility. In mammals, the primary effect is on the nervous system, producing an impairment of cerebral energy metabolism resulting in depletion of adenosine triphosphate (ATP) and phosphocreatinine in the brain (Breen and Schenker, 1972).

The effect of the ammonia on the bacterial suspension added for the infectivity trial, was not expected to be detrimental as most *Vibrio* spp can grow in a media supplemented with NH_4Cl (Baumann *et al*, 1984).

Some more mortalities were present shortly after the inoculation of the bacteria. Vanderzant *et al* (1970b), Barkate (1972) and De la Pena *et al* (1992) reported mortalities in penaeids after bacterial bath challenge, blaming these initial mortalities on toxic factors rather than on bacterial colonisation. In the present experiment bacteria had been washed prior to the inoculation, thus reducing the amount of extracellular toxic products present in the inoculum. However, more toxins could have been released once the bacteria were inoculated.

The histopathological results from the bath challenge assisted in providing an understanding of the nature, degree, chronological sequence and sites of lesions due to natural injury and infection. However, it is important to realize that histological and microscopic techniques are inadequate for the detection of low bacterial numbers. For example, Johnson *et al* (1981) stated that bacteria can be detected histologically only when their numbers approach 10^6 to 10^7CFU/g of tissue. Nevertheless, application of the immunoperoxidase technique greatly improves in the bacterial detection.

The first signs of positive material were observed in the gill lamellae where bacterial cells accumulated on the surface of the gills. This finding suggests that the gills are a possible route for bacterial entry as their soft and permeable cuticles make them more vulnerable than elsewhere in the body. This might be expected as gill cuticle had previously been altered by the effect of the ammonia making it more susceptible to bacterial invasion. Lavilla-Pitago *et al* (1990) reported after studies with scanning electron microscopy, colonisation of gills in *P. monodon* after being bath challenged with *V. harveyi.* Smith and Ratcliffe (1976) made the same observation after their studies. Thus, it is important to emphasize that the health of the gills may have a significant effect on subsequent generalised infections.

Soon after finding positive staining associated with the gills, lesions were observed in the cuticle. Some of these lesions may have existed prior to the

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Soon after finding positive staining associated with the gills, lesions were observed in the cuticle. Some of these lesions may have existed prior to the

addition of the bacteria, considering the cellular reaction and the melanization associated with them. However, some other lesions appeared to be recent enough to be caused by the challenge bacteria (figure 87). The immunoperoxidase technique has a limitation in the interpretation of the results as the resulting staining is pale brown and it can be confused or camouflaged by the dark brown colour of the melanin. Positive stained areas were distinguished from melanization by examination of the controls, However, when melanin was present, it was impossible to confirm or to rule out the concurring presence of the positive material. Older lesions were likely to be colonised by the challenging bacteria during the bath challenge as is suggested by Davidson (1986) who stated that the bacteria are unable to penetrate unabraded exoskeleton but generally cause disease only under conditions of injury.

Lesions were often found to be superficial when affecting an uninterrupted area of the cuticle, whereas penetration was observed when the affected area included the insertion of cuticular setae. In this latter case, the invasion provoked a large haemocytic response. Areas of cuticular insertion have a thinner cuticle compared to the rest of the exoskeleton as it is the case of the gill cuticle making them more susceptible to bacterial penetration.

These observations suggested that the *V. vulnificus* b. I isolate appears to have a positive chitinoclastic activity and could be a mechanism for bacterial penetration through the shrimp exoskeleton and may also result in "shell

disease". Such percuticular infections entering through intracuticular pores or damaged sites, with invasion of the tissue beneath the cuticle have been described in natural infections of farmed *P. monodon* (Jiravanichpaisal *et al,* 1994).

Shell disease was defined by Happich (1900 cited by Getchel 1989) as the degradation of a crustacean's integument by an external infection, where a variety of organisms may attack the chitin of the exoskeleton. Prior cuticular damage has been associated with many of the reports concerning shell disease. Mechanical injuries caused by difficulties during ecdysis, aggressiveness, handling and high stocking densities have been blamed for predisposing shrimps to shell diseases (Getchel, 1989). Johnson (1983) pointed out that in natural, unstressed environments, chitinoclastic bacteria caused little or no harm, however, they can be seriously debilitating in cultured animals.

The high levels of organic matter commonly found in the ponds may provide favourable conditions for chitinoclastic bacterial growth (Gopalan and Pearce 1975). Many varieties of chitin digestive bacteria have been implicated as a causative agent of shell disease, most often *Vibrio* spp, *Aeromonas* spp and *Pseudomonas* spp have also been isolated from lesions on penaeid shrimp cuticle (Lightner and Lewis 1975).

Sections of normal crustacean cuticle contain the various layers that make up

the exoskeleton, including from outside to inside: epicuticle, exocuticle, calcified endocuticle and non-calcified endocuticle (Dennell, 1960, cited by Getchell, 1989). Microscopic examination of shell disease lesions usually revealed that the calcified layers of the exoskeleton were eroded and in most cases the non-calcified endocuticle remained intact, appearing to form a barrier to the diseased shell (Rosen, 1967, Malloy, 1978 and Roald *et al*, 1981) . Nevertheless, in some instances, inner tissues were found to be necrotic beneath the eroded cuticle. Young and Pearce (1975) and Brock (1983) suggested that perhaps these further lesions were due to factors other than the chitinoclastic bacteria. However, these inner lesions could be caused by damaging products from the penetrating bacteria other than the chitinoclastic enzymes, as it appears to be the case of the challenge organism. This has been shown by Jiravanichpaisal *et al* (1994) who reported that after intramuscular injection of two isolates of *V. harveyi*, the one with chitinase enzyme had a lower virulence than the isolate without it. This demonstrated that independent of chitinase enzyme, bacteria can produce other damaging factors.

The migration of haemocytes to the subcuticular damaged areas led to phagocytosis of the invading bacteria. This finding was supported by Smith and Soderhall (1986) who reported that crustacean haemocytes are capable of recognizing and engulfing a wide variety of biotic and abiotic particles. Such haemocytes containing bacteria (HCB) appeared to migrate through connective tissue and could be found distributed along the body especially in

cephalothorax and periopods.

