Motile *Aeromonas* septicaemia of farmed *Rana* spp.

**A thesis submitted to the University of Stirling
for the degree of Doctor of Philosophy**

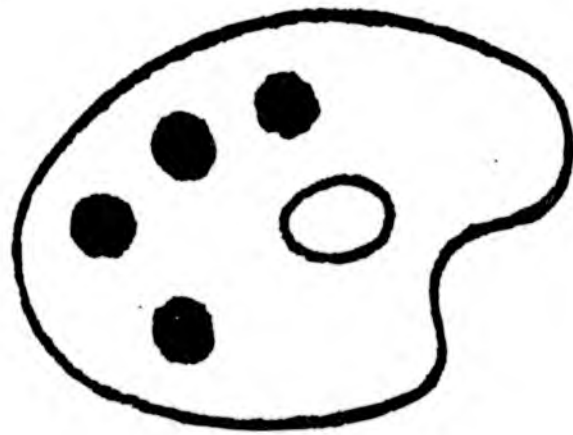**by**

Marianne D. Pearson BVM&S, MSc Aquatic Veterinary Studies, MRCVS.

**Institute of Aquaculture
University of Stirling
Scotland**

August 1998

Numerous Originals in Colour

To my parents

Declaration

I declare that this thesis has been composed by myself, that it embodies the results of my own research and has not been submitted for any other degrees. All sources of information have been acknowledged.

A portion of this work has been published in the following journals and conference proceedings:

Pearson M. D., Colquhoun D., Somsiri T. and Inglis V. (1997) Biochemical characterisation and RAPD analysis of *Aeromonas* sp isolated from septicaemic *Rana rugulosa* (Weigmann) cultured in Thailand. In: Diseases in Asian Aquaculture III. Fish Health Section, Asian Fisheries Society, Manila. Eds; Flegel T.W. and MacRae I.H. pp. 9-14.

Pearson M. D., N. Tange, I. Hirono, T. Aoki, and V. Inglis. (1998) Phenotypic and Genotypic Analysis of *Aeromonas* species isolated from Septicaemic *Rana rugulosa* (Weigmann) Cultured in Thailand. Aquatic Microbiology Symposium, Supplement to Journal of Applied Microbiology, **85** (1), p xxi.

Inglis V., Colquhoun D., Pearson M. D., Miyata M. and Aoki T. (1996) Analysis of DNA relationships among *Aeromonas* species by RAPD (randomly amplified polymorphic DNA) typing. Aquaculture International. **4**, 43-53.

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Acknowledgements

Abstract

The bacteria associated with septicaemic disease of farmed frogs in Thailand were investigated. A group of motile aeromonads, designated Au (*Aeromonas* unspeciated), was unusually homogeneous in their biochemical reactions for a geographically diverse collection, notably none of the Au isolates utilised sucrose. Bacteria conforming to the phenotypic reactions of *Aeromonas hydrophila* and *Aeromonas sobria* were isolated from the skin and intestine of diseased and clinically normal frogs but only Au isolates were found internally in septicaemic frogs.

The DNA relatedness of the collection of aeromonads was examined using randomly amplified polymorphic DNA (RAPD) and 16s rDNA sequencing. RAPD analysis produced very consistent banding patterns for the Au isolates whilst producing scattered profiles for type strains and other aeromonads from Thailand. The RAPD profiles showed that the Au isolates were all closely related to *A. hydrophila* (HG1) but were more closely related to each other. 16s rDNA sequence analysis revealed that in the hypervariable region V3 of the 16s rDNA gene all the Au isolates were identical and differed from all previously published *Aeromonas* sequences. The phenotypic and genotypic findings strongly suggest that Au is a previously unspeciated motile aeromonad.

Histological examination of tissues from frogs affected by acute Au septicaemia revealed widespread vascular congestion, severe cardiac myopathy and pulmonary, renal, hepatic and splenic necrosis. In pathogenicity studies, clinically normal animals

challenged with Au, by both injection and bath challenge, developed acute
septicaemic disease and yielded pure cultures of Au on bacteriological examination.

Haemolysin activities against frog RBC were significantly different within the
collection of aeromonads. Groups of high haemolytic activity (unspeciated
Aeromonas, Au), moderate haemolytic activity (*A. hydrophila*) and low haemolytic
activity (*A. veronii* biovar sobria, *A. veronii* biovar veronii, *A. caviae*, *A. schubertii*)
were noted. DNA colony hybridisation studies revealed that Au isolates possessed a
haemolysin gene (ASH1) which was not present in any of the other Thai aeromonads
or type strains tested. Cells from rainbow trout were extremely sensitive to Au toxins
but less so to toxins produced by other species. In contrast mammalian cells showed
very little sensitivity to Au toxins but were more sensitive to toxins produced by *A.
hydrophila*. The selection of suitable assay substrates is therefore very important; cells
from homeotherms may be insensitive to cytotoxins associated with pathogenic
processes in poikilotherms.

Abbreviations

AAHRI -	Aquatic Animal Health Research Institute
AFLP -	amplification of restriction fragment length polymorphisms
ATCC -	American Type Culture Collection
Ac -	*Aeromons caviae*
Aen -	*Aeromonas encheleia*
Aeu -	*Aeromons eucrenophila*
Ah -	*Aeromonas hydrophila*
Aj -	*Aeromonas jandaei*
Am -	*Aeromonas media*
As -	*Aeromonas sobria*
At -	*Aeromonas trota*
Asch -	*Aeromonas schubertii*
Au -	*Aermomonas* unspeciated
Avbvs -	*Aeromonas veronii* bv sobria
Avbvv -	*Aeromonas veronii* bv veronii
alc	*Alcaligenes*
bp	base pairs
CECT -	Coleccion Espanola de Cultivas Tipo
cfu -	colony forming units
CTAB -	hexadecyltrimethyl ammonium bromide
DMEM	Dulbecco's Modified Eagle's Medium
dNTP	deoxynucleotide triphosphate
dom -	dominant growth
ECP -	extracellular products
Ent -	Enterobacteria
(F) -	clinically normal frog sampled on farm
Flex	*Flexibacter* sp.
G-ve -	Gram negative
G+ve -	Gram positive
HA -	haemagglutination
HAI -	haemagglutination inhibition
HG -	hybridisation group
H&E -	haematoxylin and eosin
IASU -	Institute of Aquaculture Stirling University
ID -	identification
IPTG -	isopropylthio-β-D-galactoside
LD_{50} -	50% lethal dose
MMC -	melanomacrophage centre
MT -	metamorphosing tadpole
NAD -	no physical abnormality diagnosed
NCIMB -	National Collection of Marine and Industrial Bacteria
ND -	not determined
NR -	not recorded
NSG -	no significant growth
OD -	optical density
OTC -	oxytetracycline

PCR -	polymerase chain reaction
pers. comm. -	personal communication
RAPD -	randomly amplified polymorphic DNA
RFLP -	restriction fragment length polymorphism
rDNA -	ribosomal DNA (rRNA gene)
rRNA -	ribosomal RNA
ref -	reference (type) strain
SDS -	sodium dodecyl sulphate
TE -	tris EDTA buffer
TBE -	tris borate EDTA buffer
TEMED -	N,N,N',N'-Tetramethylethylenediamine
TSA -	tryptone soya agar
UPGMA -	unweighted pair group method with arithmetic averages
X-gal -	5-bromo-4-chloro-3-indolyl-β-D-galactoside
YT -	yeast tryptone

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Chapter 1 General Introduction

1.1 EDIBLE FROGS

Amphibians appeared about 408 million years ago. They were the first vertebrates at least partly adapted to life on land. Nine major groups of amphibians are now extinct. Only three groups exist today: salamanders (including newts), caecilians, and anurans. By far the most numerous amphibians are the frogs and toads, which belong to the group called anurans, meaning the "tail-less ones". Although anurans have developed many highly specialised forms they all retain features of the early amphibians including: respiratory physiology, reproductive strategies which take place around water and a moist and permeable skin. There are around 2600 species of anurans distributed throughout the world but many of these diverse populations, survivors from the era of terrestrial colonisation, are now in decline (Encarta, 1997).

No other vertebrates have the unusual body form or strange method of movement that marks frogs. Their long and powerful hind legs, used for jumping are much larger than their front legs, which catch the body's weight when they land. The frog's leap is an effective method of locomotion and escape. Catching a frog can be a difficult business. Those hind legs, however, which give the frog its impressive leap, also make it much sought after as food by many predators including humans.

The international market in frog meat is supplied predominantly with wild caught frogs. Just how many species contribute to this pool is unknown, but the dominant species include: *Rana catesbeiana, Rana tigerina, Rana rugulosa, Rana hexadactyla, Rana esculenta* and *Rana ridibunda* (Culley, 1984).

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In some countries new frog farming systems are contributing significantly to export earnings. The American bullfrog *R. catesbeiana*, is now being intensively cultured in Brazil, Taiwan, Thailand and Indonesia. The Chinese bullfrog *R. rugulosa* and the Indian bullfrog or Tiger frog *R. tigerina*, are also being cultured on a commercial scale in Asia (Chen 1990, Texiera 1993, Pariyanoth and Daorerk, 1995).

Frog meat has great potential in the international market because of its subtle taste and excellent nutritional value. The meat is highly digéstible, rich in protein and low in fat and calories. Frog meat can be an important product in the treatment of allergic alimentary disturbances in children. In the USA, 40% of all frog meat is consumed in hospitals. (Teixeira 1993). Farmed frogs also serve a variety of other functions, they are used for the production of medicinal compounds and are widely used for experimental and educational purposes. If the market for farmed frogs was fully exploited it could sustain a highly profitable frog culture industry .

1.2 IMPORTANCE OF FROG CULTURE.

1.2.1 CONSERVATION

Declines in the wild population of amphibians have been recognized world wide since the 1970's (Baringa 1990, Cunningham *et al.,* 1993, Hayes and Jennings 1986, Hecht 1993). The rapid fall in numbers of frogs and their kin occurred almost simultaneously in points widely dispersed across the globe (Blaustein and Wake, 1995). No adequate explanation has yet been found for such a wide ranging loss in biodiversity.
The destruction of natural habitats which affects many types of plants and animals other than amphibians, may be a significant factor. Some groups are however

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disappearing from pristine areas, such as nature preserves, where there is little human perturbation (Baringa 1990).

Exploitation of wild frogs for the frozen frog leg industry may be responsible for some declines. There are historical records of at least one wild frog population being decimated by the frogging industry. In California over a century ago *Rana aurora draytonii* were almost eliminated. Jennings and Hayes (1985) reviewed the literature between 1850 and 1940 and found evidence to suggest that this decline was due to over-harvest of *R. a. draytonii*. In California from 1860-1900 there was a steady increase in frog harvests. In 1895 frogs ranked second in value among over 60 Californian fishery products. Harvests of native stock took tens of thousands of adult frogs per annum, and continued for a number of years before the decline occurred, indicating that native populations were very abundant. After 1900, data indicate only limited frog harvests. In just 8 years California went from being the leading supplier of market frogs in the United States, to that of a supplier of quantities too small to report. Depletion of wild stocks was sufficient to require the importation of bullfrogs as substitutes because demand for frogs persisted. The natural populations of *R. a. draytonii* never recovered (Jennings and Hayes 1985).

A similar pattern of over-exploitation is now being reported in developing countries where the export of frog legs is a valuable means of earning foreign exchange. In India the industry exporting frozen frog legs has been operating since 1956 and has been solely reliant upon the natural supply of frogs. Due to indiscriminate exploitation, wild populations of these frogs are showing signs of depletion (Sukumaran 1986).

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Rana tigerina occurs naturally in rice paddies in Taiwan. Locally this frog is known as water chicken and is a highly priced food item. This frog used to be captured in the wild and sold as food. Populations however have declined following over-harvest and human destruction of the frogs natural habitat and now all market frogs are of aquaculture origin (Chen 1990).

Wild frogs are a natural method of pest control and important in the maintenance of a stable ecosystem. If frog populations are depleted, pests multiply and farmers are forced to spend more money on chemical pesticides. These often have an adverse effect on the environment and may further reduce the frog populations. Such a situation has already occurred in Bangladesh. In 1977 there were thought to be one billion frogs in Bangladesh. The paddy fields, mainly farmed by smallholding peasants, were one of the worlds greatest sources of frogs legs. By 1988 more than 50 million Bangladeshi frogs a year were being exported, mostly to the USA. One year later, Bangladesh was thought to have only 400 million frogs left (Vidal, 1994).

Taking frogs from the wild has devastating consequences. Frogs are insectivorous and fewer than 50 are needed to keep an acre of paddy field free of insects. They keep malaria and other illnesses with insect vectors at bay, provide a natural biological control agent for crops and produce waste which is a fine organic fertiliser.

By 1989 Bangladesh was importing an extra 25% of pesticides a year to compensate for its frog loss. Some of the companies exporting the wild frogs' legs to the West were also responsible for importing the chemicals. A temporary export ban was

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imposed but six years later the pesiticide companies and frog traders were lobbying to resume trading and poaching and illegal trade flourished (Vidal, 1994).

In Shanghai people are prepared to pay up to 100 yuan (£7.60) per kg for live natural frog, which can be bought in villages in the interior for as little as 2.40 yuan per kg. On January 1, 1994 the Shanghai city government published a list of protected species which may not be sold. There is however a booming black market in the protected species. In a one-month crackdown in April 1994 in the city district of Yangpu, officials seized 445 kg of frogs. Frogs stewed, steamed, or cooked with vegetables are on sale in most Shanghai restaurants. Significantly, bullfrogs bred for eating are excluded from the list of protected species indicating a potential in China for the development of frog farming. (Anon 1994).

Frog culture plays an important role in frog conservation as leads it to a reduction in the uncontrolled collection of frogs from the wild. Furthermore, frogs bred in captivity can be used to stock natural habitats, to revive or replenish depleted natural populations (Teixeira 1992).

1.2.2 SOCIO-ECONOMIC BENEFITS

Export of live frogs by developing countries earns a large amount of foreign exchange every year. In Hong Kong alone, 10 metric tons of frog meat are consumed daily (Marketing Innovation Co Ltd, pers. comm.). This market is supplied by farmed and wild caught frogs from Thailand and wild caught frogs from Bangladesh, China, Malaysia and Vietnam. It is estimated that 6,500 tons of frogs legs are consumed

every year in the Western hemisphere. In one British restaurant a plate of 12 legs costs £22 (Vidal, 1994).

Exporting frozen frog legs is an increasingly important trade for India. In the 1970s India was exporting to USA, France, Belgium, Netherlands and West Germany; a market entirely supplied by wild caught frogs (Jagadees 1979). In 1985 the frozen frog leg industry earned 64 million rupees (Sukumaran 1986).

The trade in Bangladeshi wild frogs has been environmentally and agriculturally devastating, undermining the peoples' ability to survive on the land. It pushed many Bangladeshi farmers further to the economic margins, contributing to loss of land ownership and migration to the cities (Vidal, 1994). With the decline in the wild catch of frogs, the rural poor have to turn to alternatives to supplement their incomes. The people hardest affected are women and children as this is a traditional supplement to their income. The establishment of a sustainable frog farming industry consisting of small scale family run businesses would help to avoid resource depletion, environmental damage and provide a valuable supplement to the income of small scale farmers.

The decline in the wild catch has further economic importance. Goverment figures from Bangladesh show that by 1989 $30 million a year was spent on pesticides, while only $10 million was earnt from the export of frogs. Many of the chemicals imported were of the worst quality, much came from Bhopal in India, before the accident that killed 287 people in 1984. While large farmers could buy pesticides cheaply in bulk, the traditional smallholders were paying about $5 in pesticides for every $2 they

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earned from catching 100 frogs. No one has estimated the cost of polluted waters or health effects resulting from the use of these chemicals (Vidal, 1994).

Most farms are still small scale production units (Hambalyi Supriyadi and Temdoung Somsiri pers. comm.). Individual farmers are dependant on the cost and timely supply of reliable inputs, particularly of stock, feed and services. These ancillary industries could also provide employment in rural areas.

The potential for marketing various by-products obtained from frog-processing is attractive. Frog farming generates materials used for medicine and medical research and for leather goods and manure. After tanning, the thin and soft, yet strong and elastic, frog skin gives a leather of unique quality. The fat reserves found in the abdominal cavity of the frogs can be processed into a special oil which is used in the manufacture of cosmetics, skin moisturisers and sun tan lotions. The offal from frog processing can be dried and ground into a highly nutritious flour which can be used in the manufacture of feed for frogs (Teixeira 1992). The skins of amphibians are yielding drugs useful to medicine. The full potential of such drugs has not begun to be appreciated (Anon, 1997).

Benefits from the industry farming frogs include, increased production of healthy protein rich foods for local and export markets, and increased employment in ancillary industries based in rural areas. Rational methods of farming could thus play a vital role in improving living standards of the rural population.

1.3 HISTORY OF FROG FARMING

Although man has been hunting and eating frogs for thousands of years, successful intensive culture is a recent development. Attempts at frog culture started in America at the end of the last century. During the 1890's several frog farms were established to supply *R. a. draytonii* for the San Francisco markets (Jennings and Hayes, 1985). It is unknown when the first attempts to culture the bullfrog *R. catesbeiana* began in the USA. A "frog ranch" which had been in production for ten years was reported as early as 1904 in California. Descendants of these bullfrogs are known to have been shipped to Hawaii in 1897 (Jennings and Hayes 1985).

The period 1900-1930 saw many introductions of *R. catesbeiana* to California and the start of numerous frog farming enterprises (Jennings and Hayes 1985). Early farmers collected metamorphosing tadpoles or young frogs from the wild, placed them in protected ponds and provided them with care and food through succeeding generations. Success was generally marginal due to problems with disease, predators, insufficient food and cannibalism (Culley 1991). Although there are no detailed descriptions from other parts of the world, brief notes indicate that similar systems did exist (Culley 1991).

The bullfrog *R. catesbeiana* has dominated the production for human consumption in South America and major research in Brazil has been directed towards the culture of this amphibian. In the early stages poorly developed technology was the limiting factor, making artificial frog breeding a non-viable economic activity. In the 1980's with the success of Brazilian government funded research into developing new culture systems and improved nutritionally balanced feed, prospects for frog culture

improved. During the early days it took six to seven months months for the bullfrog to attain market size (160-180g). Today the improvement of facilities and feeding methods means that the same result can be obtained in four months. As the industry evolved, production continued to improve and some farmers in Brazil now produce market weight frogs in only 70 days (Teixeira 1993).

In South East Asia, the frog culture industry has expanded rapidly in recent years. *Rana catesbeiana* culture developed in Taiwan in the late 1950's. In 30 years this industry has gone through several ups and downs. The industry failed to capture the local market in the last two decades when culture of the native tiger frog, *R. tigerina*, emerged as a successful industry. The price for the *R. catesbeiana* abroad is not attractive enough to warrant exportation so the industry is presently in decline again (Chen 1990). Culture of the tiger frog is however a thriving industry.