This finding is supported by the description of crustacean connective tissue given by Mellon (1992), who based it in the functions of vertebrate connective tissue:

> " Connective tissue is pervasive and found in association with almost every organ and can be regarded as an extensive organ that provides not only structural support, lipid storage and mechanical protection, but through which migrate elements of the circulatory and immune system."

HCB and small haemocytic nodules, presumably associated with HCB, but which could also wrap cellular debris or bacteria located in connective tissue, were soon melanized. Nash (1990) also described haemocytic activity associated with foreign material in the connective tissue of the appendages of the fresh water prawn *Machrobrachium rosenbergii*.

Bacteria penetrated deeper into the tissue causing haemocytic nodules in muscle tissue. These nodules were not positively stained as the outside layers of nodules are formed by non-phagocytic haemocytes (Nash, 1990), however in the centre positive material or melanin could be detected. Due to the limitation of the histological technique, it was not always possible to determine whether it was positive material or melanin. Studies of natural

infections in farmed *P. monodon* confirm these observations.

At the same time bacteria had entered the digestive tract through the mouth and some bacterial cells could be distinguished in the gut content at the beginning of the study. As time advanced, bacteria in the stomach content seemed to lose their integrity as individual cells and only soluble material and fine particles positively stained were observed in the hepatopancreatic tubules. Soon after that, this fine positive material was observed in between the tubules and in the haemolymph surrounding the ventral surface of the hepatopancreas mixing with the haemolymph in the ventral sinus. This positive material did not seem to provoke any haemocytic reaction as the cytoplasms of the haemocytes remained free of positive material showing no phagocytosis and there was no haemocytic aggregation present. It appears that the molecules recognised as antigens by the rabbit, source of the antiserum, were not recognised by the shrimp. *Aeromonas salmonicida* and *Renibacterium salmoninarum* infections are commonly found in salmonid fish. These organisms have evolved in such a way that they are not recognised by the immune system of the fish. *Vibrio* spp organisms could have evolved to avoid the defense mechanisms of the shrimps. It is possible that the bacterial material which entered the shrimp did not provoked a haemocytic reaction because of the small particles size rather than of its antigenic nature. However, it was still recognised as foreign material as the shrimps attempted to eliminate it.

Once the positive material had moved from the hepatopancreatic tubules, some animals were observed to have retained some of this material within the R-cell vacuoles. The R-cells function is to absorb nutrients and store and metabolize lipid and carbohydrate materials.

Classically bacteria have been considered to be decomposers, breaking down and mineralizing organic matter. However, there is increasing evidence that bacteria may play an important role not only in the regeneration and consumption of dissolved nutrients in the water column but also as a food source for direct utilization by herbivorous zooplankton (Rieper, 1981, Williams, 1981). It has also been postulated that bacteria may be used as a diet supplement for zooplankton and /or artificial diets fed to higher trophic levels (Maeda, 1988). Intriago and Jones (1993) grew *Artemia* spp to pre-adult stage on diets consisting of bacteria, bacteria plus algae and algae alone, suggesting that bacteria acted not only as food for the *Artemia* spp but also assisted in the digestion of the algae. Once the bacteria are broken down, they are reduced to protein, lipids and polysaccharides. Thus, the possibility of shrimp absorbing bacterial material as a source of nutrients can not be ruled out.

After two days, there was a consistent finding in the midgut intestine, the only area not covered with cuticle. The intestine in this area suffered an irregular modification of the epithelium with reduction of cytoplasm and apical vacuolization in some cells. This was probably due to bacterial toxins as no

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positive staining was associated with it and control animals did not show this modification. There was a case of a small positive stained lesion in the midgut epithelium that suggested damaged intestine may also be a route for bacterial penetration.

After three or four days, large cells full of a fine and uniform material of eosinophilic to brown colour appeared in the gill lamellae which coincided with the description of branchial podocytes given by Nash (1990). There was no haemocytic reaction surrounding these cells. Nash (1990) identified branchial podocyte activity after injection of *Vibrio* spp and *Aeromonas* spp. Smith and Ratcliffe (1980b) determined that the branchial podocytes removed haemocyte-derived cellular debris. Drach (cited by Johnson 1980) concluded that the foreign material, once taken up, is retained in the podocytes during an entire intermolt cycle before it is released.

At the same time cells which consistently matched the description of branchial podocytes, started to appear in the connective tissue immediately underneath the cuticle. These cells increased in number as the time advanced. They probably represent a mechanism to release foreign material at the time of moulting. These were named subcuticular podocytes, based on their appearance and possible function. However, further study should be carried out on the morphology of these cells to confirm the presence of cell extensions characteristic of the podocytes.

Eight days after the challenge, accumulations of positive material were found in the B-cell vacuoles of the hepatopancreatic epithelium. The positive material was concentrated in the luminal side of the B-cell vacuoles. The consistency of this finding raised the question of whether the positive material was being absorbed from the tubular lumen or excreted into it. Bacteria which penetrated into the shrimp, might have been destroyed in the haemolymph, moved to the hepatopancreas and be excreted through the B-cells into the gut. Alternatively, bacteria might get into the shrimp through the hepatopancreas and be destroyed in the B-cells vacuoles.

The function of the B-cells is not clearly understood. Gibson (1981) reported that the enzymatic content of the B-cell vacuoles was available for secretion and extracelluar action. However, Al-Mohanna *et al* (1985) and Al-Mohanna and Nott (1986) disagreed with the previous opinion, concluding that B-cells are concerned only with intracellular digestion and assimilation and that secretion of enzymes is confined to the F-cells. There is a second role attributed to the B-cells. Hopkin and Nott (1980) proposed that the B-cell vacuoles were a means of packing waste products of digestion for removal from the hepatopancreas. Gibson (1981) agreed, concluding that once extracelluar digestion had been completed, the B-cells by some physiological switch as yet not demonstrated, altered their role to that of waste elimination. Thus, the presence of positive material in the B-cells is likely to be a step prior to its release into the tubular lumen.

Icely and Nott (1992) have attributed to the hepatopancreas an immunological role in the removal of foreign bodies from the blood system, excretion of waste metabolites from the blood system, and detoxification of metals and foreign organic substances. Examples include the deactivation of the insecticide dimethoate in the F-cells of *P. monodon* (Voght 1987) and the removal of metals injected into the haemocoel by different routes in both R and F-cells of crayfish (Lyon and Simkiss, 1984, Roldan and Shivers, 1987). Nevertheless, B-cells have not been reported, until now, to be involved in these clearing mechanisms.

This accumulation of positive staining within the hepatopancreas was later discharged as a step towards the death of the tubular epithelial cells (bolitas) and/or septic hepatopancreas. This was supported by the observation of discharged positive material in healthy animals with no hepatopancreatic damage.

Antennal gland excretory pores were not observed as a route of entry for the bacteria.

In a few animals positive staining was associated with the nuclei of the hepatopancreatic tubular epithelium. To date there is no explanation for the presence of this material. Hepatopancreatic epithelial cells evolve from a single cell line, the E-(embryonic) cells. These cells, located at the end of the tubules, undergo intense mitosis and differentiate into B-(blister), R-

(resorptive) and F-(fibrilar) cells. It might be possible that while the nuclei undergo such an activity require or allow the penetration of external material into the nuclei. In this occasion, positive material may have been to be present and would have been carried into the nuclei.

There was a trend for the stressed animals to have more lesions and accumulate more material in the B-cells than unstressed animals. However, this observation could not be statistically validated as individuals varied greatly. The individual variations were probably due to the stress already being suffered by the shrimps subjected to captivity in laboratory conditions.

Two new challenges were designed for a better understanding of the observations made during the bath challenge, an injection and an oral challenge. The injection challenge was expected to provide information regarding the shrimps defense mechanisms once the bacteria entered the tissues and examine whether the positive material present in the B-cell vacuoles originated via the haemolymph or not.

Initial shrimp mortalities after the bacterial injection were attributed to toxic shock rather than to the bacterial colonization of the shrimp tissues or the stress of injection regime as the control animals injected with sterile saline were not affected. Adams (1991) observed high mortalities 2h after injection of shrimp with heat-killed bacteria. Leong and Fontaine (1979) reported 100% mortality within 4h of injecting the shrimp with a bacteria-free filtrate of

a *V. parahaemolyticus* culture. Mortalities reported in these two papers were more severe than those observed in this experiment. This difference may be due to the fact that the *V. vulnificus* b. I isolate injected had been previously washed twice with sterile saline, whereas in Adam's experiment, no bacterial washing was reported thus possibly maintaining larger amounts of toxins and Leong and Fontaine injected only the culture broth where the toxins accumulated during bacterial growth.

Most of the shrimp that remained alive after the challenge did not show signs of the disease after 24h. This may indicate that the bacteria were being eliminated and not colonising the shrimp tissues or producing lethal doses of toxins.

The sequential histopathological events following the injection of bacteria were described by Fontaine and Lightner (1973) as follows:

> "1. Haemocytic infiltration. 2. Encapsulation of foreign body or necrotic material by fusiform haemocytes and formation of melanin. 3. Deposition of foreign or necrotic material by haemocytes."

In this experiment, the response of the shrimp to bacterial cells and injury was found to be both rapid in onset and enduring. Only a few bacterial cells or colonies were observed immediately after the injection due to the very

efficient clearance mechanism of haemocytes which had already migrated to the inoculation site. Adams (1991) reported that more than 99% of heat-killed bacteria were cleared from the haemolymph within 4h in *P. monodon*. Some of the bacteria were broken down *in situ* as positive material was detected in the haemolymph. This observation differs from Ratcliffe *et al* (1982) who suggested that bacteria were not broken down in the haemolymph of the crab *C. maenas* as few bacterial breakdown products were detected. The different results may be due to the different methods utilised.

The presence of positive material was not only localized at or in the vicinity of the injection but also in certain target organs, especially gills, hepatopancreas, heart and lymphoid organ. Gills, heart and hepatopancreas had already been reported as target organs in the crab *C. maenas* (Smith and Ratcliffe, 1976 and 1980a; White and Ratcliffe, 1982).

Simultaneously, haemocytes started to phagocytose bacteria. HCB were first observed in the haemolymph both in the sinuses and free in the body or in association with tissue bathed by the haemolymph such as the antennal gland. However, HCB were not present in the vessels containing haemolymph until later. This was probably due to the lack of a venous system in the shrimps. The haemolymph is not transported through vessels until it passes through the heart and into the arteries. Once the haemolymph was uniformly stained with peroxidase due to the presence of bacterial material, the muscle tissue of periopods, pleopods and mouth area were

observed to have a stronger staining reaction than the rest of the tissues. It was thought to be due to a higher haemolymph circulation, because of the higher activity of those muscles.

Most of the animals showed lymphoid organ pathology (LOP). This condition is characterised by the development of hyperplastic and degenerative spherical groups of cells or spheroids and their metastasis to other parts of the body. There is still very little information available regarding the aetiology, pathogenesis or significance of this condition. The cytopathology within the spheroids including vacuolation, necrosis and inclusions, suggest that LOP is a pathological condition. Turnbull *et al* (1993) reported no evidence of viral involvement and could not determine whether LOP is a specific or non-specific reaction.

Some of the spheroids in the lymphoid organ showed small amounts of positive material. This staining might indicate that there still remained a little of its haemolymph filtering activity. However, the absence of HCB or haemocytes in the spheroids suggests that the filtering function is seriously impaired. If this was the case, the positive material might come originally from the haemolymph or the normal lymphoid tissue, both surrounding the spheroids.

No positive staining was observed in association with the ectopic lymphoid spheroids, emphasising their lack of activity as haemolymph filters.

Many of the migrating haemocytes showed pyknotic nuclei, an observation that is perhaps explained by Bauchan (1981) who described degranulated haemocytes as having degenerating nuclei, nuclear karyorrhexis or pyknotic nuclei. Released products from degranulation of the haemocytes promote the inflammatory mechanism, both cellular and humoral. The activation is rapid and the active proPo proteins released stimulate further degranulation of proPo components from the adjacent granular haemocytes.