Frog culture in Thailand has been practiced since the 1980's however commercial success was only attained in the 1990's (Somsiri, 1994, Pariyanoth and Daorerk, 1995). The species of frogs cultured here differs from South America. Although *R. catesbeiana* has been imported and cultured successfully, it is not the most important species. The frogs commonly farmed in Thailand are local species; *R. rugulosa*, the Chinese bullfrog, and *R. tigerina* the Indian bullfrog also known as the tiger frog. These two species are however phylogenically very closely related (Kao *et al.*, 1993).

1.4 CULTURE PRACTICES

Frogs may be cultured extensively and semi-extensively in earthen ponds or intensively in tanks made of concrete or fibreglass.

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Intensive culture is common practice in Thailand, Taiwan and South America. Culture is divided into two main phases: breeding and ongrowing. Breeding areas simulate the natural conditions of the frog as far as possible, water weeds are often placed in the tanks to provide appropriate habitats. Broodstock densities are low from 1 to 12 animals per square metre, usually at a sex ratio of 2 males to 3 females.

Ovulation is triggered by rains so spawning takes place during the rainy season. Egg masses are collected from the breeding areas and stocked in hatching tanks. Hatching takes place in 1-10 days depending on temperature, environment and species being farmed. Tadpoles are removed to nursing tanks where they are stocked at approximately $2000/m^2$ and fed initially on water flies or hard boiled egg yolk before being weaned onto commercial crumb.

Time to metamorphosis and length of growout period again vary depending on the location and the species cultured. For *R. tigerina* and *R. rugulosa* metamorphosis begins after 28-36 days. While for *Rana catesbeiana*, which can be cultured in temperatures from 12-37°C, metamorphosis is 100% complete at 75-90 days in warmer areas but not until 6 months in cooler areas. When metamophosis starts the tadpoles need something to cling to therefore surfaces such as water weeds or bamboo rafts are placed in the ponds.

After metamorphosis the frogs are transferred to growout ponds and weaned onto commercials pellets. Growout frogs are stocked at $50-100/m^2$. Size grading and restocking are carried out at regular intervals. The frogs are fattened until they reach market size which varies from 170g in South America to 300g in Southeast Asia. The

frogs are either marketed live (common in Asia) or processed (e.g. frogs legs) for export (Anon 1995, Chen 1990, Pariyanoth and Daorerk, 1995, Teixeira, 1992, Teixeira, 1993).

Semi-intensive systems are encountered in countries such as Indonesia where the frog farming industry is still in its infancy. The frogs are reared in earthen ponds and commonly fed on chopped snails, waste from chicken processing plants or occasionally on commercial pellets. The farmers supplement the artificial diet by hanging lamps in the pens to attract flies as natural feed (Hambali Supriyadi, pers. comm.).

Extensive culture practices are not common. Animals are reared in large field nurseries where they feed naturally on zooplankton, tubifiex worms and insects. When sufficient food is not available they are highly cannibalistic which accounts for the very poor survival rates (20-25%) in such systems. (Sukumaran, 1986).

1. 5 CURRENT PROBLEMS FACING THE INDUSTRY

There are many limiting factors to successful frog culture such as: disease, inadequate nutrition, abuse of chemicals, variable market and inadequate support structure. These problems are often inter-related and can cause recurrent severe economic losses.

Inadequate support means no structured health programmes to provide advice, no diagnostic facilities and no manuals or workshops to train farmers in basic health management techniques. Poor nutrition reduces growth rates, causes nutritional imbalances and increases susceptibility to disease. Repeated outbreaks of disease have

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led to widespread abuse of chemotherapeutics. Frog farmers in Thailand and

Indonesia routinely use large amounts of antibiotic during the culture cycle. This

frequent and indiscriminate use of drug therapy can cause severe problems with the

emergence of resistant pathogens. Antibiotic use can also result in residues in the flesh

rendering the product unacceptable to the export market.

1.6 HEALTH STATUS OF CULTURED FROGS

Poor husbandry leads to continual disease problems. In Thailand outbreaks of

bacterial septicaemias have killed 80-90% of tadpoles and growout frogs. Protozoal

infections can cause 50-70% mortalities in tadpole nurseries. Thus epizootics of

bacterial or protozoal diseases can result in huge financial losses for farmers (Somsiri,

1994).

Diseases reported by Thai frog farmers include in tadpoles: columnaris disease due to

Flexibacter spp.; *Aeromonas* septicaemia; external protozoal infections, especially

with *Trichodina* spp. and oedema disease. In growout frogs the following problems

have been recorded: red-leg; ascites; gas in the intestine; red spots on toes; ulceration

(Somsiri, 1994). Some Thai frog farmers claim to have few problems with husbandry

and disease. Others report frequent disease outbreaks but levels of expectation vary.

One farmer in Thailand reported 10 to 50 frogs suffering from oedema disease per

pond per day as an acceptable level of disease.

Diseases reported by Indonesian frog farmers include in tadpoles: red-leg and

subcutaneous oedema (large vesicles below the skin). In growout animals red-leg,

ascites and ulceration have been reported (Hambalyi Suprayadi pers. comm.).

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In *R. catesbeiana* culture, disease is a continuing serious problem and preventive measures are often ineffective. Bullfrogs frquently succumb to bacterial infections and die, even under sanitary conditions (Culley 1986). The reasons for these outbreaks of infection are not well understood. The pathogens are in the frogs gut but cause no problems until they invade the circulatory system. What triggers this invasion is not known, but stress and poor nutrition are implicated (Culley 1991).

The scientific study of the aetiology and pathology of infectious diseases in intensively cultured frogs has scarcely begun. The existing knowledge of frog bacterial diseases comes from wild populations and from laboratory populations of many different species.

1.7 BACTERIAL DISEASES OF FROGS

Frogs are constantly at risk from bacterial infections. Many potentially pathogenic bacteria are widespread in the environment and can enter the frog with food and air and also with water diffusing through the permeable amphibian skin. Bacterial invasion is even more likely when frog defences are compromised, for example by damaged skin, which is a common occurrence in farmed animals.

Bacterial septicaemias have been described in laboratory populations of frogs since the last century; they cause mass mortalities in wild amphibian populations and major losses in farmed frogs (Bradford 1991, Dusi 1949, Gibbs 1973, Glorioso *et al.*, 1974a, Nyman 1986). The understanding and subsequent development of successful treatment of septicaemic frog disease depends on identification of the bacteria involved in the disease process.

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Various bacteria have been isolated from septicaemic frogs (see Table 1.7). Red-leg

disease, an epizootic form of bacterial septicaemia, poses a most serious threat to frog

health at all life stages (Glorioso *et al*, 1974a). There is however some confusion over

what the term "red-leg" actually means. Simple irritation of the skin can cause

reddening. Such irritation, if not treated, may allow invasion by opportunistic

pathogens but is not a disease syndrome in itself. Some authors attribute red-leg

septicaemias to those caused by the bacterium *Aeromonas hydrophila* alone (Hird *et

al.*, 1981, Bradford 1991, Dusi 1949, Nyman 1986, Gibbs 1973). Red-leg symptoms

(cutaneous haemorrhages, ulcerated skin on toes and feet) are however linked to

bacterial septicaemias caused by other bacteria as well as *A. hydrophila*. To add

further confusion many frogs that are killed by bacterial septicaemias, never have

reddened legs. In this review red-leg will be taken to mean any epizootic bacterial

septicaemia.

Frogs suffering from red-leg are lethargic and after a day or two develop capillary

dilation and petechial haemorrhages of the inner thighs and abdomen. The final stages

of the disease can be accompanied by extreme oedema of the thighs and the

abdominal region (Reichenbach-Klinke and Elkan 1965).

The natural occurrence of red-leg epidemics in wild populations of frogs has been

reported by several authors. Dusi (1949) recorded the occurrence of an epidemic of

red-leg due to *Pseudomonas hydrophila* in a population of American toads, *Bufo

americanus*. The population of over 300 was wiped out in three days. Although many

dead toads were observed, bacteriological samples were only taken from one

moribund toad, two healthy *B. americanus* tadpoles and one healthy *Rana pipiens*

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Table 1.7 **Bacteria identified as causative agents in diseased amphibians (adapted from Culley 1978).**

Date	Investigator	Bacterial isolates
1898	Russell	*Bacillus hydrophilus fuscus* *(Aeromonas hydrophila)*
1905	Emerson and Norris	*Bacillus hydrophila fuscus* *(Aeromonas hydrophila)*
1942	Kulp and Borden	*Bacillus hydrophila fuscus* *(Aeromonas hydrophila)*
1949	Dusi	*Pseudomonas hydrophila* *(Aeromonas hydrophila)*
1952	Kaplan	*Pseudomonas hydrophila* *(Aeromonas hydrophila)*
1963	Gibbs	*Aeromonas hydrophila* *Staphylococcus epidermis* *Citrobacter freundii*
1966	Gibbs *et al.*	*Aeromonas hydrophila* *Mima* sp. *(Acinetobacter* sp.)
1974a,b	Glorioso *et al.*	*Aeromonas hydrophila* *Aeromonas shigelloides* *Citrobacter freundii* *Flavobacterium* sp. *Mima polymorpha* *(Acinetobacter* sp.) *Proteus mirablis* *Proteus morganii* *Proteus retgerii* *Proteus vulgaris* *Pseudomonas aeruginosa* *Pseudomonas fluorescens* *Pseudomonas putida*
1974	Keymer	*Aeromonas liquefaciens* *Escherichia coli* *Proteus morgani* *Pseudomonas aeruginosa*
1975	Eisinger	*Corynebacterium* sp.
1976	Elkan	*Pseudomonas aeruginosa*
1976	Carr *et al.*	*Aeromonas hydrophila* *Aeromonas* sp. *Pseudomonas* sp. *Acinetobacter* sp.
1986	Nyman	*Aeromonas hydrophila*
1991	Bradford	*Aeromonas hydrophila* *Enterobacter aerogenes* *Enterobacter agglomerans*

which was also in the pond. *Pseudomonas hydrophila (Aeromonas hydrophila)* was isolated from the toad, one of the tadpoles and from two water samples.

Bradford (1991) recorded the mass mortality and extinction of a population of *Rana mucosa*. The population declined from around 800 individuals in early summer to nearly zero in late summer. Many carcasses were found during the time of obvious population decline. Live frogs were occasionally found in a sick or moribund condition. Affected individuals were emaciated, sluggish and poorly coordinated and the ventral surface of the thighs and sometimes the forearms and toes were abnormally red with enlarged capillaries or haemorrhages. The author states that these symptoms were consistent with red-leg disease.

Three affected individuals were collected for bacteriological examination. Analysis of blood from two specimens revealed *A. hydrophila* and *Enterobacter aerogenes* in one specimen and *E. aerogenes* and *E. agglomerans* in the other. From the evidence of the clinical signs in affected animals and the isolation of *A. hydrophila* from one individual, the author concluded that the dramatic population decline was due to red-leg disease caused by *A. hydrophila.*

Nyman (1986) described an epizootic form of bacterial septicaemia among larval *Rana sylvatica* in Rhode Island which eliminated the population within two weeks. Bacterial cultures of tissue from dead and moribund tadpoles were positive for *A. hydrophila*. Infected animals showed conspicuous petechial haemorrhages concentrated ventrally on the abdomen and the tail. Apparently healthy animals also frequently exhibited these symptoms. In later stages of the disease, larvae were

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emaciated, lethargic and anorexic. Nyman concluded that deaths were attributable to

A. hydrophila.

These papers illustrate the confusion which surrounds reports of red-leg epizootics. In each case although a bacterial septicaemia may have caused the deaths, there may have been other predisposing factors which were not investigated.

Dusi and Bradford sampled only one and three frogs respectively. It is not possible to draw conclusions from the results of such small samples. Nyman sampled only dead or moribund tadpoles. He did not give details on how many tadpoles were sampled, nor on the numbers of tadpoles from which *Aeromonas hydrophila* was isolated. Nor did he state whether other bacteria were present. It is not possible therefore to assess the significance of the *A. hydrophila* isolates.

Reports such as those described above may have over-estimated the involvement of *A. hydrophila* in wild frog population declines. The bacterium is however the most commonly identified cause of mortality in laboratory frog populations. Gibbs (1973), in a review of health and disease in laboratory *Rana pipiens,* described the average laboratory frog as infested with numerous parasites, infected with bacteria and viruses, malnourished and possibly shocked from extremes suffered during shipment. Most, the author stated, died within days or weeks of receipt and the cause of death in the overwhelming majority of cases was septicaemia caused by *A. hydrophila.* While acknowledging that other organisms had been implicated in spontaneous disease Gibbs considered the incidence of such infections to be low. He concluded that if

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bacterial infection with *A. hydrophila* could be stopped, the majority of laboratory frogs survived and regained their health under optimum conditions.

Glorioso *et al.* (1974a) studied both pond and laboratory populations of septicaemic bullfrogs (*R. catesbeiana*) and described in detail the clinical signs associated with the disease. Septicaemic metamorphosing tadpoles were lethargic, and oedematous with haemorrhages of the tail and newly formed appendages. Death usually occurred within 24 hours of development of these signs. Seventy per cent of the tadpoles died during metamorphosis.

The disease in adult frogs was insidious, sometimes lasting as long as six months before death. The clinical signs included oedema, haemorrhaging, loss of appetite and lethargy. Affected frogs usually had diffuse or petechial haemorrhaging of the dermis from the mandible to the toes. Muscular, intermuscular and periosteal haemorrhages were seen in the hind limbs, accompanied by flaccid atrophy of the muscle.

The authors isolated bacteria from the heart blood of diseased frogs. Between 2 and 8 different species of pathogens were isolated from most individual specimens. No one organism was found in every frog and no discernible patterns were seen in their distribution. The pathogenicity of bacterial isolates was tested by inoculation of healthy frogs and tadpoles. Pathogenic isolates are listed in Table 1.7. Members of the pathogenic group including *Proteus* sp., *Pseudomonas* sp., *Aeromonas* sp. and *Flavobacterium* sp. were isolated from the maintenance water and from the intestinal tracts of both healthy and diseased frogs. These bacteria are ubiquitous in the aquatic

environment and have been demonstrated to be opportunistic pathogens for other aquatic animals.

Glorioso *et al.,* (1974a) found that even though individual organisms proved lethal under laboratory conditions pure cultures of a single suspect pathogen could be isolated from the blood of only 15% of naturally infected frogs. This contrasts with the pure cultures of *A. hydrophila* reported by earlier workers. The authors suggest as an explanation that primary bacterial isolation steps are difficult and in previous studies slow growing species such as *Mima* were over grown by *Aeromonas* spp. They postulate that improved techniques in bacterial isolation resulted in more mixed bacterial isolates.

Although *Aeromonas hydrophila* is frequently cited as the cause of red-leg in frogs (Hird *et al.,* 1981, Bradford 1991, Dusi 1949, Gibbs, 1973, Gosling 1996, Nyman, 1986) the bacterium can be isolated from many apparently healthy frogs and tadpoles in their natural environment. Hird *et al.,* (1983) found *A hydrophila* to be widely spread in clinically healthy, wild caught adult and larval *Rana pipiens* in Minnesota. The authors isolated *A. hydrophila* from the intestines of 46% of the 222 frogs sampled. They also demonstrated the presence of mixed bacterial cultures in the heart blood of 11 clinically healthy animals. These cultures included three isolates of *A. hydrophila.*

Recent research at the Institute of Aquaculture (Margaret Crumlish unpublished data) has revealed the presence of mixed populations of bacteria in phagocytes of clinically healthy farmed tropical frogs. The bacteria, visible by microscopy in macrophage

preparations, were viable but often a resuscitation step was necessary to effect their recovery. In addition mixed populations of bacteria resembling those observed in the macrophages could be isolated from the internal organs of stressed animals without a phagocyte concentration step.

Stressors have long been considered predisposing factors in disease production by opportunist pathogens found in the aquatic environment. Any number of physical, chemical or biological factors could exert physiological changes in the host which would favour tissue colonisation by bacteria. If viable bacteria are already present internally then stressors which compromise the defence mechanisms of an animal may allow such bacteria to mulitply and cause septicaemic disease.

The literature examined suggests that septicaemic frog disease is the result of a complex interaction between the frog and one or more members of a group of bacteria ubiquitous in the aquatic environment (Glorioso *et al.,* 1974a), but that variant, virulent pathogens may exist (Gibbs, 1973, Nyman, 1986, Rigney, *et al.,* 1978).This body of literature is however based on laboratory populations of frogs, with occasional reference to outbreaks of disease in wild populations.

Enzootics and epizootics of bacterial septicaemias are the most frequently reported cause of mortalities in farmed frogs, however the prevalence and significance of bacterial pathogens in farmed frog populations has yet to be documented. The environmental conditions found on frog farms (Figure 1.7.1) are very different to those found in laboratories (Figure 1.7.2) or under natural conditions. Data drawn from laboratory situations should not therefore be extrapolated to the culture situation.

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Figure 1.7.1 Environmental conditions on the farm

Figure 1.7.2 Environmental conditions in the laboratory

Research into these problems must be a priority if the frog culture industry is to be a success. The following research project was therefore undertaken:

1.8 AIMS OF THE STUDY

1. To undertake field research in Thailand to isolate and identify bacteria consistently associated with frog septicaemic disease.

2. To establish whether those bacteria are part of the flora of clinically normal frogs.

3. To describe the histopathology associated with frog septicaemic disease.

4. To investigate variation between selected bacterial isolates from diseased frogs, in particular the production of extracellular enzymes and toxicity factors.

5. To select a virulent isolate and set up a model disease system for frogs.

6. In view of the findings from the field studies an additional area was included in the study i.e. the DNA typing by RAPD analysis and 16s ribosomal DNA sequencing of an unspeciated aeromonad repeatedly isolated from farmed frogs displaying signs of acute septicaemia.

Chapter 2. Field Studies

2.1 INTRODUCTION: FROG FARMING IN THAILAND

The following information was obtained in interviews with staff at Phet Buri Fisheries
Station, in Central Thailand and from Dr Temdoung Somsiri at the Aquatic Animal
Health Research Institute in Bangkok. It reflects the opportunistic way this branch of
aquaculture has developed, the intuitive approaches used to tackle problems and the
fundamental absence of a scientific basis.

There are four edible frogs currently being marketed in Thailand: *R. rugulosa, R.
tigerina, R. macrodon* (wild caught only) and *R. catesbeiana*. In addition *Rana bythii*
the giant frog is being experimentally cultured in the North. Frogs are cultured in
Southern, South Western, Northern and Central Thailand. The province of Phet Buri,
in Central Thailand, has one of the highest concentrations of frog farms, although
culture only started there in 1991. Farms vary in size from small, consisting of 1 or 2
ponds, to large farms covering 0.5 hectares (Figures 2.1.1 to 2.1.4). Broodstock,
nursery and growout operations are normally performed within a single, small scale
"backyard" operation. Most commercial farms are family owned. Frogs are usually
sold on the local market in small quantities (20-30kg at a time), farmers also export
live frogs to Hong Kong, Malaysia and Singapore.

On average the farmer has about 20 concrete tanks (or ponds). The pond size varies
but averages 12m^2. These units usually contain polystyrene floats or bamboo benches

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Figure 2.1.1   Small-scale frog farm

Figure 2.1.2   Small-scale frog farm

Figure 2.1.3 Large-scale frog farm

Figure 2.1.4 Large-scale frog farm

for the frogs to sit on and feeding trays. Tadpoles are stocked at $2000/m^2$ and growout

frogs are stocked at $50\text{-}100/m^2$. Broodstock densities are kept low at around $20/m^2$.

Recently (1996-97) many farmers have changed from concrete tanks to large earthen

ponds which are fenced off into smaller areas. These ponds are still intensively

managed at similar stocking densities to the concrete tanks but the farmers feel that, in

them, disease outbreaks are less frequent.

Farms use various water sources including underground springs, irrigation canals,

rivers and rain water. A very few farms use dechlorinated tap water. Many farms can

share the same canal water source. Waste water is released into drainage canals. Some

farms drain waste water from the frog pens into fish ponds, for the culture of tilapia or

catfish. The waste water may also be used as tree fertiliser or be drained into a settling

pond.

Water management practices differ greatly between farms. Depth in tanks varies from

10-30cm. The number of times pond water is changed depends on the type of feed

used, source of water and water quality, some farmers change water every 1-2 days,

others every 7-10 days. Many farmers suggest that when stocking densities are high

i.e. 100 growout frogs/m^2, water should be changed every day. In tadpole ponds with

good quality water 50% is exchanged each time. In the growout ponds some farmers

drain 100% of the water, others maintain that this stresses the frogs and only change

70%.

Frogs require good quality feed. Tadpoles are normally fed for four days on water flies before being weaned onto pellets. Eighty per cent of the growout farms use pellets. There are some purpose formulated frog pellets but these are expensive and are generally only fed to post metamorphic frogs. Later stages are fed with catfish pellets which are cheaper. In Petchburi many farmers use trash fish with a vitamin and mineral premix added once weekly. Tadpoles are fed three times daily, small frogs are fed twice daily, growout frogs are fed once per day and broodstock are fed once every two days.

The spawning season lasts for 6-7 months from March to September. Spawning success depends on the weather, the frogs like it hot and wet, low temperatures reduce the success rate. The broodstock ratio is 2 males to 3 females. Originally broodstock were caught from the wild but now all stock are commercially produced. At the start of the season the female will produce 500-2000 eggs, at the peak she will produce 5000-10000 eggs. In one season a female may spawn 3-10 times, the fewer eggs she produces in each spawn the more frequently she will spawn.

Hatching takes place after 24 hours. The survival rate of the eggs depends on the season. Initially hatch rates are low, at the peak of the season i.e. from May to July the hatch rate is 80-90% successful. Metamorphosis starts after 3-4 weeks. The growout period from metamorphosis to market size (250-400g) takes 4 months.

Survival rate from tadpole to frog is about 80% if husbandry is good. If an outbreak of disease occurs then mortalities can reach 100%. Antibiotics in use in the industry include; oxytetracycline (OTC), sulphonamides, chloramphenicol and amoxycillin.

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These are usually mixed in the feed, although OTC is occasionally given as a bath. Antibiotics are used prophylactically every week on some farms. In addition there are general frog medicines on the market which do not list ingredients. These medicines are also often used prophylactically and sometimes the same frog medicines are used for different diseases. Salesmen give the farmers advice on how to use the drugs but this is commercially orientated and therefore their advice is unlikely to be impartial.

2.2 COLLECTION OF STUDY MATERIAL

Five field trips were made in August 1994 to frog farms in different geographical locations in Thailand; farm 1 in Kamphaeng Phet, farm 2 in Nakon Ratchasima, farm 3 in Phet Buri, farm 4 in Pathum Thani and farm 5 in Chon Buri (Figure 2.2). On each farm as much history as possible was recorded; however the amount of information available on each farm differed, translation from Thai into English was occasionally difficult and the farmer was not always present during the visit.

Bacteriological and histological samples were taken from 10 diseased frogs and 10 clinically normal frogs. Originally the plan was to complete all sampling processes on the farm, however on the first farm this proved impossible in the time available. Therefore ten diseased animals were sampled on site while the clinically normal animals were transported to the laboratories in Bangkok and sampled the following day. Thereafter on each trip the diseased animals and two clinically normal animals were sampled on site and the remaining clinically normal animals were transported to the laboratory. This stressed the frogs but was the best compromise available and ultimately provided interesting data.

Figure 2.2 Sampling Sites in Thailand

Kamphaeng Phet

Nakon Ratchasima

Pathum Thani

Chon Buri

Phet Buri
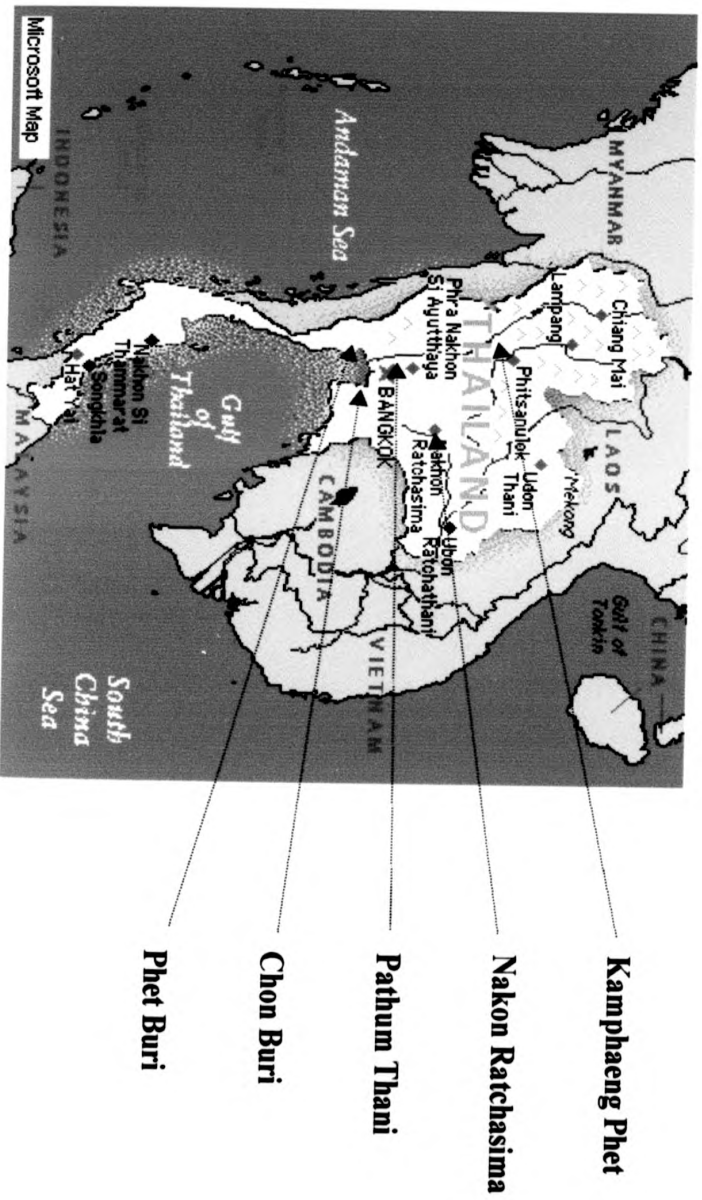
2.2.1 SELECTION OF SAMPLE ANIMALS

Animals were considered diseased if they showed external lesions such as rectal prolapse, skin ulceration and/or were lethargic i.e. failed to move away from stimuli. Animals were considered clinically normal if they showed no external signs of disease and actively attempted to evade capture. Both diseased and clinically normal animals were selected randomly from ponds distributed throughout the farm site.

2.2.2 SAMPLING PROTOCOL

Using a sterile wire loop direct smears were taken from frog: inner thigh skin, lesions, thigh muscle, heart blood, kidney, liver, spleen and intestinal contents. A small area of tissue $0.5cm^2$ was excised from each organ listed and fixed in chilled 10% buffered formalin. Aseptic techniques were used; instruments were cleaned, dipped in 95% ethyl alcohol and flamed between each sample from each animal.

2.2.3 BACTERIAL CULTURE MEDIA AND METHODS

Samples were streaked directly onto tryptone soya agar (TSA, Oxoid), Pseudomonas selective media (Oxoid) and Aeromonas selective media (Oxoid) and incubated at 30°C. On the first farm visit samples were also streaked onto MacConkey's agar and Cytophaga agar but these provided no valuable information and as the number of subcultures was becoming unmanageable their use was not continued. Suspected pathogens were subcultured on TSA. The biochemical characterisation of bacteria is described in detail in chapter 3. Preliminary differentiation using essential first stage tests provided family and some genus groupings. Selected isolates were further identified to genus and species level using second stage characterisation tests and stored on TSA slopes and in stab cultures.

2.3 RESULTS

2.3.1 FARM HISTORIES

2.3.1.1 Farm 1. Kamphaeng Phet

At the time of the visit this farm had been in production for two years (1992-1994). The broodstock were originally imported from Chanburi. However initially too few animals were used and in-breeding resulted in reduced growth, skeletal abnormalities and decreased resistance to disease. The frogs were stocked at 100 frogs/m^2, in 10cm of water and fed twice a day (morning and evening) on catfish pellets. Antibiotics were used on the farm from the beginning, drugs used included oxytetracycline (OTC) and an undefined general frog medicine. A survey carried out by staff at the Aquatic Animal Health Research Institute (AAHRI) in Bangkok, the previous year, revealed the presence of bacterial isolates from this farm which were resistant to all antibiotics used in Thai aquaculture. In 1993, during the rainy season, the farmer reported frequent outbreaks of disease on the farm, which he found difficult to treat.

2.3.1.2 Farm 2. Nakon Ratchasima

This farm was established in August 1992 and had been in production for two years at the time of the visit. Tadpoles had been brought from a neighbouring farm and local broodstock were caught from the wild. After the first year the farmer selected broodstock animals from his own stock. He found it easy to breed frogs but difficult to maintain them. Both *R. catesbeiana* and *R. rugulosa* were reared on the farm. The stocking density for the frogs was generally 80/m^2 or if there were no disease problems 100/m^2. The water supplying the ponds was collected from two reservoir ponds which were limed regularly. Frequency of water changes depended on the feeding regime. When the frogs were fed twice daily the water was changed once

every 3-4 days, when the frogs were fed once per day the water was changed every 7-10 days. About 70% of the water was changed depending on the feeding levels (100% changes stressed the frogs). The waste water from the frogs drained into a lily pond from which lotus blossoms were harvested. The farmer admitted that the frogs suffered frequent disease problems which he described as oedema of the leg muscles and abdomen and also gas in the abdomen. The number of frogs with oedema varied from 10-50 per pond, per day. When the incidence of disease increased the farmer coated the frog pellets with a preparation called Frog 100, if that did not work he used Frog 200. The farmer did not know what was in this commercial preparation and no details were given on the container. The drug did not give complete recovery but the farmer reported that if diseased frogs were thrown into an adjacent canal, where water quality was much superior to the ponds, they appeared to recover within 48 hours. Approximately 3 OTC treatments were used in the 6 month cycle from egg to market in response to signs of disease. OTC was usually administered at a rate of 3-5g/kg of feed/day for 7-14 days. Formalin was used to remove ectoparasites and piperazine was administered to remove intestinal worms. Potassium permanganate was applied to the ponds prophylactically to improve water quality. The farmer judged the dose of potassium permanganate by the colour of the water. Sodium chloride treatments were also used for minor bacterial problems.

2.3.1.3 Farm 3. Phet Buri

In 1993 many frog farms in this area suffered from disease outbreaks and were forced to close down. In 1994 there were fewer farms, frog stocking densities were kept lower, and consequently disease outbreaks were reduced. On this farm stocking densities were maintained at 100 adult frogs/m^2. In 1993 the farmer treated disease

problems with OTC, in 1994 he tried treatments of salt (sodium chloride) and frequent water changes as alternatives to antibiotic therapy. Following this strategy at the first sign of disease problems the farmer changed the water and added 2% salt. Only two thirds of the water was changed each time to avoid stressing health compromised animals. Water changes were repeated on the following two days if clinical signs persisted. The farmer felt that this regime was more effective than the OTC treatments he had used the previous year. He was of the opinion that generally in 1993 disease was much more prevalent than in 1994, however there were no written records to support this statement. The farmer at the same time stated that he found it difficult to control red-leg. On his farm 3-5 frogs died per day per pond. The frogs showed symptoms of leg oedema, ascites and also haemorrhages on thighs. The farmer often found that frogs with ulcers had abscesses in their livers. He had kept many of the mortalities from the last septicaemic episode in a deep freeze which when examined revealed large abscesses in the livers. These were not sampled as histological examination of the tissue would have been impossible and it would have been difficult to assess the relevance of bacterial cultures from mortalities which had been kept frozen for several weeks.

2.3.1.4 Farm 4. Pathum Thani

It was difficult to obtain an accurate history for this site as the farmer was not present and his wife was not sure of the details. The farm was 3 years old, stock was originally collected from the paddy fields and then bred on the farm. The frogs were all fed on pellets and the pond water was part exchanged each day. Antibiotics were used prophylactically when ponds were completely drained i.e. for grading or transfer. On average 2-3 dead frogs were found in each pond daily but mortalities had been

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much higher the previous month. Red-leg symptoms were reported occasionally. When signs of red-leg first appeared salt treatments were applied. Antibiotics were also used which were sometimes effective and sometimes ineffective.

2.3.1.5 Farm 5. Chon Buri

History was very difficult to obtain at this site. The farm had been established for 24 years. The farmer both bred and bought in frogs which were grown on for export. For the first five years no antibiotics were used on the farm, oxytetracycline treatments were then used intermittently. The worst frog health problems were caused by infestation with large numbers of intestinal protozoa, although red-leg was occasionally seen. Frogs were fed on catfish pellets or frog pellets.

2.3.2 RESULTS OF BACTERIAL SURVEY

A wide range of bacteria were isolated from both diseased and clinically normal frogs. This data is summarised in Tables 2.3.2.1 to 2.3.2.10. Bacteria from the following genera were isolated: *Aeromonas*, *Flexibacter*, *Pseudomonas* and many members of the family Enterobacteriaceae. Bacteria isolated in predominately pure cultures from internal organs were considered potential pathogens and subcultured. On plates where mixed growth occurred only dominant colonies were subcultured.

As the survey progressed the isolates to be identified began to reach unmanageable numbers. A decision was therefore made to concentrate on identifying and pursuing similar isolates which appeared in more than one frog on more than one farm. While several bacteria were obtained in pure cultures, the only isolates consistently appearing in the internal organs of diseased animals from different farms were the motile aeromonads. Attention therefore became focused on this genus. A unique isolate with characteristics biochemically identifying it as a motile aeromonad was frequently observed but could not be speciated. This isolate was designated *Aeromonas* unspeciated (Au).

A *Flexibacter* sp. (Flex) was consistently isolated from the skin, and occasionally from the intestine of diseased and clinically normal frogs. This bacterium was frequently isolated in pure cultures from the skin, intestine and all internal organs of frogs which had appeared healthy on the farm but had been stressed by transport to Bangkok.

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Table 2.3.2.1 Farm 1. Clinical signs and bacterial cultures from diseased frogs

Frog	Length (cm)	Clinical Signs	Internal organs	Isolate ID	Skin	Isolate ID	Intestine	Isolate ID
1	13	Died before sampled, no obvious signs or lesions	Most heavy pure cultures. Spleen mixed but dominant growth	Au	Heavy very mixed	NSG	Heavy very mixed	NSG
2	11	Moribund Liver friable and bronzed	Most heavy pure Muscle mixed, dominant growth as other organs but also yellow colonies as on skin	Au Flex from muscle	Heavy almost pure	Flex	Heavy very mixed	NSG
3	5	Ulcer on right leg, small, weak, low body fat, non-feeder	Light mixed	NSG	Light dominant	Flex	Moderate very mixed	NSG
4	7	Ulcer on left hind, normal activity, low body fat.	Very light mixed	Flex	Moderate mixed	NSG	Light mixed	NSG
5	13	Ascitic, ulcers on head, weak, pericarditis and peritonitis, liver enlarged and mottled grey green colour.	Very heavy pure growth	Au	Moderate mixed	Au (from ulcer) Flex	Moderate mixed	NSG
6	6	Very poor frog. Ulcers left hind calf and dorsal surface left hind foot. Weak movement. Loss of digits and one eye. Non-feeder.	Most no growth, liver very mixed similar to skin possible contamination	NSG	Moderate mixed	NSG	Heavy dominant	NSG
7	6	Very thin and weak. Loss of digits and one eye. Skin dark, Stomach empty, intestinal obstruction	Moderate pure growth kidney and muscle, no growth other organs	Au	Moderate mixed	Flex	Moderate mixed	NSG
8	NR	Ascitic. Thin and weak. Abdomen slightly distended. Lower intestine full of gas	Moderate very mixed	Ent Flex	Moderate mixed	Flex	Heavy dominant	Ent
9	5	Thin and weak. Stomach distended with solid material. Liver very pale.	Light pure growth heart, liver, spleen	G-ve rod no ID	Moderate very mixed	NSG	Heavy mixed but discrete growth identifiable	Ah
10	5.5	Thin, liver small and grey. Petechial haemorrhages in left thigh.	Heavy very mixed	NSG	Heavy mixed	NSG	Heavy mixed	NSG

Abbreviations listed in key at the end of table 2.3.10

Table 2.3.2.2 Farm 1. Clinical signs and bacterial cultures from clinically normal frogs

Frog	Length (cm)	Clinical Signs	Internal Organs	Isolate ID	Skin	Isolate ID	Intestine	Isolate ID
1	7.5	NAD	Very light mixed dominant	Flex	Heavy dominant	Flex	Heavy very mixed	NSG
2	7.5	NAD	Moderate pure	Flex	Heavy mixed dominant	Flex	Heavy very mixed	Flex
3	7.5	NAD	Moderate mixed dominant	Flex	Moderate mixed	NSG	Heavy mixed dominant	Flex
4	8	NAD	Most organs no growth but kidney moderate pure	Flex	Heavy mixed dominant	Flex	Moderate mixed	NSG
5	7	NAD	Light pure growth	Flex	Heavy mixed dominant	Flex	Moderate mixed	NSG
6	7	NAD	Heavy pure growth	Flex	Moderate mixed	NSG	Heavy mixed	Ah
7	8	NAD	Most organs no growth liver light mixed	NSG	Heavy dominant	Flex	Heavy mixed	NSG
8	8	NAD	Light mixed	Flex	Heavy very mixed	NSG	No growth	-
9	8.5	NAD	Moderate mixed	Flex	Heavy mixed dominant	Flex	Heavy mixed	NSG
10	8	NAD	Most no growth muscle, kidney light mixed	Flex	Heavy dominant	Flex	Heavy mixed	NSG