Bacteria and the HCB appeared to migrate from the site of injection into the connective tissue or through the haemolymph into other tissues. There was a large concentration of HCB in the tunica propria surrounding the posterior midgut caecum, midgut intestine and hepatopancreas. This concentration of haemocytes was similar to a haemocytic enteritis which was described by Brock (1983) as a haemocytic infiltration of the midgut mucosa, dorsal caecum and hindgut gland. Haemocytic enteritis described in natural conditions has been associated with toxins in the water, mainly algae toxins.

The HCB located in the hepatopancreas tunica propria appeared to penetrate between tubular spaces. Icely and Nott (1992) in their description of the hepatopancreas refers to the tunica propria of the hepatopancreas as discontinuous in EM studies therefore it would not prevent the migration of the HCB into the organ.

At the same time haemocytic aggregation started to appear together with the HCB and were found in large quantity in gills, heart and lymphoid organ. The aggregation of haemocytes around injected material is in itself considered to indicate the participation of humoral factors (Fontaine and Lightner, 1974). A cell adhesive protein has been reported to be released along with opsonins on degranulation of the semi-granular and granular haemocytes (Soderhall, 1986). This results in the haemocytes becoming sticky (Smith and Ratcliffe, 1980b) leading to the formation of aggregations.

Encapsulation and nodule formation closely followed the aggregation during the process where the rather disorganized arrangement of haemocytes becomes more organized (Nash 1990). HCB and necrotic cellular debris were encapsulated.

Lewis (1973) reported that after challenging *P. aztecus* with *V. anguillarum* beneath the dorsal carapace, the phagocytic activity in the haemolymph was most apparent during the initial phase after the injection. As the infection progressed beyond 8h, phagocytic activity was rarely demonstrable. It has been proved that with the attachment of haemocytes to capsules or nodules, there is a corresponding decline in the number of circulating cells in the haemolymph (Smith and Ratcliffe, 1976). This is only temporarily detrimental to the host as mobilization or haematopoiesis of new haemocytes returns the circulating cell count to normal within 48h (Smith and Soderhall 1986). Challenged animals had an increased compensatory haematopoiesis

compared with the controls in the haematopoietic nodules which was observed as early as 6h after injection *Vibrio* spp in *M. rosembergii* (Nash 1990).

It appeared that the HCB released the phagocytosed material or were broken down in the lymphoid organ and heart as large amounts of positive material accumulated in these organs. White and Ratcliffe (1982) support this observation after studies of the defense mechanisms of *C. maenas*. Faye (1978) and Abu-Hakima and Faye (1981 cited by Adams 1991) found a similar mechanism in insects suggesting that phagocytes containing bacteria moved to the fat body where they disintegrated as appears to occur in the heart and lymphoid organ of *P. monodon*. Adams (1991) proposed that this is a phenomenon whereby bacteria are quickly cleared to tissues where bactericidins and lectins have been stimulated to deal with their disposal.

Positive material appeared in very close association with the muscle fibres of the heart. Similarly, Johnson (1987) described phagocytic fixed cells in the heart of penaeid shrimps. He referred to their less remarkable phagocytic activity, as the fixed phagocytes must wait until the circulation brings foreign particles into their vicinity, whilst the greater number of extremely mobile haemocytes can respond immediately wherever foreign material is introduced into the haemocoel. The challenged animals did not show any organised retention of positive material in the heart, fixed phagocytes were not observed even long after the injection challenge or the bath challenge .

Subsequent melanization of the encapsulating nodules was initiated. Melanisation is one of the mechanisms involved in the proPO system which had been activated by the presence of the bacteria

Haemocytic response, including aggregation, persisted up to 4 days post-challenge and probably would have been observed to persist even longer had the experiment been extended for a longer period.

Fibroplasia or fibroblast proliferation with the elaboration of collagen was observed to play a significant role in the chronic inflammatory response, commencing as early as six hours. The characteristics of the dense fibrous tissue as being well organized, stable and not resorbed reported by Fontaine and Dyjak (1973) were also noted in the muscle injection site. It is also claimed to remain as a "permanent scar" (Fontaine and Dyjak, 1973 ; Fontaine and Lightner, 1975).

Shrimps injected with the high dose of the bacteria were found to have a positive stained necrosis of the connective tissue surrounding posterior midgut caecum, midgut intestine and the stroma of the hepatopancreas. The epithelium of these structures and other organs were not stained. This observation suggests that the bacteria injected overwhelmed the shrimp defense mechanism and would have been lethal had the animal not been sacrificed for examination and that the connective tissue is the first and main vehicle for HCB and probably bacteria as well. Bacteria together with HCB

that may have died during their migration and released the bacteria contained in them into the connective tissue colonising it. Phagocytosis alone does not insure death of the infectous agent, as seen in gafkemia, a bacterrial disease where the bacterium (*Aerococus viridans)* rapidly divides within the haemocytes and is a fatal condition for lobster (Cornick and Stewart, 1968). However, the positive staining was so intense and generalised, that the presence of intact organisms could not be confirmed. Owens (1993, personal communication) observed that the connective tissue was the most susceptible tissue to bacterial invasion after experimental injection of penaeid shrimp. However, connective tissue appears to be more susceptible not because of its nature but because if its immunological role in the migration of elements of the circulatory and defense mechanism system.

Description of natural infections of farmed shrimp agree with the findings described previously. Anderson *et al* (1987) observed small colonies most commonly located within the heart, gill filaments and at the periphery, or in the distal intertubular tissues of the hepatopancreas. The presence of these colonies in the heart, gill filaments and around and in the distal intertubular tissue of the hepatopancreas coincide with the location described for the HCB.