Abbreviations listed in key at the end of table 2.3.10

Table 2.3.2.3. Farm 2. Clinical signs and bacterial cultures from diseased frogs

Frog	Length (cm)	Clinical Signs	Internal organs	Isolate ID	Skin	Isolate ID	Intestine	Isolate ID
1	10	Ulcer upper left snout and jaw. Thin and weak. Yellow liver.	No growth	-	Moderate very mixed	NSG	Heavy very mixed	NSG
2	9	Ascites, weak. Body fat OK, feeding OK.	No growth	-	Moderate very mixed	NSG	Moderate very mixed	NSG
3	7	Moribund, liver very enlarged but normal colour other wise no abnormalities	Heavy pure	Au	Heavy mixed	Au Flex	Heavy very mixed	NSG
4	10	Moribund, ascites, liver enlarged and engorged with blood, stomach swollen, red flush on thighs	Moderate pure	Au ·	Heavy very mixed	NSG	Heavy dominant	As
5	9.5	Thin and weak, pale liver, non-feeder.	Moderate mixed	NSG	Heavy very mixed	NSG	Heavy very mixed	NSG
6	8.5	Thin and weak, ascites, liver pale grey, feeding OK.	No growth	-	Heavy mixed	NSG	Heavy very mixed	NSG
7	9.5	Thin and weak, ascites, rectal prolapse, non-feeder	Heavy pure	Au	Moderate dominant	Aer sp.	Heavy dominant	Ah
8	7.5	Thin and weak, ulcer right inner thigh, pale mottled liver	Light very mixed	NSG	Moderate dominant	As	Heavy dominant	NSG
9	9	Thin and weak, pale grey liver	Light very mixed	NSG	Moderate very mixed	NSG	Moderate very mixed	NSG
10	10.5	Thin and weak red flush on thighs	Heavy pure	Au	Moderate very mixed	Flex	Moderate very mixed	NSG

Abbreviations listed in key at the end of table 2.3.10

Table 2.3.2.4 Farm 2 Clinical signs and bacterial cultures from clinically normal frogs

Frog	Length (cm)	Clinical Signs	Internal organs	Isolate ID	Skin	Isolate ID	Intestine	Isolate ID
1 (F)	9	NAD	No growth	-	Light mixed	NSG	Heavy dominant	NSG
2 (F)	10	NAD	No growth	-	Moderate mixed	NSG	Moderate mixed	NSG
3	8.5	NAD	Moderate mixed	Flex	Heavy mixed	Flex	Light mixed	As
4	9	NAD except slight red flush on thighs	Light mixed	Flex	Moderate mixed	NSG	Moderate dominant	As
5	10	Liver bright yellow, lots of abdominal fat	Light mixed	NSG	Moderate very mixed	NSG	Moderate very mixed	NSG
6	8.5	Liver green/brown colour	Very light mixed	NSG	Heavy mixed	Flex	Light mixed	NSG
7	8.5	Red flush on thighs but very active	Moderate mixed	Flex	Heavy mixed dominant	As	Moderate mixed	NSG
8	9	Liver pale, haemorrhages on surface and in kidney	No growth except liver very mixed	NSG	Heavy dominant	Flex	Moderate mixed	NSG
9	8	NAD	Light dominant	Flex	Heavy mixed but discrete growth identifiable	As	Heavy mixed	NSG

Abbreviations listed in key at the end of table 2.3.10
One frog escaped from the holding tank therefore only nine animals were sampled

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Table 2.3.2.5 Farm 3. Clinical signs and bacterial cultures from diseased frogs and

metamorphosing tadpoles.

Frog	Length (cm)	Clinical Signs	Internal organs	Isolate ID	Skin	Isolate ID	Intestine	Isolate ID
1	9	Ascites, skin dark, flush to inner thighs	Heavy pure growth except liver and heart	G-ve rod no ID	Heavy mixed	Flex	Heavy mixed	NSG
2	1.5	MT no obvious clinical signs but lethargic	Moderate mixed dominant	Ah	Moderate mixed dominant	As	Moderate mixed dominant	Ah
3	2.5	MT ascites	Light mixed	NSG	Heavy mixed dominant	Flex	Not sampled	-
4	2	MT haemorrhages on inner thighs	Heavy mixed	As Ah	Heavy very dominant	A h	Heavy very dominant	As
5	2	MT haemorrhages on inner thighs liver very yellow	Heavy very mixed	NSG	Heavy mixed	Ah	Not sampled	-
6	2	MT good body fat, eating OK, liver very red all previous tadpoles had pale yellow livers	Heavy mixed	Ah	Heavy mixed	Ah	Moderate very mixed	NSG
7	2	MT subcutaneous oedema both thighs very small pale yellow liver	Moderate very mixed	NSG	Moderate very mixed	NSG	Moderate very mixed	NSG
8	9.5	Ascites, good body fat but petechial haemorrhages all organs, weak, red flush to thighs	Light mixed	NSG	Moderate mixed dominant	NSG	Light mixed	NSG
9	10.5	Lethargic, small abscess in liver, organ very congested with large splash haemorrhages on surface	Very heavy pure	Au	Heavy mixed dominant	NSG	Light mixed	NSG
10	2	MT no obvious clinical signs but lethargic	Light mixed	NSG	Moderate mixed dominant	As Ah	Light mixed	NSG

Abbreviations listed in key at the end of table 2.3.10
The majority of animals exhibiting signs of disease on this farm were metamorphosing tadpoles (MT) therefore a selection of these were sampled in addition to three diseased adult frogs. The tadpoles were very much smaller than the animals normally sampled and as a consequence it was difficult to obtain samples of internal organs aseptically.

Table 2.3.2.6 Farm 3. Clinical signs and bacterial cultures from clinically normal

frogs.

Frog	Length (cm)	Clinical Signs	Internal organs	Isolate ID	Skin	Isolate ID	Intestine	Isolate ID
1(F)	10.5	Liver marbled, deep red but fern like white pattern	Very light mixed	NSG	Heavy very mixed	Flex	Very light mixed	NSG
2(F)	9.5	Liver pale, several fibrous tracks	No growth	-	Moderate mixed	NSG	Light mixed	NSG
3	10	Lively, feeding well but liver grey colour	No growth	-	Very light pure	NSG	Light mixed	NSG
4	10	NAD	No growth except heart very light mixed	NSG	Very light mixed	NSG	Light mixed	NSG
5	9	Lively but liver mottled and grey	No growth	-	Heavy very mixed	NSG	Heavy mixed	NSG
6	9.5	Lively but liver mottled and pale	No growth	-	Moderate mixed	As	No growth	-
7	9	NAD	No growth	-	Moderate mixed	NSG	Moderate mixed	As
8	10	Lively but liver small grey/green colour	No growth except liver very light pure	NSG	Moderate mixed	NSG	Moderate mixed	NSG
9	9	Lively but some fluid in abdomen	No growth	NSG	Heavy mixed	NSG	No growth	-
10	10	NAD	No growth except muscle very light mixed	NSG	Moderate mixed dominant	NSG	Moderate mixed	As

Abbreviations listed in key at the end of table 2.3.10

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Table 2.3.2.7 Farm 4 Clinical signs and bacterial cultures from diseased frogs.

Frog	Length (cm)	Clinical Signs	Internal organs	Isolate ID	Skin	Isolate ID	Intestine	Isolate ID
1	11.5	Lively but severe ulcers on hind feet	No growth	-	Moderate mixed	NSG	No growth	-
2	10.5	Moribund pale pink flush to thighs	Moderate pure growth	Au	Heavy mixed dominant	As dom Flex	Heavy mixed dominant	NSG
3	10.5	Lively but severe ulcers on hind feet	No growth	-	No growth	-	Moderate mixed	NSG
4	9.5	Severe ulcers right hind	No growth	-	Moderate mixed	As	Moderate mixed	As
5	10	Severe ulcers hind feet, liver small dark and green less body fat than other frogs	No growth	-	Moderate mixed dominant	As dom Flex	No growth	-
6	10	Severe ulcers on hind feet	Light mixed	Citrob acter	Heavy mixed dominant	NSG	Light mixed	NSG
7	10	Severe ulcers on hind feet	No growth except kidney light mixed	NSG	Moderate mixed	As	Light mixed	NSG
8	NR	Abscess in right hip joint and muscle. Thigh bone eroded at top, either fracture or dislocation	Heavy mixed most organs but abscess and liver pure growth small white colonies	g+ve coccus no ID	Moderate very mixed	NSG	Moderate almost pure	Alc?

Abbreviations listed in key at the end of table 2.3.10
In error only eight diseased animals were sampled on this farm.

Table 2.3.2.8 Farm 4 Clinical signs and bacterial cultures from clinically normal frogs

Frog	Length (cm)	Clinical Signs	Internal organs	Isolate ID	Skin	Isolate ID	Intestine	Isolate ID
1(F)	12	Liver very yellow and possibly enlarged, lots of body fat	No growth except liver one colony	NSG	Moderate very mixed	NSG	No growth	-
2(F)	10.5	Liver bronzed and enlarged	No growth except kidney very light mixed	NSG	Moderate very mixed	NSG	No growth	-
3	9.5	Ulcers on end of hind digits	No growth	-	Heavy mixed but discrete growth identifiable	Flex	Heavy mixed	Alc?
4	10.5	Some fluid in abdomen	No growth	-	Heavy mixed dominant	Flex	Heavy mixed	NSG
5	11.5	Liver bronzed with fibrous tracts on surface. White raised plaques on surface of small intestine	No growth except kidney light pure	Flex	Heavy mixed	Flex	Light dominant	NSG
6	10.5	Liver mottled one obvious fibrous tract	No growth except muscle light mixed	NSG	Moderate mixed	Flex	Light mixed	NSG
7	10	Thin non-feeder, ulcers on jaw and feet	Liver and kidney heavy pure, muscle and spleen light pure, heart heavy dominant	Ent sp.	Heavy mixed	Flex	Moderate mixed	NSG
8	12	Lethargic, fly strike on abdomen, ascites bronzed liver.	Light mixed	Flex	Very heavy mixed	NSG	Light mixed	NSG
9	12	Lethargic gas in intestine, haemorrhages on wall of intestine, ascites.	Light mixed dominant	Flex	Very heavy very mixed	Flex	light mixed	NSG
10	12	Lethargic, ascites.	Heavy mixed except heart and spleen no growth	Flex	Heavy mixed	Flex	Moderate mixed	Flex

Abbreviations listed in key at the end of table 2.3.10

Table 2.3.2.9 Farm 5 Clinical signs and bacterial cultures from diseased frogs

Frog	Length (cm)	Clinical Signs	Internal organs	Isolate ID	Skin	Isolate ID	Intestine	Isolate ID
1	11.5	Severe ulcers on head left fore arm and hind leg, liver grey and mottled	No growth	-	Heavy mixed	NSG	Light mixed	NSG
2	12	Severe ulcer over right eye and on right foot, liver grey and mottled	Light mixed	NSG	Moderate mixed	NSG	Light mixed	NSG
3	9.5	Thin, small, liver very dark	No growth	-	Moderate mixed	NSG	Light mixed	NSG
4	11	Severe ulcers on head	No growth except liver 2 colonies and spleen 1, mixed	NSG	Moderate mixed	NSG	Light mixed	NSG
5	14	Severe ulcers around mouth and on hind feet, liver bright yellow and mottled	No growth	NSG	Moderate mixed	NSG	Moderate mixed	NSG
6	11.5	Severe ulcer on head and left hind foot, liver green/yellow and mottled	Light mixed	NSG	Light mixed	NSG	Light mixed	NSG
7	12	Severe ulcers on head, liver very yellow	No growth except liver and spleen light mixed	NSG	Light mixed	NSG	No growth	-
8	12.5	Severe ulcer around right eye, liver grey and shrunken	No growth except liver 1 colony	NSG	Heavy dominant	NSG	Light mixed	NSG
9	13	Severe ulcers on head and feet, liver very yellow and mottled	No growth	NSG	Heavy dominant	NSG	Moderate mixed	NSG
10	9.5	Small, thin, severe ulcers hind feet, liver small dark and mottled	Heavy pure growth except muscle	G-ve rod no ID	Moderate mixed	NSG	Light mixed	NSG

Abbreviations listed in key at the end of table 2.3.10

Table 2.3.2.10 Farm 5. Clinical signs and bacterial cultures from clinically normal

frogs

Frog	Length (cm)	Clinical Signs	Internal organs	Isolate ID	Skin	Isolate ID	Intestine	Isolate ID
1	12	NAD	No growth	-	Moderate mixed	NSG	Moderate dominant	NSG
2	12	NAD	No growth	-	Moderate mixed	NSG	Moderate dominant	NSG
3	11.5	NAD	No growth	-	Heavy almost pure	NSG	No growth	-
4	12	NAD	No growth	-	Heavy mixed	NSG	No growth	-
5	12.5	NAD	No growth	-	Heavy mixed	As	Light mixed	NSG
6	13	NAD	No growth	-	Moderate mixed	NSG	1 small white colony	G+ve coccus
7	11	Very swollen abdomen, ulcer on left inner thigh, intestine and oesophagus distended with gas and fluid, muscle haemorrhages in area of ulcer	Liver and spleen 1 small white colony each, heart no growth, kidney light mixed but small white colonies frequent, muscle very heavy mixed, dominant	G+ve coccus Au (muscle only)	Very heavy mixed	NSG	Light mixed small white colonies frequent	G+ve coccus
8	13	Ulcers on face and feet	No growth	-	Moderate mixed	NSG	Heavy mixed	As
9	11.5	Moribund, ulcers on feet	No growth	-	Moderate very mixed	NSG	Light mixed	NSG
10	12	Dead	Heavy pure growth liver and kidney, heart dominant, muscle no growth	G-ve rod no ID	Heavy mixed	Flex	Heavy mixed	G+ve coccus

Alc = *Alcaligenes*
Ah = *Aeromonas hydrophila*
As = *Aeromonas sobria*
Au = unspeciated Aeromonad
dom = dominant growth
Ent = Enterobacteria
(F) = clinically normal frog sampled on farm
Flex = *Flexibacter* sp.
G+ve = Gram positive
G-ve = Gram negative
ID = identification
MT = metamorphosing tadpole
NAD = no physical abnormality diagnosed
NR = not recorded
NSG = no significant growth from culture plate

2.3.3 PRELIMINARY TESTS OF BACTERIAL PATHOGENICITY

The results of the bacterial survey indicated two isolates which were potentially pathogenic for frogs: the *Flexibacter* and the unspeciated aeromonad. Two pilot virulence studies were arranged in collaboration with AAHRI staff to assess the pathogenicity of the selected bacterial isolates. Death of any frog within 2 weeks of challenge was considered indicative of pathogenicity of the individual isolates. Pathogencity was confirmed at a later date in larger scale challenges described in detail in Chapter 7.

2.3.3.1 *Flexibacter* sp.

The protocol for bacterial challenges is described in detail in Chapter 7. The pilot studies were based on this protocol however viable counts were not carried out as this was a very small scale study to determine the potential virulence of the isolate for frogs. A suspension of an isolate from the liver of a septicaemic frog was prepared with an optical density of 1 at 610 nm. Three frogs were injected intramuscularly at a dose rate of 0.1ml/100g body weight. The experimental frogs showed no sign of ill health over a three week period. The frogs were kept under optimal conditions at very low stocking densities i.e. three animals per 25l tank..

2.3.3.2 Unspeciated Aeromonad

Three groups of three frogs were placed in separate aquaria under optimal conditions. Three suspensions of the challenge organism were prepared with optical densities of 1, 0.5 and 0.1 at 610nm (following the protocol given in Chapter 7 but again viable

counts were not performed). Each group of frogs received intraperitoneal injections of one of the suspensions at a dose rate of 0.1ml suspension/100g body weight. The trial was terminated after 24 hours as 6 of the animals had died and the remaining 3 were moribund, see table 2.3.3.

Table 2.3.3 Clinical signs and bacterial cultures recovered from frogs challenged with unspeciated Aeromonad.

	Clinical signs	Liver	Isolate ID	Kidney	Isolate ID	Spleen	Isolate ID
OD 1							
Frog 1	Dead	Heavy pure	Au	Heavy pure		Heavy pure	
Frog 2	Dead	Heavy pure		Heavy pure	Au	Heavy pure	
Frog 3	Dead	Heavy pure		Heavy pure		Heavy pure	Au
OD 0.5							
Frog 1	Dead	Heavy pure		Heavy pure	No ID	Heavy pure	
Frog 2	Dead	Heavy pure		Heavy pure		Heavy pure	No ID
Frog 3	Moribund, peritonitis, fat adherent to abdominal wall at injection site, haemorrhages in thigh muscle, peritoneum and liver	Moderate pure	No ID	Moderate dominant		Light pure	
OD 0.1							
Frog 1	Dead	Heavy pure	No ID	Heavy pure		Heavy pure	
Frog 2	Moribund, Haemorrhages in thigh muscle.	Very light mixed		Moderate dominant	Au	3 cols Flex	
Frog 3	Moribund, Ascites +++.	No growth		No growth		1 col Flex	

Identification of bacterial isolates with the API 20 E system revealed that Au was isolated from all of the High dose group (OD 1) and from one frog in the low dose

group (OD 0.1). The remaining cultures could not be identified by these limited

biochemical tests but as this was a pilot study no further tests were undertaken.

2.4 DISCUSSION

The frog farms visited in this study were all of comparable size. Most were self

contained units maintaining broodstock, tadpoles and growout animals on the farm,

only farm 5 bought in post-metamorphic frogs to grow on. All the businesses were

family owned and provided a valuable source of income in addition to more

traditional farming concerns.

Farmers 1 and 2 reported frequent outbreaks of epizootic disease which were difficult

to treat. On both these farms an aeromonad which proved difficult to identify (Au)

was isolated in heavy pure cultures from the internal organs of four animals. Farm 3

had also suffered from severe disease problems the previous year, indeed most farms

in the area had been affected and some had been forced to close down. Farmer 3 felt

that his new management strategy of frequent water changes and salt treatments had

improved the general frog health but he did admit that the frogs still suffered from

epizootic disease. He had a freezer container full of dead animals from a recent

outbreak, many of which had abscesses in their livers, a problem which often occurred

in the frogs on this farm.

At the time of the visit to farm 3 the post metamorphic stock appeared quite healthy,

and the farmer could only supply three adult frogs to sample, the remaining diseased

animals were all metamorphosing tadpoles. These yielded several aeromonads from

the internal organs but all cultures were mixed. This may have been due to the fact

that the animals were much smaller than the frogs usually sampled so aseptic

procedures were more difficult. Heavy pure cultures of Au were found from only one

postmetamorphic animal, however that frog had an abscess in its liver similar to those seen in the frozen animals.

On farm 4, which reported red-leg symptoms occasionally, Au was isolated in pure growth from the internal organs of one frog. Other animals, which were suffering from severe ulceration, revealed very little growth from internal organs. The level of daily mortalities were similar on farm 3 and farm 4 i.e. 2-3 per pond and 3-5 per pond respectively as opposed to 10 -50 per pond reported by farmer 2. Both farm 3 and 4 were employing frequent water changes whereas farm 2 was part exchanging the water at most once every three days.

Farmer 2 had noted that there appeared to be an environmental component to the aetiology of septicaemic disease. When he found frogs showing signs of lethargy or oedema he flung them into the supply canal, where the water quality was much superior to the ponds, and the frogs appeared to recover. Although removal of diseased animals from the rest of the crop was a sensible procedure, disposing of them in a supply canal that may be shared by other farmers shows a worrying lack of concern for disease control and may indicate a more relaxed management style than on the other farms. This in turn could explain the higher rates of mortality accepted by the farmer and the greater numbers of septicaemic animals from which potentially pathogenic bacteria were isolated.

Details on water management were not available for farm 1 however strains of bacteria which were resistant to all the antibiotics used in Thai aquaculture had been isolated from frogs on the farm by staff at AAHRI the previous year. This suggests

that a variety of antibiotics had been employed on the farm and calls into question

their management practices.

It is interesting that red-leg was not considered a problem by farmer 5, this farmer was

more concerned with protozoal infections in the frog intestine. Heavy pure bacterial

growth was only obtained from the internal organs of a single diseased animal and this

was not an aeromonad. However an isolate of Au was found in mixed culture from the

muscle of one of the apparently normal frogs which was transported to Bangkok and

subsequently developed signs of septicaemia. It is difficult to comment on the

significance of this isolate. Although growth was heavy from the muscle and the

aeromonad was the dominant culture, it was not isolated from any of the other organs,

which produced only light mixed growth. On all the other farms where red-leg had

been a problem, animals were found with pure cultures of aeromonads from the

internal organs and on two of the farms where management practices were

questionable more than one animal was affected.

It should be noted that some of the animals from which Au was isolated displayed

very few external clinical signs. These frogs were frequently large and fat, the

presenting symptom was lethargy rather than ulceration or reddening of the legs. The

animals which were sampled because they were severely ulcerated revealed light

mixed or no growth at all in the internal organs. This suggests that although damaged

skin may facilitate the invasion of the frog by pathogenic bacteria other factors are

also involved.

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The normal frogs produced very different flora to the diseased animals. The

Flexibacter was present systemically in every normal frog sampled from farm 1. This

may be due to the fact that they were transported to AAHRI and held in a tank, for

over 24 hours before sampling, the stress may therefore have precipitated subclinical

infections. On subsequent field trips two normal frogs were sampled on the farm and

the remaining frogs were transported to Bangkok. The cultures from the internal

organs of the two frogs sampled on the farm were not markedly different from the

majority of those sampled after transport, some yielded light mixed growth from the

internal organs while in others there was no growth at all. However none of the

animals sampled on the farm showed signs of septicaemia nor did bacterial samples

produce pure or dominant growth.

In contrast although most of the frogs which were transported looked healthy and were

extremely active, some animals developed signs of septicaemia the following day.

One clinically normal animal from farm 5 died overnight after transport. Heavy pure

bacterial cultures were isolated from the liver and kidney but the organism could not

be identified using first stage tests and API strips.

Problems of decreased survival after shipment have been recorded by several authors

(Miles, 1950, Gibbs, 1963, 1973, Hird *et al.,* 1983,). Deaths and clinically apparent

disease presumably occur because host resistance is impaired to potentially pathogenic

bacteria. Miles (1950) reported a uniformly fatal attack of red-leg in tree frogs (*Hyla*

arborea) imported into England from Italy. The animals were packed in a very

crowded container and soon after arrival many deaths occurred. Samples of lymph and heart blood yielded profuse, pure cultures of *Bacterium alkaligenes.*

Hird *et al.*, (1983) surveyed the patterns and occurrence of *A. hydrophila* and *Enterobacteriaceae* in frogs and tadpoles in Minnesota. They found *A. hydrophila* and 29 species of *Enterobacteriaceae* isolated from the intestines. They also isolated mixed cultures from the heart blood of eleven frogs. Most of the frogs with isolates in the heart blood were collected in North Dakota, the most distant collection site, suggesting an association between septicaemia and transit time to the laboratory.

Gibbs (1963) describes a study of over three hundred *R. pipiens* obtained from a commercial supplier. Cultures of blood and a variety of tissues from these frogs revealed that two thirds were seriously infected with combinations of *A. hydrophila, Staphylococcus* spp., and *Citrobacter freundii* however many animals showed few gross clinical signs.

Gibbs (1973), in a review of the health status of laboratory frogs, states that there are no adequate means of defining frog health and suggests as a minimum standard that a healthy frog must survive for an extended period under optimal laboratory conditions. He comments that very few laboratory frogs meet this standard and most die within days or weeks of receipt, supporting the theory that transport stress can precipitate bacterial septicaemias.

Classic diagnostic microbiology dictates that bacteria should not be recovered from the internal organs of healthy animals. However in this study mixed cultures were frequently isolated from the internal organs of apparently healthy animals. At the start of this study it was assumed that unless the growth was heavy and one species was dominant these cultures were likely to be contaminants, as sampling conditions in the field and even in the tank room were often difficult. However, as remarked in the introduction, recent work at the Institute of Aquaculture (Maragret Crumlish, unpublished data) has revealed the presence of mixed populations of viable bacteria in the macrophages of apparently healthy frogs. The mixed bacterial populations recovered in surveys of healthy and diseased animals (Gibbs, 1963, Glorioso *et al.,* 1974a, Hird *et al.,* 1981, Hird *et al.,* 1983) support the hypothesis that frogs have a permanent population of bacterial flora. It is possible that the stress of transport compromised the defenses of the animals sufficiently to allow such bacteria to multiply within the internal organs but that these were not primary pathogens and therefore did not possess the virulence factors necessary to produce acute septicaemic disease alone.

The presence of mixed cultures makes it very difficult to determine which organisms are responsible for causing disease. Gibbs (1963), Glorioso *et al.* (1974a), Hird et al. (1981), Hird *et al.* (1983), have all found mixed cultures from the internal organs of septicaemic and clinically normal frogs. Purification by subculture is essential for accurate identification, however deciding which isolates to subculture from a mixed plate is difficult, particularly when there is no dominant growth. Potential pathogens and contaminants encompass a large number of genera. A single plate could yield four

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or more different isolates of possible interest. The numbers of subcultures from each frog can therefore increase exponentially.

The aim of this study was not to provide a comprehensive description of frog bacterial flora but rather to identify bacteria which kill frogs, establish whether those bacteria were part of the flora of clinically normal frogs, and select a virulent isolate for further analysis. Therefore criteria for selecting potential pathogens from mixed plates had to be established. The presence of a particular colony type in more than one organ of an animal, and colonies which produced dominant or pure heavy growth from one or more organs were selected as potential pathogens. Colonies from the skin and intestine which resembled potentially pathogenic isolates from other samples were also subcultured.

The organisms which grew most frequently in pure or dominant cultures and were isolated from more than one frog on more than one farm were the motile aeromonads. In particular the group of unspeciated aeromonads appeared to be associated with septicaemic disease. The pilot virulence studies supported this finding, as all the challenge animals were either dead or moribund with signs of severe septicaemia within 24 hours of exposure.

Some of the isolates from mixed plates may also be important secondary pathogens. In particular the *Flexibacter* sp. appeared to invade stressed animals although it was part of the normal skin bacterial flora. However the results from the pilot virulence studies

did not indicate the pathogenic potential displayed by the unspeciated aeromonad. In fact the challenge frogs conformed to the criteria for good health suggested by Gibbs (1973) in that they survived an extended period in the laboratory under optimal conditions. It is probable that the *Flexibacter* sp. is only pathogenic to stressed animals under poor environmental conditions.

Further study of these isolates to assess their virulence potential would be interesting. However this project did not have the resources for an in depth investigation of many different bacterial genera. The results of the pilot virulence studies and the fact that the *Flexibacter* sp. was not isolated from diseased animals on the farm suggested that it was of less significance as a primary cause of septicaemic disease in farmed frogs than the motile aeromonads.

The motile aeromonads were therefore selected for further investigation. Members of this genus cause disease in many farmed aquatic species and have often been reported as the cause of red-leg in laboratory and wild populations of frogs. The frequent isolation of the unspeciated Aeromonad was of particular interest and is discussed in much greater detail in the following chapters.

Chapter 3. Histological Studies

3.1 INTRODUCTION

3.1.1 NORMAL HISTOLOGY

It is important to understand the normal structure and function of animal tissue before trying to interpret pathological changes. The normal histology of man and other mammals has been studied since early in the nineteenth century and is well described in many standard texts (Junqueira *et al.,* 1992; Ross *et al.,* 1995; Wheater *et al.,* 1990). In recent years, owing to the increasing economic importance of farmed fish, many texts on normal histology of various fish species have been published (Anderson and Mitchum, 1974; Chinabut *et al.,* 1991; Groman 1982; Hibiya, 1982). Descriptions of amphibian histology appear in comparative texts (Leake 1975, Welsch and Storch, 1976), but there are no complete studies on the microscopic stucture and function of farmed frog tissues. However the general principles of vertebrate histology apply to amphibians, therefore knowledge of mammalian and fish histology provides a useful background. Brief summaries of the normal histology of organs most likely to be affected by bacterial septicaemias i.e. well vascularised structures, and possible portals of entry for bacteria, the skin and the small intestine, are presented in this section. These are based on descriptions of amphibian anatomy from comparative texts and supported by details from the more familar teleost and mammalian structures.

3.1.1.1 Liver

The liver of the adult frog is a bi-lobed organ, located in the anterior part of the abdominal cavity (Leake, 1975). Vertebrate liver functions include; gathering,

transforming and accumulating metabolites and neutralizing and eliminating toxic

substances (Junqueira *et al.,* 1992). The organ is unusual in that it has a dual blood

supply: the portal vein carries oxygen-poor nutrient-rich blood from the abdominal

viscera; and the hepatic artery which supplies oxygen-rich blood (Junqueira *et al.,*

1992). The portal system branches within the liver, and eventually divides into long

wide blood capillaries known as sinusoids (Hibiya, 1992). The liver cells

(hepatocytes) are concentrically arranged around the sinusoids and are seperated from

the blood by a thin epithelium. After the blood has passed through the liver

parenchyma it is collected by the hepatic vein and transported to the heart.

The histological structure of the frog liver is similar to that of the teleost fish (Leake,

1975), which differs from the mammalian in that the hepatocytes are not organised

into well defined lobules (Roberts, 1989). However the basic cell structure is similar

for all vertebrates. Hepatocytes are large, polyhedral cells containing a spherical

nucleus which may be centrally located or peripheral depending on the amount of fat

and glycogen stored. The nucleus contains densely staining chromatin along the

nuclear membrane and a prominent nucleolus (Wheater *et al.,* 1990). In sections

stained with haematoxylin and eosin (H&E), the cytoplasm of the hepatocytes is

eosinophilic, mainly because of the presence of large numbers of mitochondira and

some smooth endoplasmic reticulum (Junqueira *et al.,* 1992). In well-nourished

individuals, hepatocytes store significant quantities of glycogen and process large

quantities of lipid; both these metabolites are partially removed during routine

histological preparation thereby leaving irregular, unstained areas within the

cytoplasm (Wheater *et al.,* 1990). In farmed fish in particular, cytoplasmic appearance

can be variable depending on nutritional status. Hepatocytes are often swollen with

glycogen or neutral fat even when nutrition is marginally less than ideal and during cyclical starvation phases the cells may be shrunken and the entire liver loaded with yellow ceroid pigments (Roberts 1989).

Bile secretion is the main digestive function of the liver. Bile is synthesised by all hepatocytes and secreted into a system of minute canaliculi which form an anastomosing network between the hepatocytes (Wheater *et al.*, 1990). The canaliculi are tubular spaces formed by the plasma membranes of two hepatocytes and have no discrete structure of their own (Junqueira *et al.*, 1992). Intrahepatic bile ducts collect bile from the bile cannaliculi, these are lined by cuboidal or columnar epithelium and have a distinctive connective tissue sheath (Junqueira *et al.*, 1992). They gradually enlarge and fuse to form the common bile duct. This in turn opens into the gall bladder which is a large spherical sac situated on the underside of the liver. The bile is stored and concentrated before being returned to the common bile duct which delivers bile to the intestine.

Teleost livers differ from mammalian in that haemopoeitic tissue, complete with melanomacrophage centres (MMC), is found in varying amounts around the larger vessels of the liver. The melanomacrophage centres contain specialised cells with phagocytic activity which store tissue breakdown products in the form of pigments such as ceroid. Large deposits of melanin are also prominent within these phagocytyic centres (Roberts 1989).

3.1.1.2 Kidneys

In adult frogs the kidneys are the chief excretory organs (Leake, 1975). The paired
kidneys are compact, light brown organs which unlike the fish kidney are relatively
mobile within the abdominal cavity.

The nephron is the main functional and structural unit of the vertebrate kidney. Each
nephron consists of a dilated portion, the renal corpuscle, and the renal tubule which
connects the corpuscle with the collecting ducts (Welsch and Storch, 1976).

The renal corpuscle is composed of a tuft of capillaries, the glomerulus, surrounded by
a two layered capsule formed from the closed end of the renal tubule (Junqueira *et al.,*
1992). Despite their adaptation to the terrestrial environment, amphibians have
retained a fish-type nephron in which a large glomerulus filters a considerable amount
of water without much tubular reabsorption (Hourdry, 1993). The amphibian renal
tubule is divided into five sections: neck piece, proximal tubule, transitory piece,
distal tubule and connecting piece (Welsch and Storch, 1976). It therefore lacks the
loop of Henle portion which is responsible for the formation of hypertonic urine.

The neck piece is short, narrow and lined by ciliated cuboidal epithelium (Leake
1975). The proximal tubule is lined by simple cuboidal or columnar epithelium with a
thick brush border. The transitory piece of ciliated cuboidal epithelium leads into the
distal tubule which is lined by low unciliated columnar epithelium (Leake 1975). The
connecting piece is poorly defined and more or less forms part of the terminal part of
the distal tubule (Welsch and Storch, 1976). Urine passes to collecting tubules that
join each other to form larger straight collecting ducts. The collecting ducts drain by

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paired nephric ducts into a urinary bladder which develops as a pocket in the cloacal wall (Leake 1975).

In histological sections of mammalian kidneys the distinction between the proximal and distal tubules is based on the following characteristics. Cells of proximal tubules are larger than the cells of the distal tubules; they have brush borders which distal cells lack, are more acidophilic due to an abundance of mitochondria and possess numerous apical canaliculi and pinocytic vesicles. The lumens of the distal tubules are larger and the epithelial cells are flatter and smaller than those of the proximal tubule, therefore more cells and more nuclei are seen in the distal tubule wall than in the proximal tubule wall in the same histological section (Junqueira *et al.*, 1992).

The mammalian kidney can be divided into an outer cortex and an inner medulla. The cortex contains the glomerulus and the proximal and distal convoluted tubules. The medulla contains part of the renal tubules known as the loop of Henle (Junquiera *et al.*, 1992). The kidney of the teleost fish is a more mixed organ comprising haemopoietic, reticuloendothelial, endocrine (chromaffin cells and inter-renal tissue) and excretory elements (Roberts 1989). The excretory portion of the fish kidney is not divided into discrete areas, the glomeruli and tubules are distributed throughout the interstitium.

Like the mammalian kidney the main function of the frog kidney is excretory. Adult frogs excrete chiefly urea. A copious urine flow is maintained to prevent hydration of the body but mechanisms for reabsorption of water in the kidney tubules, cloaca and large intestine allow adjustment of amounts of fluid excreted. Chromaffin tissue and

inter-renal tissue are normally associated in amphibians into a distinct adrenal gland close to the anterior end of the kidney (Leake 1975).

3.1.1.3 Spleen

The spleen is a large lymphoid organ, dark red in colour. It is the largest accumulation of lymphoid tissue in the vertebrates (Leake 1975). Chief functions of the mammalian spleen are: removal of debris and other particulate matter from circulating blood; production of immunological responses against blood borne antigens; removal of aged or defective blood cells from the circulation (Wheater *et al.*, 1990). The amphibian spleen fulfills all these functions and is also the primary site of erythrocyte production (Leake 1975).

In mammals the spleen has a dense, fibro-elastic outer capsule giving rise to supporting connective tissue trabeculae which conduct larger blood vessels throughout the spleen. In some mammals the capsule and trabeculae contain smooth muscle and are contractile allowing for the rapid expulsion of red blood cells from the spleen reservoir (Wheater *et al.*, 1990). In teleosts the splenic capsule is fibrous, devoid of muscle and does not have the dense trabeculae extending into the tissue (Roberts, 1989).

The main components of the vertebrate spleen other than the capsule and trabeculae are the ellipsoids and the splenic pulp. The splenic pulp is divided into white pulp and red pulp based on the colour seen in a fresh section. The white pulp consists of lymphatic tissue, mostly lymphocytes. In H &E stained sections the white pulp appears as basophilic areas due to the presence of dense heterochromatin in the nuclei

of the numerous lymphocytes (Ross *et al.*, 1995). In the mammalian spleen the lymphocyte-rich white pulp is clearly distinguishable from the erythrocyte-rich red pulp. Although the teleost spleen has lymphocyte-rich and erythrocyte-rich portions, the lymphoid tissue tends to be more diffuse thus the boundary is not clear (Hibiya, 1982).

Essentially the red pulp consists of splenic sinusoids separated by the splenic cords. The splenic cords consist of a loose network of reticular cells and reticular fibres that contain large numbers of erythrocytes, macrophages, lymphocytes, plasma cells and granulocytes. Blood fills both the sinusoids and the cords of the red pulp, often to a degree that obscures the underlying structure and makes it difficult, in histological sections, to distinguish between the cords and the sinusoids (Ross *et al.*, 1995).

Ellipsoids are thick walled filter capillaries which result from the division of splenic arterioles. The splenic artery enters the organ at its hilus, courses through part of the capsule and branches into the trabecular arteries, which are vessels of various sizes that follow the course of the connective tissue trabeculae.When they leave the trabeculae to enter the red pulp, the arteries are immediately surrounded by a sheath of lymphocytes which constitute the white pulp. These vessels are known as central arteries or white pulp arteries. After leaving the white pulp the central artery subdivides to form straight penicillar arterioles. Near their termination some of the penicillar arterioles are surrounded by a sheath of reticular cells and fibres, these are the ellipsoids (Junquiera *et al.*, 1992). The walls of the ellipsoids contain erythrocytes and phagocytic cells and are capable of trapping large quantities of particulate matter from the circulation (Roberts, 1989). The vessels then continue as simple capillaries

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that carry blood to the sinusoids. From the sinusoids blood proceeds to the red pulp veins and eventually flows through trabecular veins into the hepatic vein.

In the teleost spleen MMC occur, and are usually located close to a vessel (Roberts 1989). The MMC are discrete encapsulated structures which serve as "repositories" for the end products of cell breakdown such as phospholipids and also for antigens and other particulate material (Ferguson 1989).