Jiravanichpaisal *et al* (1994, personal communication) described natural percuticular infections of farmed *P. monodon* where bacteria invaded epidermis, connective tissue and musculature, leading to a systemic

dissemination of bacteria. These animals showed extensive bacterial invasion and invasion of phagocytic haemocytes in the heart and lymphoid organ. Moreover bacteria migrated to the systemic haemolymph sinuses and invaded the connective tissue in the hepatopancreas, stomach, midgut and musculature where haemocytes aggregated. Jiravanichpaisal *et al* report does not agree with the time sequence observed after both the bath and the injection challenge. In these challenges haemocytic reaction was first observed in haemolymph sinuses, connective tissue of the hepatopancreas and midgut and later on in the lymphoid organ and heart.

Control animals showed a lower number of haemocytes in the injection site. No encapsulation or melanization was observed in the shrimp injected with sterile saline.

The injection challenge carried out with the *V. anguillarum* isolate resulted in very high mortality. This may be due to the number of cells injected which could be larger than estimated probably as a result of the use of *V. vulnificus* b. I isolate growth curve or it could also be possible that the *V. anguillarum* isolate had a more pathogenic effect. Only one sample of two animals 4h after the injection was possible.

For the purpose of this study the antiserum proved to be sufficiently specific for immunohistochemistry and ELISA.

The pathology observed in the only two animals sampled, was very similar to the pathology described after injection with *V.vulnificus* b. I isolate. Two main observations were revealed. Most haemocytes involved in the defense mechanisms, including haemocytes present around the site of injection, in aggregations, present in heart , lymphoid organ or surrounding posterior midgut caecum, midgut intestine or hepatopancreas had pyknotic nuclei. This was a common finding with *V.vulnificus* b. I isolate injection but not to such a large extent possibly due to a milder reaction of the shrimp to the inoculum used.

The other main observation was the loss of midgut intestine and hepatopancreatic epithelium cells. These cells have been referred to as "bolitas" due to their shape. Such cells are present in some natural episodes associated with toxins of chitinolitic bacteria present in the mouth and oesophagus. The loss of epithelial cells in the experiment appears to be related to the injected and not as a result of ingested toxins.

The injection of sonicated bacteria was expected to produce information regarding the metabolism of bacterial material whilst avoiding the pathology and mortalities associated with a bacterial cell injection. However, one of the shrimp was found dead, probably due to the traumatic effect of the injection itself.

As was observed in the injection of intact cells of *V. vulnificus* b. I isolate, the muscle of periopods, pleopods and mouth area were more heavily stained.

Soon after the injection, haemolymph transported the positive material to the gills in large amounts. The muscle of the injection site was soon free of positive material leaving a small haemocytic reaction, probably for the repair of the mechanical damage caused by the injection.

As was observed when bacterial cells were injected, the positive material appeared to concentrate in heart and lymphoid organ as if these two organs filtered the haemolymph retaining the positive material. Spheroids in lymphoid organ showed some traces of positive material.

In spite of the sonication procedure a few haemocytic nodules and a few HCB were found in two of the shrimps. This suggests that some bacterial cells had resisted the sonication procedure and had been injected into the shrimp provoking the haemocytic reaction.

In neither of the two injection challenges, intact nor the sonicated bacterial cells, was positive material found in the B-cell vacuoles. This may have been due to the shorter duration of the experiments when compared with bath and oral challenge. Therefore it is not possible, from the available evidence to comment on the route by which the bacterial material enters the B-cell vacuoles.

Finally, a third challenge was designed, an oral challenge. The purpose of this challenge was a) to check the possibility of bacterial entry into the hepatopancreas through the oral route. b) To investigate whether the presence of the positive material in the B-cell vacuoles might have been absorbed through the tubules. And finally c) to attempt to reproduce a septic hepatopancreas which is a very common presentation of vibrio infection, but was not observed in the shrimp from the bath challenge.

The addition of ammonia to the water to induce stress in the shrimps caused a small number of mortalities as expected similar to the bath challenge. No animals died after the inoculation of the bacteria in contrast to the report by Leong and Fontaine (1979) who challenged juvenile brown shrimp (*P. aztecus*) by feeding them with shrimp meat inoculated with *V.parahaemolyticus* and incubated for 24 or 48h. Shrimps were fed with infected meat for 4 days. The experiment revealed a 16% mortality within the first 96h which did not resume until repeated doses of bacteria were fed. It was suggested that the death of the shrimps probably resulted from the action of toxins associated with the bacteria and retained in the shrimp meat, rather than the multiplication effect of the bacteria. This may explain the lack of mortality in these experiments.

In the first sampling, 30min after inoculation, oesophagus and stomach were full of intensely stained material. However, this staining was not associated with entire bacterial cells, but to an uneven material. The same sample

stained with H&E showed a small amount of basophilic material that appeared to be part of the bacterial inoculum. These bacterial cells appeared modified having lost their typical appearance. The hepatopancreatic enzymes were thought to be related to this effect on the bacteria as is discussed in page 201.

Lavilla-Pitogo *et al* (1990) showed that the colonization by the bacteria occurred specifically on the feeding apparatus and oral cavity of *P. monodon* larvae bath challenged with *V. harveyi*, suggesting the oral route for the initiation of the infection. Such findings were not recorded in these experiments.

A set of setae in the foregut was observed to filter the stomach content. Homogenous material, less intensely stained passed through, while coarser material was retained and released through the intestine.

The foregut is a complex structure specialized in grinding the food and mixing it with enzymes (Powell 1974). Food in the stomach is reduced to a semi-fluid state through the concerted mechanical and chemical effects of the gastric mill, digestive enzymes and emulsifying agents. The stomach contents are then sorted by the setae, fluid and microparticulated material entering the hepatopancreas for further stages of digestion and residual substances passing directly to the midgut (Gibson 1981)

The filtration process is carried out by the cardiac setal screen and the gland filter, both present in all decapods.

The cardiac setal screen prevents access of solid material into the ventral lumen of the cardiac chamber. The mesh size is estimated to be smaller than 1μm (Powell 1974, Kunze and Anderson, 1979; Ngoc-Ho, 1984).

The gland filter occupies the ventral half of the pyloric chamber of the stomach and opens directly into the antechamber of the hepatopancreas. The gland filter consists of the ampullary setal screen and the filter press. The ampullary setal screen mesh size can be as small as 0.05μm (Ngoc-Ho, 1984) and the mesh size is also reduced by the overlap between the rows of setae. The filter press restricts the lumen of the upper ampullary chamber. Kunze and Anderson (1979) suggested that the gland filter acts as a means of final abrasion and reduction of particulate material, and not as a sieving mechanism. It appears that the gland filter ensures vigorous mixing of the filtrate of the cardiac chamber with digestive enzymes from the hepatopancreas.

Hopkin and Nott (1980) concluded for the crab *C. maenas*, that only particles with a diameter of less than 0.1μm can pass through the filtering system of setae at the opening of the primary ducts of the hepatopancreas. The coarse fraction containing all particles greater than 0.1μm in diameter was passed directly into the midgut.

From this information and considering the size of *Vibrio* spp bacterial cells, 0.5-1.5 by 1.5-4μm (Austin and Austin, 1988), it was believed that the chemical action of the enzymes together with the mechanical action of the sieves prevented ingested bacteria getting into the hepatopancreas. Song *et al* (1993) challenged *P. monodon* with *V. damsela* using anal intubation. Hepatopancreatitis and shrimp death was achieved when using 10^5CFU/ml of the mentioned organism. Bacteria might gain access to the hepatopancreas because they were introduced with pressure crosscurrent against the anatomical arrangement of the filtration systems.

Despite this mechanism, a few bacterial cells were found in two tubules of the hepatopancreas of one of the shrimps, suggesting that the mechanism described previously failed at some point. Later sampling showed another animal which developed an infection in one of the tubules. Bacterial cells which managed to penetrate inside the hepatopancreas might be responsible for the infection of hepatopancreatic tubules, or there may have been pre-existing infections

The positive material present in the stomach filtered into the hepatopancreas filling the tubules and diffusing through the tubular wall. Immediately after that, the positive material was observed in the haemolymph between the tubules and surrounding the ventral surface of the hepatopancreas. Later on it was present in a diluted form in the ventral sinus.

As mentioned before there was no visible haemocytic reaction to this soluble positive material.

Again the R-cell vacuoles were observed to contain positive material. This supports the suggestion made after studying the bath challenge that the cells absorbed the positive material as if it were nutrients. Again a few animals showed positive staining of the hepatopancreatic tubular epithelium nuclei. The observation of this phenomenon in two separate challenges where bacterial material entered the hepatopancreas, reduces the possibility that it was a processing artefact.

Two animals were observed to have their hepatopancreatic epithelium affected with loss of B and R-vacuoles, after the positive material entered the hepatopancreas and passed through the epithelium. This was probably due to some toxic effect of the bacterial material passing through the hepatopancreatic tubular walls. However, the reduced number of animals available for the experiment did not allow the confirmation of the consistency of this observation.

Positive material in the haemolymph was carried to the gill lamellae and was also seen to bath the antennal gland. Later on, this positive material seemed to accumulate in lymphoid organ and in close association with the muscular fibres of the heart as happened in the bacterial cell injection challenge and sonicated bacteria injection challenge.

Twenty four hours after inoculation, positive material was found in the B-cell vacuoles supporting the hypothesis derived from the bath challenge that foreign material present in the haemolymph appears to be excreted through the hepatopancreas and also through gills. Animals which showed positive material in the tubular lumen had a reduced number of B-cell vacuoles in the tubular epithelium. The remaining B-cells did not contain positive material inside their vacuoles, suggesting that foreign material is released to the tubular lumen within the vacuoles.

Stress did not affect the enzymatic or mechanical barrier against the introduction of bacteria in the oral challenge, but was associated with a slower clearing of the positive material present inside the shrimp.

The small number of bacterial cells present in the stomach soon after inoculation was surprising and it was thought to be an artefact of the fixation procedure. However, the results from the bacterial fixation trial proved that Davidson's fixative did not disrupt the bacterial cell wall. This conclusion was expected as the bacterial wall is more resistant because of its rigidity than higher animal cell walls.

Once the absence of bacterial cells was ruled out as an artefact, the enzymes of the hepatopancreas were thought most likely to be involved in the break down of the bacterial cells. However, after culturing the bacteria with hepatopancreatic extracts, no effect on bacterial survival was detected. Mori

and Stewart (1978) described bactericidal activity in the hepatopancreas, to be greater than in the haemolymph in the lobster (*Homarus americanus*) two days after the administration of a *Pseudomonas peroleus* vaccine. This finding was probably caused by the decrease of haemocyte numbers in the haemolymph and their accumulation in the hepatopancreas. In the present study the mechanical action of the cardiac setal screen and the gland filter appeared to be the main cause for the rupture of the bacterial cells and the chemical action of the hepatopancreatic enzymes were responsible for breaking down or degrading the components of the bacteria.

Comparing the results from the three challenge methods it was concluded that bacteria were cleared through converging routes. Bath challenge proved to be a combination of the oral and the injection challenge. Between the three of them a bacterial clearing mechanism is proposed for *P. monodon* and in general for decapod crustaceans.

Bacterial cells can penetrate through damaged gill cuticle, body cuticle or through the insertion of cuticular setae. Haemocytes respond to this invasion phagocytosing the bacterial cells or breaking them down *in situ*. The HCB and free bacteria migrate through connective tissue and are transported with the haemolymph, accumulating around hepatopancreas, midgut, caecum, gills and antennal gland. Once HCB reach the heart, they are distributed to lymphoid organ.

Bacteria entering through the mouth are broken down in the stomach and filtered through gastric sieves. Only soluble bacterial material and fine particles pass into the hepatopancreas. The coarse component of this material is discharged through the gut. The soluble material that enters the hepatopancreas diffuses through the hepatopancreatic tubules into the haemolymph and spreads through the haemolymph sinuses into the gills and the antennal gland. This material reaches the heart and distributes through the vessels to lymphoid organ, gills and antennal gland.

Heart and lymphoid organ appeared to retain and accumulate the bacterial material.

Finally, bacterial material is released to the outside in two steps. An immediate one, through the gills and a later step through the hepatopancreatic B-cell vacuoles and branchial and subcuticular podocytes.