3.1.1.4 Skeletal muscle

In vertebrates skeletal muscle is composed of extremely elongated, cylindrical, multinucleated cells, commonly called muscle fibers and arranged together in parallel array (Wheater *et al.*, 1990, Junqueira *et al.*, 1992). The individual fibers are bound together by connective tissue into bundles which collectively form the whole muscle. Each of these whole muscles, bundles and fibres is surrounded by connective tissue containing collagen fibres. The envelope around the muscle is called the epimysium, that around the bundles the perimysium and that around the fibre the endomysium. In these connective tissue sheaths numerous blood capillaries and nerve fibres occur (Welsch and Storch, 1976). The muscle fibres ordinarily contain several oval nuclei, usually found at the periphery of the cell under the cell membrane. This characteristic nuclear location is helpful in distinguishing skeletal muscle from cardiac muscle which has centrally located nuclei (Junqueira *et al.*, 1992). Regular cross-striations are the characteristic feature of skeletal muscle fibres and can be seen in longitudinal sections. The cross striations result from the arrangement of contractile proteins within the muscle fibres (Wheater *et al.*, 1990).

3.1.1.5 Heart

The adult frog heart possesses a thin walled sinus venosus, paired atria (one receiving blood from the lungs and the other from the body) and a thick walled ventricle. The ventricle wall has numerous trabeculae on its inner surface which probably help to keep separate streams of blood returning from the pulmonary circuit and the body. The first part of the truncus (bulbis cordis) is composed of cardiac muscle and is contractile; the second part comprises the short ventral aorta. The heart including the bulbis but not the ventral aorta is enclosed in a thin pericardium (Leake 1995).

In mammals, teleosts and amphibia the walls of all parts of the heart consist of three basic layers: an internal membrane, an intermediate layer and an external membrane. The endocardium is the internal membrane lining the internal surface of the heart and consists of a single layer of endothelial cells supported by loose connective tissue which accomodates movements of the heart muscle without damage to the endothelium (Wheater *et al.*, 1990). The external membrane is the epicardium, which is composed of a simple flat epithelium and an underlying layer of connective tissue. The intermediate layer is the thickest layer and is composed of muscle in the atrium and ventricle. The atrium wall is thin and contains few muscle fibers. The ventricle wall however is thick and rich in cardiac muscle (Hibiya, 1982). In many teleosts the ventricular muscle is divided into two distinct layers. There is an outer compact layer in addition to an inner spongy component with numerous trabeculae. The thickness of the compact layer is related to scope for activity, being almost absent in less active species such as pleuronectids (Ferguson 1989, Roberts 1989).

In vertebrates cardiac muscle fibres are essentially long, cylindrical cells with one or at most two nuclei, centrally located within the cell. The ends of the fibres are split longitudinally into a small number of branches the ends of which anastamose with similar branches of adjacent cells (Junqueira *et al.*, 1992, Wheater *et al.*, 1990). Cardiac muscle fibres have a similar arrangement of contractile proteins to that of skeletal muscle and are consequently striated in a similar manner. However in routine H&E preparations this is often difficult to visualise in cardiac muscle due to the irregular branching shape of the cells (Wheater *et al.*, 1990). A unique and distinguishing feature of cardiac muscle is the presence of darkly staining transverse lines that cross the chains of cardiac cells at irregular intervals. These intercalated discs represent the site of attachment of a cardiac muscle cell to its neighbours. The junctions may appear as straight lines or may exhibit a steplike pattern (Junqueira *et al.*, 1992). Between the muscle fibres, delicate connective tissue supports an extremely rich capillary network (Wheater *et al.*, 1990).

3.1.1.6 Lungs

Many small animal species do not possess any particular respiratory organs; in them the exchange of gases takes place through the body surface. In larger organisms special respiratory organs develop and integumentary respiration is of reduced importance (Welsch and Storch, 1976). The frog which lives in water or damp terrestrial conditions, still uses its moist, well vascularised skin for respiration and in fact most oxygen uptake is through the skin (Rankin and Davenport, 1981). The lining of the buccal cavity is also used and shows evagination of capillaries into the buccal epithelium. The adult frog also possesses simple paired saccular lungs opening directly into the pharynx. The vascularised epithelial lining of the lungs is smooth in

very young frogs, but as the animal grows older this is thrown into folds (septae). The septae separate infundibula which are lined with thin squamous respiratory epithelium and thus form blind ended alveoli (Leake, 1975).

3.1.1.7 Small Intestine

In the small intestine of vertebrates the processes of digestion are completed and the products of digestion are absorbed. In all vertebrates the surface area of the intestine is increased by the formation of villi in the mucosa (Welsch and Storch, 1976). The epithelial lining of the mucosa is composed of two main cell types. The majority of cells are absorptive and are termed enterocytes. These are tall columnar cells with a basally located nucleus. The presence of extensive microvilli on the surface of each enterocyte further increases the absorptive surface. Mucus producing goblet cells are scattered between the enterocytes. These cells possess a distended apical half which is filled with mucus. A connective tissue layer extends into the core of each villus and contains a rich vascuclar and lymphatic network into which digestive products are absorbed (Wheater *et al.*, 1990).

The small intestine is the principal site of digestion in the adult frog. This shows a greater degree of specialisation and localisation of function than in teleosts. The amphibian small intestine is differentiated into a proximal duodenum and a distal ileum. The duodenum has thick muscle layers, submucosal glands and receives the bile and pancreatic ducts. The ileum has a folded epithelium of columnar and mucous cells bearing a striated border (Leake, 1975).

3.1.1.8 Skin

The vertebrate skin consists of an epithelial layer, the epidermis, and a layer of connective tissue, the dermis (Junqueira *et al.,* 1992). There is however great diversity in the structure and function of skin between different vertebrate groups. In mammals the external layer of the skin is relatively impermeable to water, which prevents extreme water loss by evaporation and allows for terrestrial life (Junqueira *et al.,* 1992). The outermost layers consist of dead keratinised cells which are continuously shed and replaced by cells arising from the basal layers (Leeson and Leeson, 1979). The teleost skin must be impermeable to water to allow fish to maintain their osmotic balance, however unlike mammals, the fish skin is living and capable of mitotic division at all levels even at the outermost squamous layer (Roberts, 1989).

The skin is extremely important for the survival of the amphibian. It interacts with biotic and abiotic factors in the environment, but unlike mammals and teleosts it does not in general serve as a barrier to the inward or outward flow of water to and from the body (Toledo and Jared, 1993). Water is absorbed passively through the skin, mainly the ventral surface, as the animals sit in pools of fresh water or even in some species, by merely pressing the abdomen against moist soil (Rankin and Davenport, 1981).

The amphibian epidermis consists of a stratified squamous epithelium usually divided into three morphological layers:
1. The stratum corneum, on the outside and frequently composed of a single layer of flattened and keratinised cells.

2. The stratum intermedium, or transition layer, lying directly beneath the stratum corneum and made up of various layers of polyhedral cells.

3. The stratum germinativum, the germinal layer of the epidermis, made up of a single layer of cells which are frequently columnar and stand on a basal lamina (Toledo and Jared, 1993).

The epidermis is supported and nourished by the dermis, a thick layer of connective tissue which is composed of two layers; the stratum spongiosum and the stratum compactum. The stratum spongiosum lies closer to the surface and is made up of loose connective tissue, containing collagen, elastic fibres, nerves, blood vessels and granular and mucous glands. The stratum compactum lies beneath and is made up of dense connective tissue. The collagen fibres in this layer are often arranged in bundles parallel to the body surface (Toledo and Jared, 1993).

3.1.2 HISTOPATHOLOGY OF MOTILE AEROMONAD SEPTICAEMIA

There have been many papers written on the gross appearance of frog septicaemic disease and records of outbreaks go as far back as the 19th century (Russell, 1898, Emerson and Norris, 1905, Kulp and Borden, 1942, Gibbs, *et al.,* 1966, Nyman 1986, Bradford 1991). However no detailed histological descriptions of the naturally occurring disease have been published.

Septicaemia is the disease state compounded of toxaemia, hyperthermia (in mammals) and the presence of large numbers of infectious micro-organisms in the blood stream. Many infections including viral, bacterial and protozoal produce septicaemias. The difference between septicaemia and bacteraemia is one of degree. In bacteraemia

bacteria are present in the blood stream for only transitory periods and do not produce clinical signs. In septicaemia the causative agent is present throughout the course of the disease and is directly responsible for the clinical signs which appear (Blood *et al.*, 1983).

The term motile *Aeromonas* septicaemia is used to describe motile aeromonad infections of warm water fish. Clinical signs of the disease in fish include rupture of minor blood vessels and haemorrhages caused by haemolysins. The haemorrhages may be associated with ulcerative skin lesions which vary in size. Internally the principal feature is hyperaemia of the viscera with haemorrhage over the mesentery and peritoneum. The spleen and kidney are often enlarged (Roberts, 1993).

Histologically a generalised necrotising septicaemia with focal lesions is observed. Capillary beds are generally hyperaemic, haemorrhagic and contain numerous macrophages and other leucocytes. Focal areas of acute liquefactive necrosis are present in many organs and are particularly apparent in the spleen and kidney where haemopoietic tissue may be completely destroyed. The kidney tubular cells are frequently sloughed into the lumen, and often only the glomerulus remains intact. The intestinal vascular bed is highly congested, the submucosa is oedematous and infiltrated by macrophages and lymphocytes. The overlying mucosa is generally necrotic and large expanses of mucosal epithelium may have sloughed into the gut lumen (Roberts, 1993).

Although there are no complete histological descriptions of naturally occurring aeomonad infections in frogs, there have been investigations into the histological

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appearance of laboratory induced infections. Russell, in 1898, undertook an extensive

investigation into the bacterium he described as *Bacillus hydrophilus fuscus, a*

bacterium later renamed *Aeromonas hydrophila.* The author inoculated the bacterium

into frogs (species not stated) in the thigh, in the pertioneum and in the lymph sac on

the back. Histological examination of affected animals revealed areas of congestion

and necrosis which were particularly marked in the spleen.The thigh muscles also

presented areas of degenerative change (although it is not stated whether these were in

all animals or only those where the muscle was the site of injection). In cross section

there were areas of necrosis in the muscle fibres, always accompanied by

haemorrhage. In densely congested areas some of the fibres underwent pressure

atrophy. In other areas the fibres underwent degenerative change with seperation of

the muscle bundles, and loss of cross striations. The author noted little change in the

lungs and kidneys of diseased frogs, however the livers showed severe, diffuse

degenerative change. The staining of the hepatocytes was uneven and the cytoplasm

appeared granular. In some areas there was loss of cell outline. The nuclei showed all

stages of degeneration with frequent karryolysis and karryorhexis. There was

considerable capillary dilation and congestion. The capillaries as well as being

engorged with erythrocytes contained much cellular debris. The walls of the

capillaries were fragmented in many places allowing haemorrhage into surrounding

tissue. Many bacteria were observed within the capillaries.

Gibbs *et al.,* (1966) made a detailed histological study of the sartorius muscle in

laboratory frogs (*Rana pipiens*) naturally suffering from bacterial septicaemias. The

authors compared newly arrived septicaemic animals with those which had undergone

antibiotic treatment. Oedema, necrosis and haemorrhages were observed in the muscle

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of septicaemic animals. The muscle fibres also showed a reduced affinity for the eosin dye in H&E stained sections and, as reported by Russell (1898), the muscle striations were difficult to discern. These authors made no histological examination of any other organs.

These two studies indicate that the changes occurring in frogs affected by *Aeromonas hydrophila* septicaemias are similar to those seen in fish suffering from motile aeromonad septicaemias. The available studies on frog septicaemic disease are however incomplete. The most extensive investigations undertaken by Russell almost a hundred years ago (1898) were of artifically induced infections. The histological studies of natural infections have been limited.

The aims of this part of the study were therefore to provide a more detailed picture of the pathological changes taking place during the bacterial septicaemic disease process in farmed frogs.

3.2 MATERIALS AND METHODS

Collection of tissue for histological examination is described in Field Studies Section

2.2.2. The fixed blocks of tissue from each frog were dehydrated and paraffin wax

embedded by standard histological methods in a Reichert-Jung Histokinette 2000

automatic tissue processor. Processed tissues were sectioned at 5μm and stained with

Haematoxylin and Eosin (H&E).

Routine H & E (Drury and Wallington, 1980).

1. Take section to water -   xylene 5 minutes
                            100% alcohol 2 minutes
                            methanol 1½ minutes
                            wash in tap water

2. Mayers Haematoxylin 5 minutes

3. Wash in tap water

4. Acid alcohol 4 quick dips

5. Wash in tap water

6. Scott's tap water 1 minute

7. Check stain under microscope

8. Wash in tap water

9. Eosin

10. Wash in tap water

11. Methanol 30 seconds

12. 100% alcohol 2 minutes

13. 100% alcohol 1½ minutes

14. Xylene 5 minutes

15. Blot dry and mount (pertex mountant).

3.3 RESULTS

3.3.1 GROSS PATHOLOGY

The common presenting sign in frogs which yielded Au on culture, was lethargy. Some frogs presented with a red flush to the thighs however this was not a consistent feature. Some were ulcerated around the mouth and digits but again this was not a consistent finding and animals with no bacterial septicaemia also presented with ulcerations. Internally many of the animals were ascitic and small haemorrhages were observed on the surface of the viscera. The organs were frequently enlarged and hyperaemic. Two frogs which showed signs of more chronic disease, one with an abcess in the liver, the other with severe pericarditis and peritoneal adhesions, also yielded heavy pure cultures of Au.

3.3.2 HISTOLOGICAL FINDINGS

Histologically Au infection in frogs was characterised by extensive vascular congestion, haemorrhage and vascular dilation, destruction of the haemopoeitic tissue and focal areas or zones of acute liquefactive necrosis.

The livers in particular showed severe hyperaemia, with enlargement of the sinusoids (Figures 3.3.3 and 3.3.4). Many necrotic erythrocytes and leucocytes were observed in the sinusoids and blood vessels (Figure 3.3.4) and numerous replete macrophages were present. Hepatocyte cytoplasm often contained bile pigments indicating bile stasis (Figure 3.3.4). Haemorrhages and areas of focal necrosis were observed throughout the parenchyma (Figure 3.3.4). Hyperaemia was again very prominent in the kidneys, particularly in the glomerular capillaries (Figure 3.3.8). The glomeruli and tubules were frequently necrotic and haemorrhages were observed between the

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tubules (Figure 3.3.9). In the spleens there was massive destruction of the

haemopoeitic tissue producing a large amount of cellular debris (Figures 3.3.13,

3.3.14 and 3.3.15). In some areas oedema and necrosis of the white pulp and reticular

fibres lining the sinusoids resulted in the formation of spaces in the tissue (Figure

3.3.15). Many replete macrophages were observed loaded with tissue breakdown

products (Figure 3.3.14). The hearts showed severe cardiomyopathy with extensive

sarcoplasmic necrosis, vacuolation, inflammatory cell infiltration, and haemorrhages

(Figures 3.319 and 3.3.20). Large colonial foci of bacteria were occasionally observed

in the muscle, surrounded by necrotic debris from the inflammatory cell infiltrate and

muscle tissue (Figure 3.3.21). There was extensive necrosis of the respiratory

epithelium in the lung with pooling of blood in the capillary channels and blood

vessels (Figures 3.3.27, 3.3.28 and 3.3.29). Bacterial colonial foci were also observed

in this organ localised in the blood vessels and associated with necrotic blood

products (Figure 3.3.29).

The changes in the small intestine were less severe, there was very little vascular

congestion, although the smooth muscle was oedematous and degenerative with

occasional small areas of haemorrhage (Figure 3.3.33). Some macrophages were

observed infiltrating the overlying mucosa and necrotic changes were observed in

some enterocytes (Figure 3.3.34). The skeletal muscle from the thigh, in sharp contrast

to the cardiac muscle, showed a minimal amount of change. A few necrotic muscle

fasiculi were observed within the muscle but unlike the widespread myonecrosis

observed in the heart, these were isolated and the surrounding fasiculi appeared

healthy (Figure 3.3.24). No large scale inflammatory infiltration or signs of

haemorrhage were observed. The skin from the inner thighs showed a similar lack of

pathological change (Figure 3.3.36).

The changes occuring in each organ are illustrated in the following figures. Examples

of normal tissue are also presented to illustrate the severity of the changes

Figure 3.3.1 Liver from a normal well-fed frog. The lack of well-defined lobulation and the presence of melanomacrophage centres (MMC) scattered throughout the parenchyma indicate that the histological structure of the ranid liver more closely resembles that of the teleost than the mammal (H&E x 298).

Figure 3.3.2 High power view of melanomacrophage centre (MMC) and cords of fatty hepatocytes (H) containing peripheral nuclei (arrowed) separated from sinusoids (S) by a thin epithelium (H&E x 587).
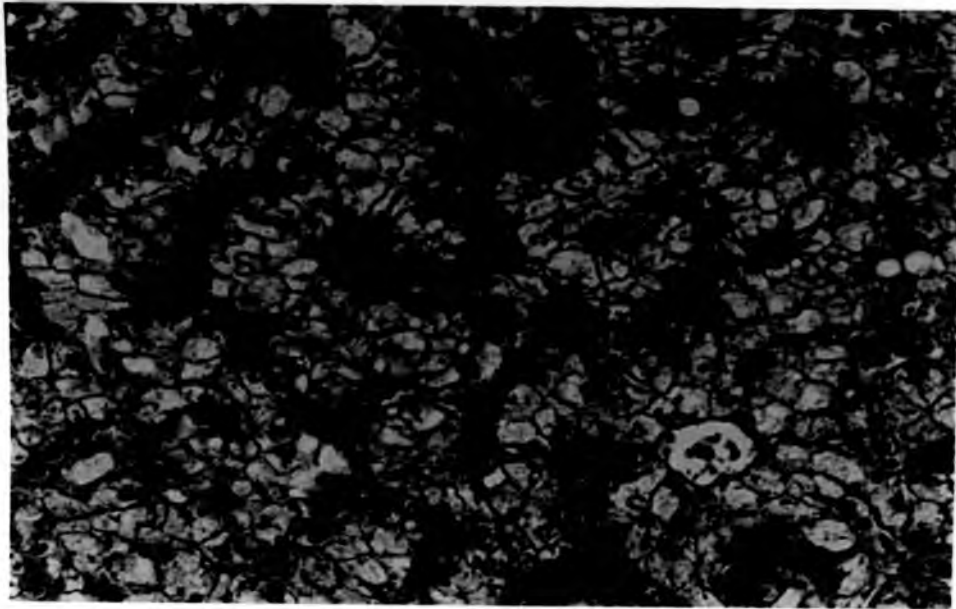
Figure 3.3.3 Liver from septicaemic frog, showing severe congestion of sinusoids (S) with bile pigments (arrowed) accumulated in cytoplasm of hepatocytes (H&E x 287).

Figure 3.3.4 Liver from septicaemic animal, the sinusoids are packed with blood and cellular debris. In some places, the walls of the sinusoids are damaged causing haemorrhage (arrowed) between the hepatocytes. Many of the hepatocyte nuclei are pyknotic (P) others appear quite healthy (N). There are accumulations of bile pigments in the cytoplasm of some hepatocytes (BP) (H&E x 599).