The defense mechanism response has proved to be extremely efficient in quickly immobilizing bacteria and preventing the spread of infection (Smith and Ratcliffe, 1980a) and also facilitates the degradation and ultimate disposal of the material from the body (Smith and Ratcliffe 1980b). However, the defense mechanism may be overcome when the gastric sieving is damaged or the percuticular penetration surpasses the shrimp's defense mechanism. This may result in an active infection.

Material from bacterial walls gains access to the shrimp as part of whole bacterial cells or once broken down through the hepatopancreas. Gram negative bacterial wall is known to trigger the proPO system, consequently stimulating the defense mechanism of the shrimp. Presence of bacteria in the environment may have an enhancing effect on the defense mechanisms of the shrimp. Therefore, it is possible that larvae reared in "sterilized" environments may be less able to cope with the microbial ecosystem present in the rearing ponds.

Septic hepatopancreas was not reproduced in any of all these experiments, coinciding with Lightner's opinion, who suggested that the initial cause of septic hepatopancreas might not be infectious (1992 personal communication). Jiravanichpaisal *et al* (1994) reported septic hepatopancreas associated with *V. harveyi* in farmed *P. monodon* without the presence of any other pathogens such as baculovirus and parasites and concluded it was an oral infection.

There may be three variable parameters that might be related to the occurrence of a septic hepatopancreas between farm and laboratory conditions: the water quality of the environment, the bacterial agents and the food or feeding procedure. In the first case is the environmental conditions which may be the cause of stress. The pond water quality may be worse than the conditions used in the experiment in spite of the addition of ammonia. However, the level of stress is believed to be higher in the animals

kept under laboratory conditions, even before the addition of ammonia.

Another factor to consider is the different bacterial species involved in the condition. However, this is unlikely as different *Vibrio* spp have been cited in association with septic hepatopancreas. Jiravanichpaisal *et al* (1994) reported that juveniles and adult shrimps had hepatopancreatic invasions irrespective of the bacterial species involved.

The last factor which may influence the acquisition of septic hepatopancreas is the shrimp feeding. The animals used for the experiment were fed on fresh mussel once a day. Farmed shrimp are fed on artificial pelleted food whose quality is judged on survival of the shrimp and growth rate. This food might not be adequate unless it is used in an extensive culture which allows the shrimp access to natural foods to balance otherwise imperfect compounded feed. The B-cells have been found to reflect differences in diet. Al-Mohanna and Nott (1987) reported that in comparison between shrimp fed prawn flesh and others fed on artificial formulation, the B-cell vacuoles of shrimp fed on shrimp flesh were translucent and practically devoid of residues, with the artificial formulated food, the vacuoles contained residues which they hypothesised were undigested food. Thus, the prawn flesh appeared to be utilised more efficiently.

Histological examination of farmed *P. monodon* shows a rougher content in the digestive tract than the animals fed with mussel. This could suggest that

this rough residual material might damage the setae system of the foregut allowing the entrance of bacteria.

In addition, shrimps are fed during the day although as adults *P. monodon* tend to feed at night (Moller and Jones, 1975). Feeding easily degradable pellets at a time of day when the shrimp are not particularly active or hungry may lead to waste of feed and subsequent pollution.

Apparently healthy looking shrimps have been reported to harbour bacteria in the haemolymph, including *Aeromonas* spp, *Pseudomonas* spp and especially *Vibrio* spp the most common isolates (Lightner, 1977, Ruangpan *et al,* 1994, personal communication), similar finding are described in lobsters (Stewart *et al,* 1966) and crabs (Welsh and Sizemore, 1985). The sterility of the blood is considered to be the normal condition in vertebrates, however, this findings suggests that a low-level bacteraemia may be more prevalent phenomenon among marine invertebrates.

Brady and Lasso de la Vega (1992) reported low levels of bacteria in the haemolymph of *M. rosenbergii* but no bacteria were found in the haemolymph of the animals without lesions. Similar finding was described by Welsh and Sizemore (1985) in crabs.

It appears that bacteria present in the environment are constantly threatening the shrimp gaining access into it through small lesions. Sterility of the

haemolymph is a condition the animal strives to achieve on a continuous basis throughout most of its life. Shrimps try to control these organisms with the defense mechanisms already described. However, physiological stress may facilitate an increase in bacterial numbers by reducing the effectiveness of the host defense mechanisms rendering way to an infection.

Despite the evidence for the oral route of infections, it is still possible that the septic hepatopancreatic necrosis may result from the haematogenous spread of bacteria. The shrimp hepatopancreas is a target organ of most of the viral infections and is also affected by the toxins carried in the water. When bacteria present in haemolyph overcome the defense mechanisms, they may find damaged tissue the ideal location where they can proliferate, developing a septic hepatopancreas.

CHAPTER V

CONCLUSIONS

CONCLUSIONS

The intensification of culture methods enlarges the risk of *Vibrio* spp infection by increasing the chances of cuticular injuries due to aggression and cannibalism, of horizontal transmission of infectious diseases and of poor water quality. These conditions encourage the penetration of bacteria into the body and the onset of the disease due to the stressful rearing condition. The control of outbreaks should involve all aspects of management.

In the present study, bacterial material has been observed to penetrate into the shrimps through the digestive system. Bacterial cell components such as peptidoglycans are reported to trigger the proPo system. Bacteria naturally present in the water could possibly have an enhancing effect in the nonspecific defense mechanism of the shrimp. Further research into the potential use of bacteria, the appropriate range of bacterial species and the concentration which would have a beneficial effect without overwhelming the shrimp defense mechanism, would be a relevant area of study which results could contribute to new management measures of disease control.

A new type of cells, not reported before, have been detected after histological examination of challenged shrimps. They were named subcuticular podocytes. Morphological studies using electron microscope may produce new information on the description of these cells.

The possibility of damage of the digestive filtering system caused by artificial foods which would lead to hepatopancreatic infections has been discussed. Further research on the effect of feeding pellet food feeding on the anatomical structure of the filters system could produce useful information for the shrimp industry.