Figure 3.3.5 Normal frog kidney. The lobules are roughly divided into inner areas, which contain predominantly glomeruli (G) and distal tubules (DT), and outer areas, which contain predominantly proximal tubules (PT). These areas are less well defined than the cortex and medulla of the mammalian kidney, probably because frog nephrons do not possess a loop of Henle (H&E x 60).
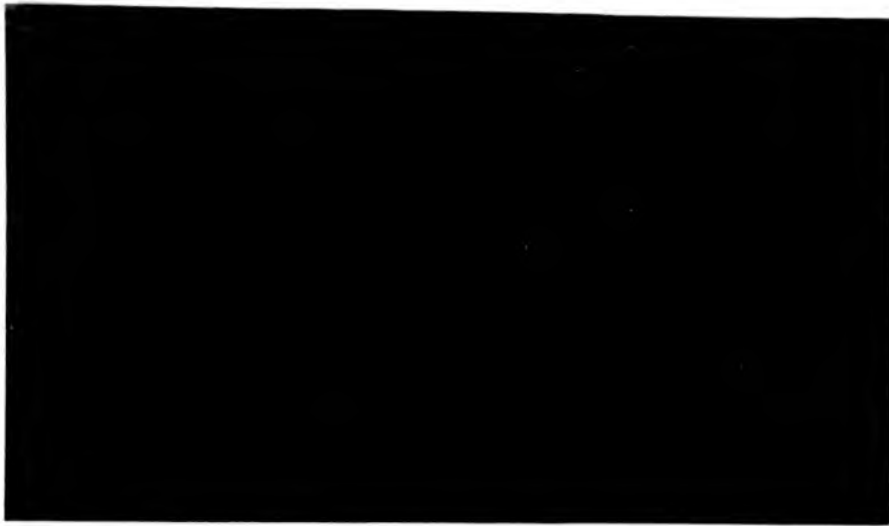
Figure 3.3.6 High power view of frog renal corpuscle. The structure is typical of a vertebrate renal corpuscle, and consists of Bowman's capsule (BC), glomerulus (G), vascular pole (VP) and urinary pole (UP) which connects to the ciliated neck piece (NP) (H&E x 591).

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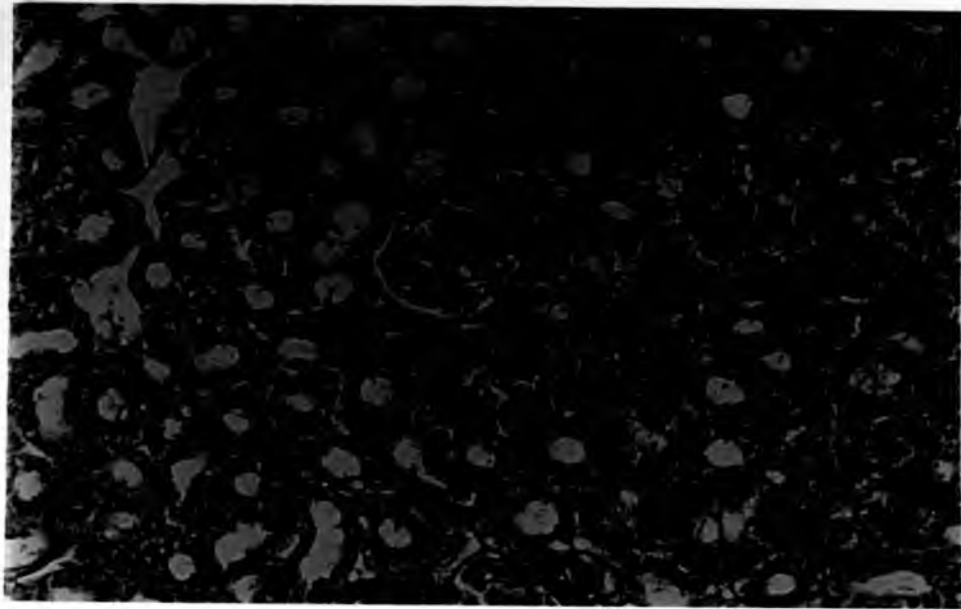
Figure 3.3.7 Kidney from clinically normal frog, demonstrating proximal (PT) and distal tubules (DT), glomerulus (G). The epithelial cells of the proximal tubules are larger than those of the distal tubules have brush borders and are more eosinophilic (H&E x 298).
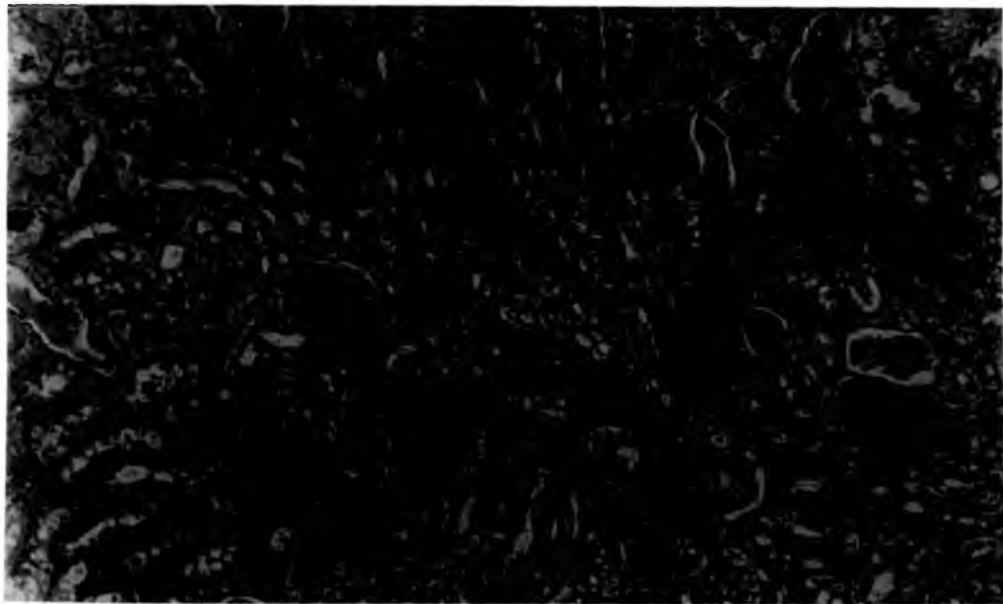
Figure 3.3.8 Kidney from septicaemic frog, demonstrating congestion of glomerular capillaries (G) and haemorrhages (arrowed) between the tubules (H&E x 152).
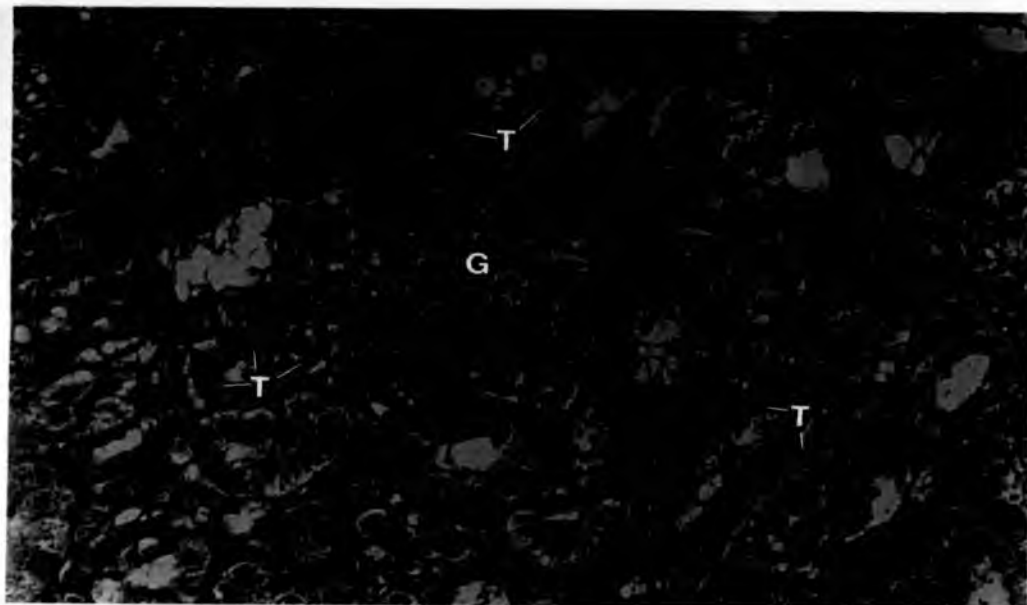
Figure 3.3.9 High power view of glomerulus from septicaemic frog, demonstrating necrosis of glomerulus (G) and tubular epithelial cells (T). The blood cells in the glomerular capillaries and between the tubules also show signs of degenerative change (H&E x 301).
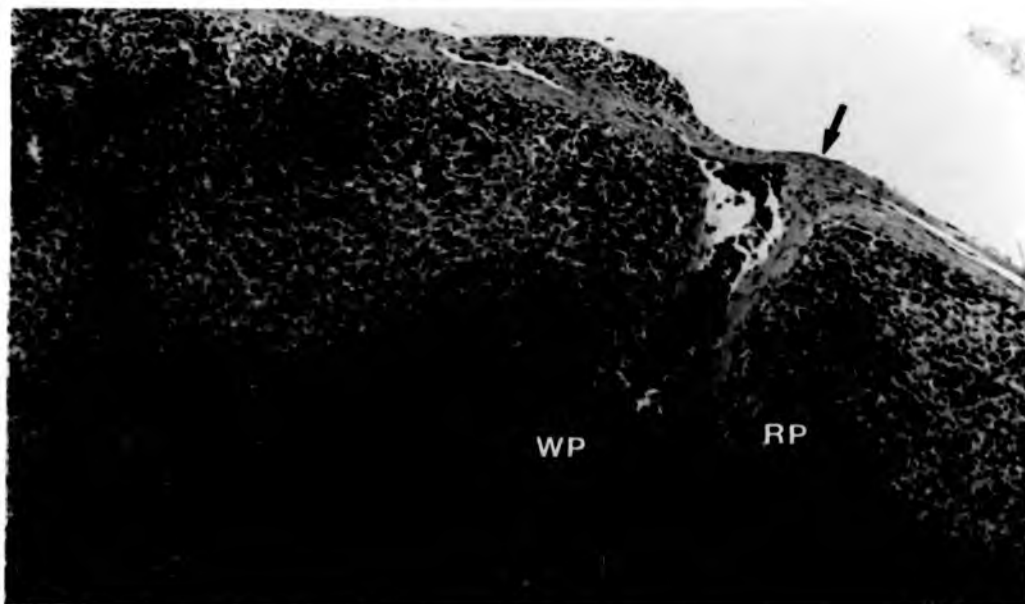
Figure 3.3.10 Spleen from clinically normal frog demonstrating thick connective tissue capsule (arrowed), white pulp (WP) and red pulp (RP) (H&E x 149).
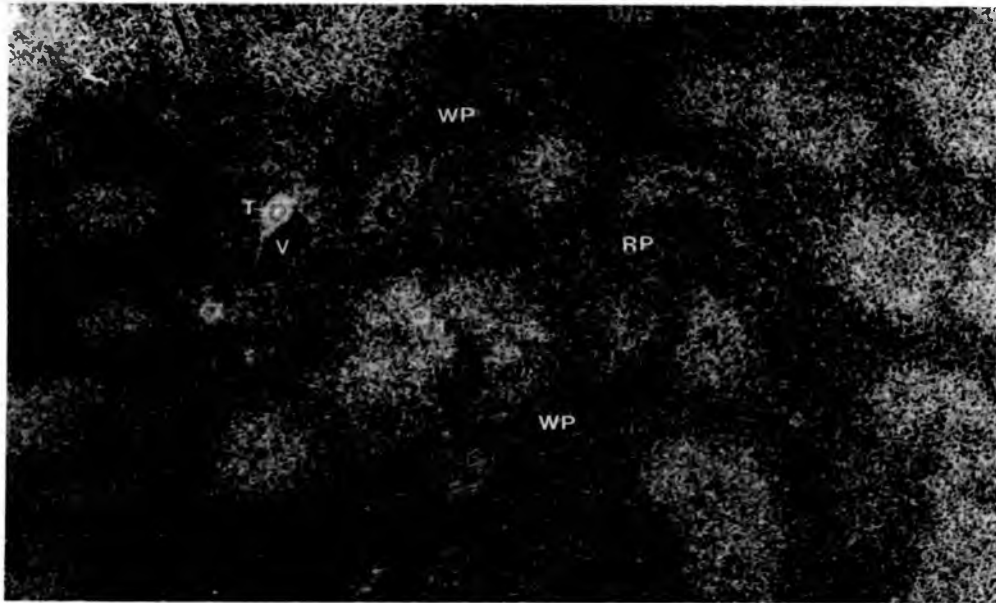
Figure 3.3.11 Spleen from clinically normal frog. Unlike mammals there are no splenic nodules along the arteries, however the definition between the lymphocyte rich white pulp (WP) and erythrocyte rich red pulp (RP) is clearer than in the teleost. Many of the red blood cells normally stored within the red pulp have been mobilised in this animal possibly due to the stress of capture; therefore the pale areas of supporting reticular network (N) are clearly visible within the parenchyma. To the left there is a connective tissue trabecula (T) carrying blood vessels and nerves through the pulp, the large vein (V) is particularly prominent (H&E x 60).
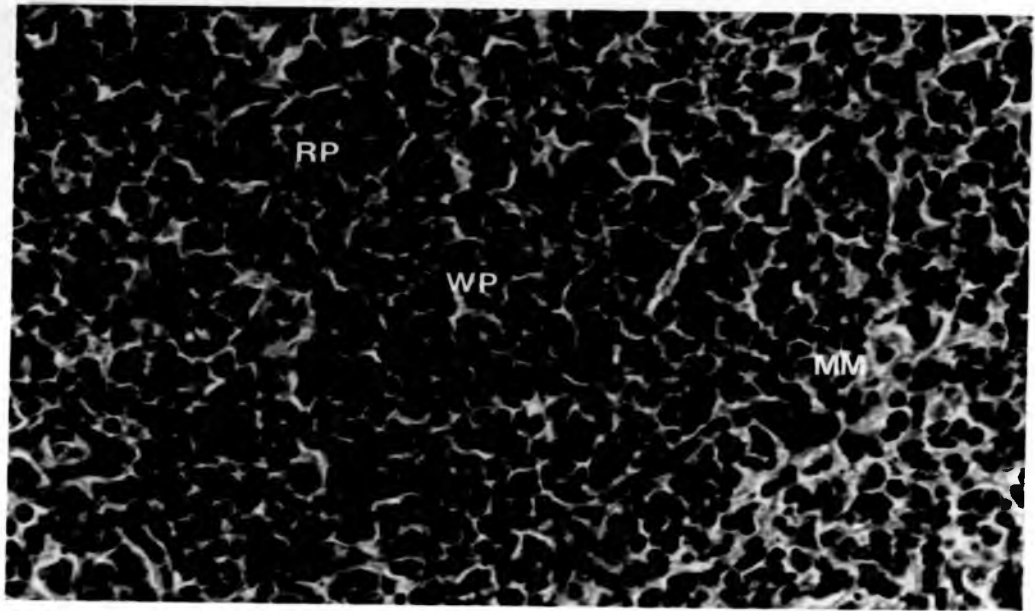
Figure 3.3.12 High power view cells making up splenic pulp of a clinically normal frog. White pulp lymphocytes (WP), red pulp red blood cells (RP), melanomacrophage centre (MM) (H&E x 596).