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APPENDIX I

GIEMSA STAINING METHOD

APPENDIX I

GRAM'S STAIN

The Gram's stain is a differential reaction that divides all bacteria in two categories based on the different chemical composition of the cell wall and membrane.

Method:

1. Place a drop of sterile saline onto a clean glass slide with the help of a sterile loop.

2. Remove a minute quantity of culture using a sterile loop. Emulsify the culture in the liquid on the slide and spread evenly.

3. Allow the slide to dry naturally and pass the slide, with the film upwards through the flame three times to fix the film. Allow the slide to cool.

4. Cover the slide with crystal violet solution and leave it for 1 minute.

5. Wash off the residual stain with iodine. Cover the slide with fresh iodine and leave it for 1 minute. Tip off the iodine.

6. Pour the alcohol/acetone (95%/5%) mixture over the slide until no more

colour runs off the slide freely.

7. Wash the slide with water.

8. Flood the slide with the safranine counterstain and leave it for 2 minutes.

9. Wash the slide with water and dry.

Interpretation:

.Gram-positive: resist decolorisation by ethanol or acetone and stain **blue/purple.**

.Gram-negative: are decolorised by ethanol or acetone and are stained **red/pink**.

APPENDIX II

SDS-PAGE METHOD

APPENDIX II

SDS-POLYACRILAMIDA GEL ELECTROPHORESIS METHOD

To make up the gel:

Glassplates were wiped with acetone and clamped together with the book clamps, making sure that the sponge sealed the bottom of the glass plates to avoid leakage.

Separating gel was prepared with : 2.5ml of separating buffer, 4ml of acrylamide solution and 3.5ml of distilled water. The mixture was deaerated with a gentle vacuum until bubbles raised from the bottom of the bottle, then 7.5μl of TEMED and 31.5μl of ammonia persulphate solution were added. Gel mixture was poured into the glass container to within 1.5cm of the top of the small plate. 200 μl of water saturated iso-butanol were layered on top of the gel mixture to ensure a straight surface line and to isolate the gel from atmospheric oxygen which impedes polymerization. It was allowed to set for 30 minutes. A small sample was kept in the pipette tip to test when the gel was set.

Iso-butanol was rinsed off the gel with plenty of distilled water and water dried from the top by inserting filter paper without touching the gel surface.

The gel could be stored at 4°C covered with distilled water and sealed with parafilm for 24h if it was necessary. Gel was brought to room temperature before use.

Stacking gel solution was prepared with: 1.25ml of stacking gel buffer, 0.67ml of acrylamide solution and 3.05ml of distilled water, then 5μl of TEMED and 25μl of ammonia persulfate were added. Mixture was poured onto the surface of the separating gel right up to the top of the glass plate. The plastic comb was inserted into the gel and allowed to set for 20 minutes. A small sample of the gel was kept in the pipette tip to test when the gel was set.

Preparation and running the gel:

Reservoir buffer was diluted 1 in 5 and the upper tray of the gel apparatus filled with it making sure there was no leaking between glass and tray. Combs were taken out carefully, avoiding bubble formation. The lower tray was filled with water and bubbles which had accumulated at the bottom of the gel were removed with a bent syringe needle and syringe.

Sufficient buffer was added to submerge the top edge of the gel to a depth of approximately 1 cm.

Outer membrane suspension diluted 1:1 in sample buffer was boiled for 5 minutes and 5μl of bromophenol solution added to the samples in order to visualize its progression along the gel. Boiling the samples denatured the protein mixture into their individual polypeptide subunits. The SDS contained in the sample buffer is an ionic detergent used as a dissociating agent and 2-B-mercaptoethanol is a thiol

reagent that cleaves disulphide bonds of the proteins. Most polypeptides bind SDS in a constant weight ratio and the intrinsic charges of the polypeptide are insignificant compared to the negative charges provided by the bound reagent.

Samples were applied with a microsyringe as well as 5μl of the molecular weight standard. The proteins included in the standard were as follows:

-Rabbit muscle phosphorylase b:	97,400 Daltons
-Bovine serum albumin:	66,200 Dalton
-Hen egg white ovalbumin:	45,000 Dalton
-Bovine carbonic anhydrase:	31,000 Dalton
-Soybean trypsin inhibitor:	21,5000 Dalton
-Hen egg white lysozyme:	14,4000 Dalton

Electrodes were connected and run at 200 volts for approximately 30 minutes, until the marker dye ran almost to the bottom of the bulk separating gel. Gels were removed from the glassplates and placed in a plastic container for staining or use in Western blots.

APPENDIX III

HISTOLOGICAL PROCCESSING OF THE SHRIMPS

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HISTOLOGICAL PROCCESSING OF THE SHRIMPS

The following procedure provides a gut-gill panorama.

Bisect shrimp trasversely (for shrimp greater in length than 3 cm) at the junction of the cephalotorax and abdomen. Longitudinally bisect the cephalotorax just lateral to the midline (or the whole specimen for shrimp less than 3 cm in lenght). From the half of the cephalotorax without the mid-line, remove, the branchiostegal region containing the gills. Separate abdominal segments 1-3 and 6 from the remainder of the abdomen and bisect this last one as in the manner of the cephalotorax.

APPENDIX IV

HEMATOXYLIN AND EOSIN STAINING METHOD

APPENDIX IV

HAEMATOXYLIN AND EOSIN STAINING

In order to improve the examination of the different tissues of the sections, these were stained with haematoxylin and eosin.

Method

1.	Xylene	5 minutes
2.	Alcohol	2 minutes
3.	Methylated spirits	1.5 minutes
4.	Tap water	
5.	Haematoxylin	5 minutes
6.	Tap water	
7.	Acid alcohol	4 quick dips
8.	Tap water	
9.	Scott's tap water	1 minute

Check staining microscopically at this stage.

10.	Wash well in tap water	
11.	Eosin	5 minutes
12.	Quick wash in tap water	
13.	Methylated spirits	30 seconds
14.	Alcohol	2 minutes
15.	Alcohol	1.5 minutes
16.	Xylene	5 minutes
17.	Xylene	

Sections must not be left out of the xylene as they will dehydrate. Sections are coversliped after the last xylene dip.