Figure 3.3.13 Low power view of spleen from septicaemic frog, demonstrating loss of definition between white and red pulp and large areas of haemorrhage (H) (H&E x 60).
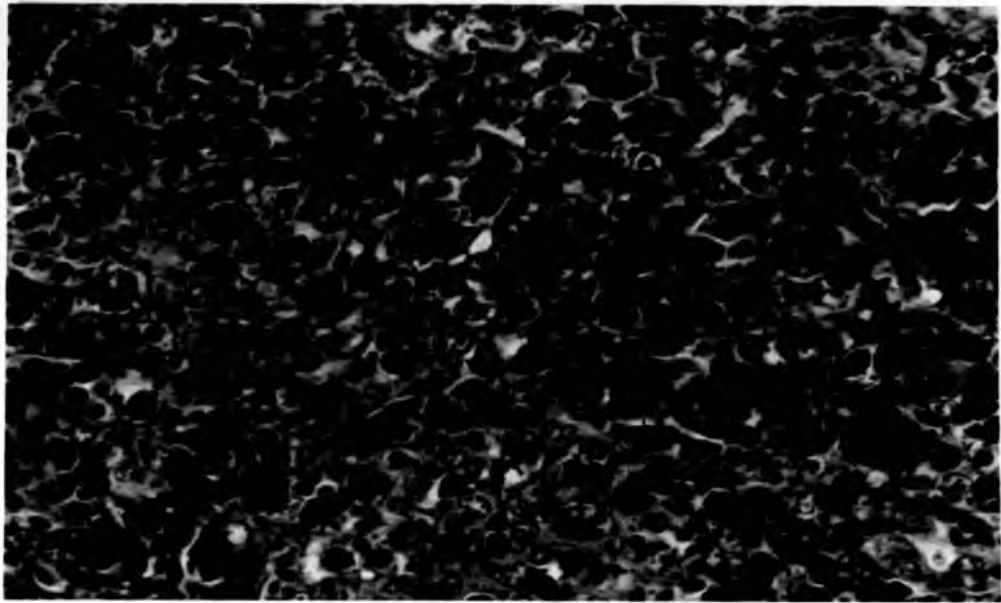
Figure 3.3.14 High power view of spleen from septicaemic frog demonstrating widespread destruction of red and white pulp. Pyknotic nuclei (arrowed) and replete macrophages (M) are scattered throughout the field (H&E x 595).
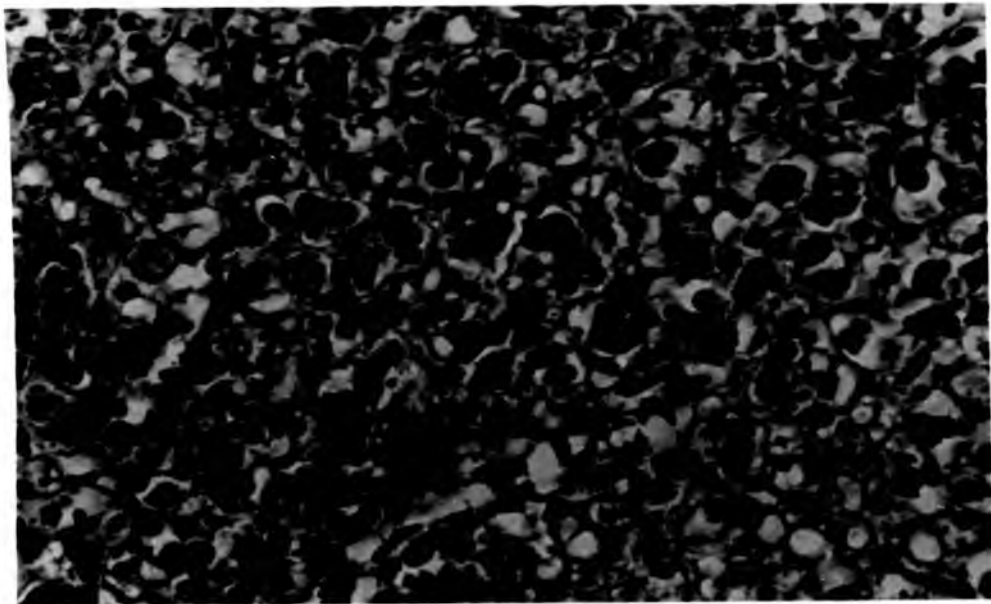
Figure 3.3.15 High power view of spleen from septicaemic frog. In some areas the destruction of the pulp and mobilisation of red blood cells exposed the underlying reticular structure which was also degenerating (H&E x 599).
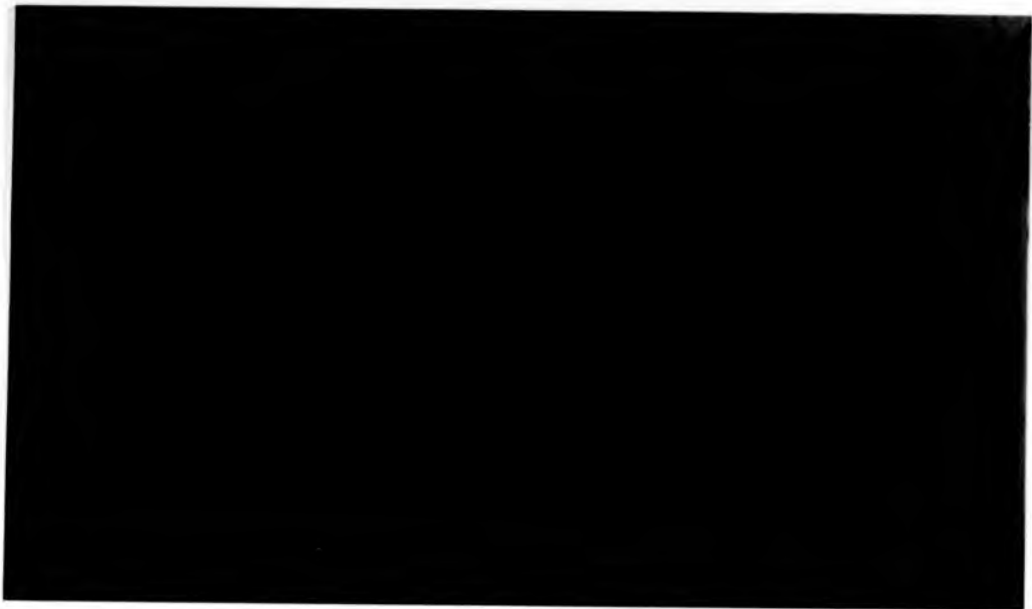
Figure 3.3.16 Low power view of heart from clinically normal frog demonstrating thin walled atrium (A) and thick walled ventricle (V). The ventricular muscle is folded into numerous trabeculae (arrowed) (H&E x 60).
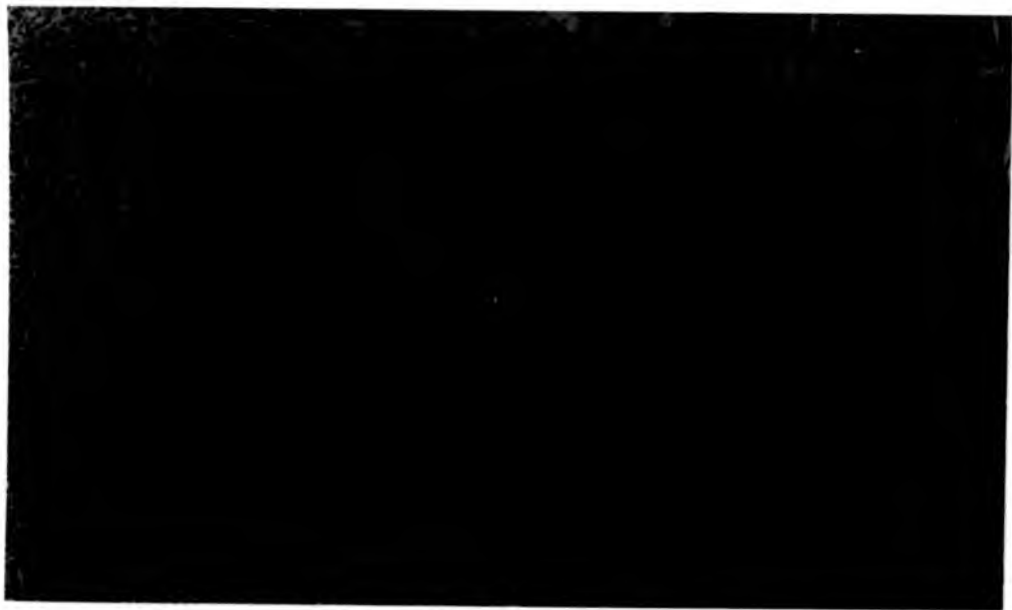
Figure 3.3 17 Compact cardiac muscle of ventricle wall from normal frog heart. Unlike the teleost fish there is no spongy layer (H&E x 300).

Figure 3.3.18 High power view of heart from clinically normal frog demonstrating transverse and longitudinal fibres. Nuclei (N) are located at the centre of the fibres (H&E x 586).

Figure 3.3.19 Low power view of heart from a septicaemic frog. There is widespread necrosis and vacuolation of the muscle fibres. Inflammatory cells (I), mainly macrophages, are infiltrating between the muscle bundles and there are small areas of haemorrhage (H) (H&E x 152).
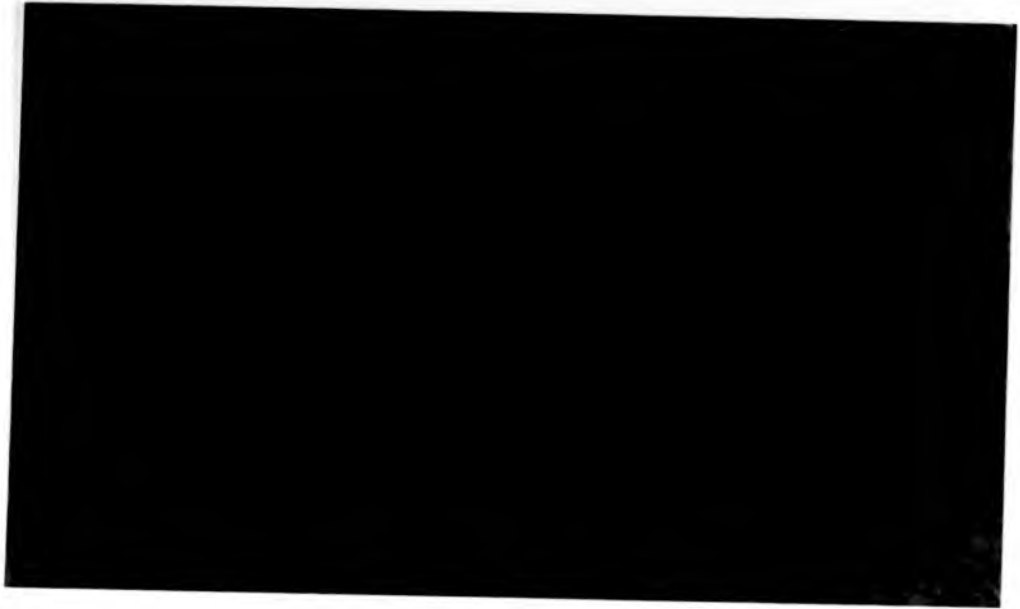
Figure 3.3.20 High power view of septicaemic frog heart demonstrating necrotic muscle fibres (N), vacuolation (V), inflammatory cell infiltration, mostly macrophages many of which are necrotic with pyknotic nuclei (P) (H&E x 596).

Figure 3.3.21 Colonies of bacteria (C) were occasionally observed surrounded by numerous necrotic inflammatory cells and muscle tissue debris (H&E x 303).

Figure 3.3.22 Transverse section of thigh muscle from clinically normal frog, demonstrating muscle fibre bundles containing a number of myofibrils (H&E x 151).
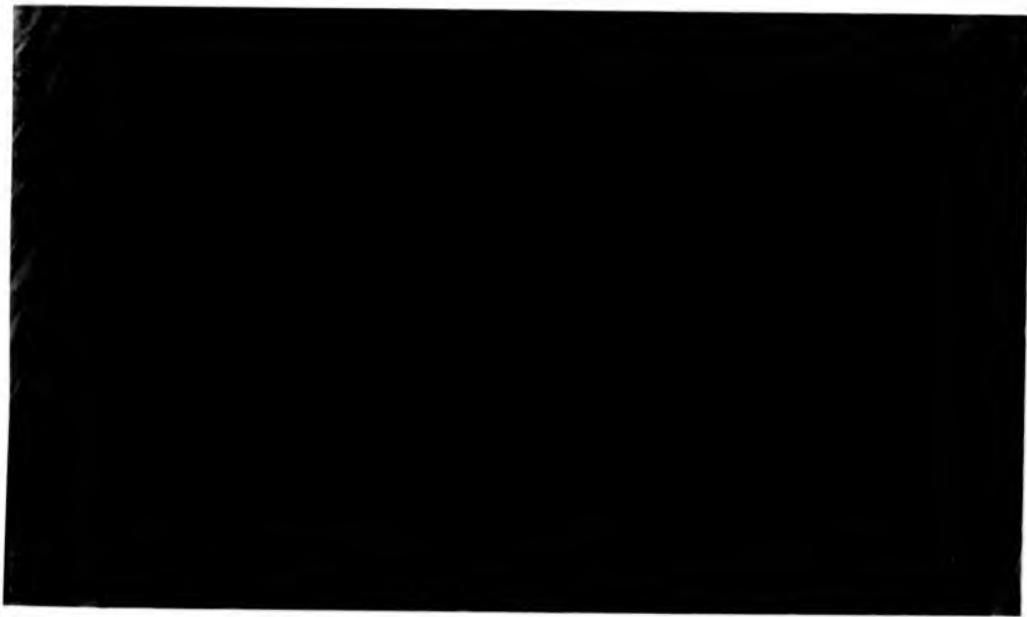
Figure 3.3.23 Longitudinal section of thigh muscle from clinically normal frog (H&E x 607).

Figure 3.3.24 High power view of necrotic muscle bundle surrounded by healthy tissue. The damaged muscle fibres are being phagocytosed by active healthy macrophages (arrowed) (H&E x 598).

Figure 3.3.25 Low power view of lung from clinically healthy frog demonstrating cavity of the lung (C), septae (S), infundibula (I), blood vessels (BV), respiratory epithelium (arrowed), melanin (M). Unlike mammals, the frog has no trachea or bronchial tree and hence no cartilage is found in the respiratory tract, the simple lungs open directly from the pharynx (H&E x 60).

Figure 3.3.26 High power view of lung from clinically normal frog demonstrating respiratory epithelium (RE) and capillary channel (CC) (H&E x 593).

Figure 3.3.27 Low power view of lung from septicaemic frog revealing severe congestion of blood vessels and capillary channels in the respiratory epithelium (H&E x 149).
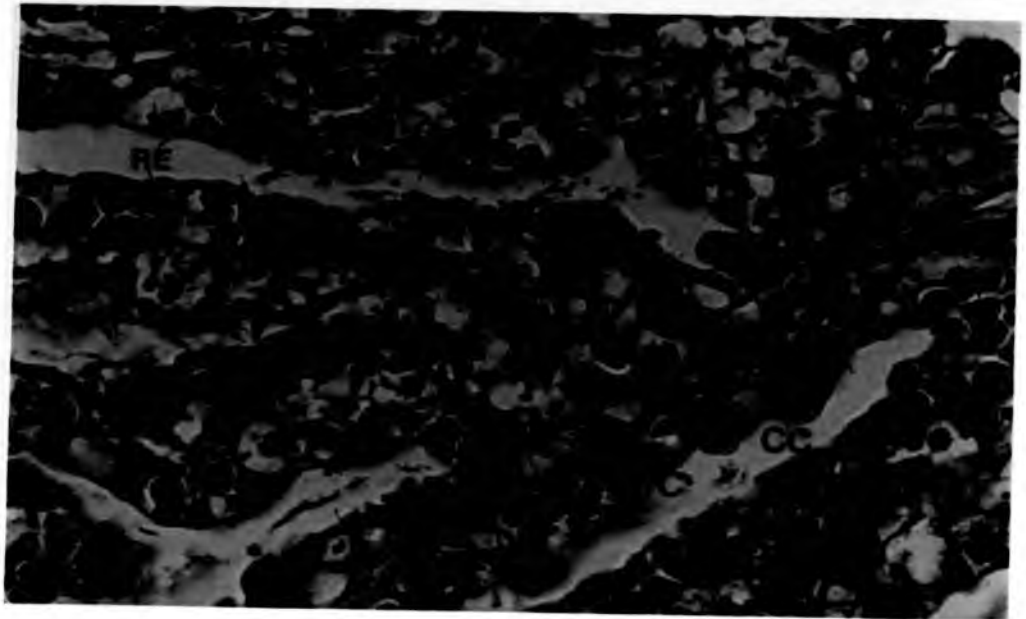
Figure 3.3.28 High power view of lung from septicaemic frog, demonstrating widespread necrosis of respiratory epithelium (RE) and supporting smooth muscle (SM). Capillary channels (CC) are congested and many blood cells have escaped causing haemorrhages (H). The field is full of pyknotic nuclei (arrowed) from epithelial, muscle and blood cells (H&E x 601).
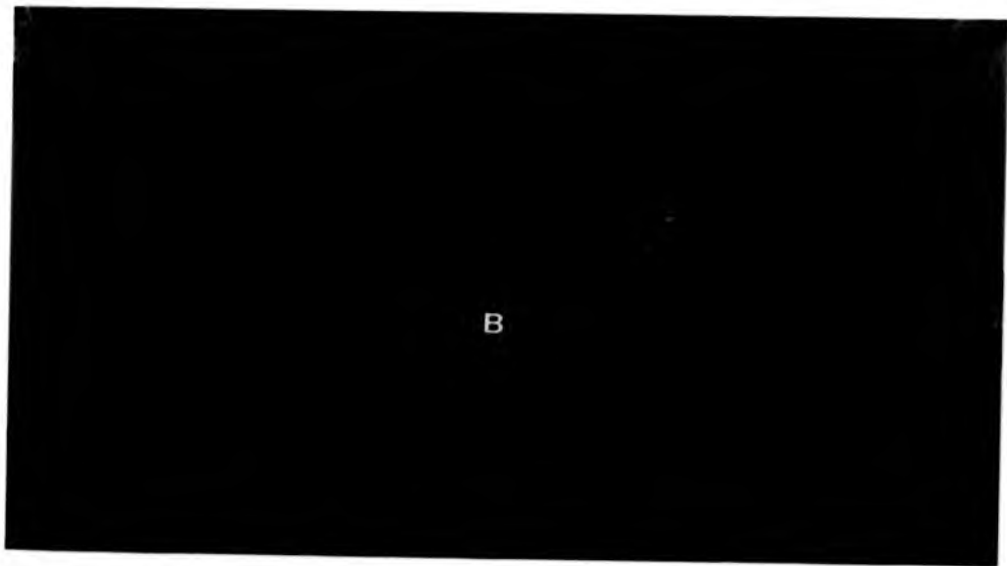
Figure 3.3.29 High power view of lung from septicaemic frog demonstrating large clump of bacteria (B) situated within the blood vessel (H&E x 298).

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Figure 3.3.30 Small intestine from clinically normal frog. Smooth muscle (SM), villi (V) (H&E x 152).

Figure 3.3.31 Longitudinal section of healthy intestinal villi. The striated border (S) of tall columnar enterocytes is formed by microvilli, numerous goblet cells (G) are present (H&E x 600).
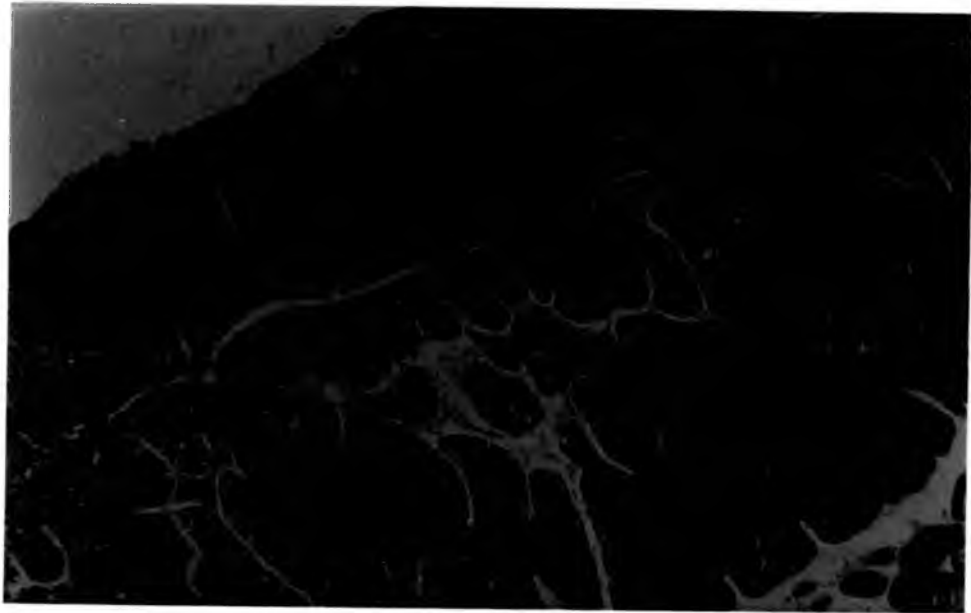
Figure 3.3.32 Small intestine from septicaemic frog. The smooth muscle is oedematous and the division into inner circular (C) and outer longitudinal (L) fibres is more noticeable than in the normal frog. Unlike other organs, there is no sign of vascular congestion. The mucosa is relatively undamaged, but appears slightly more cellular than in the normal specimen, due to inflammatory cell infiltration. This frog also had a large number of protozoal parasites (P) in the intestinal lumen but these were also frequently observed in clinically normal animals and are probably an incidental finding (H&E x 150).

Figure 3.3.33 Small intestine from septicaemic frog. Small areas of haemorrhage (H) were occasionally observed in the muscle. Inflammatory cells (arrowed) can be observed migrating through the lamina propria (H&E x 303).

Figure 3.3.34 High power view of haemorrhage in the smooth muscle. The blood cells are undergoing degenerative changes and some replete macrophages are present (arrowed). Some enterocytes (E) are showing signs of degenerative changes, others appear normal (N). The smooth muscle is oedematous and necrotic. Inflammatory cells (I) can be seen migrating through the lamina propria (H&E x 606).

Figure 3.3.35 Transverse section of thigh skin from clinically normal frog. Epidermis (E), mucous gland (M), granular gland (G), stratum spongiosum (Ss), stratum compactum (Sc) (H&E x 149).
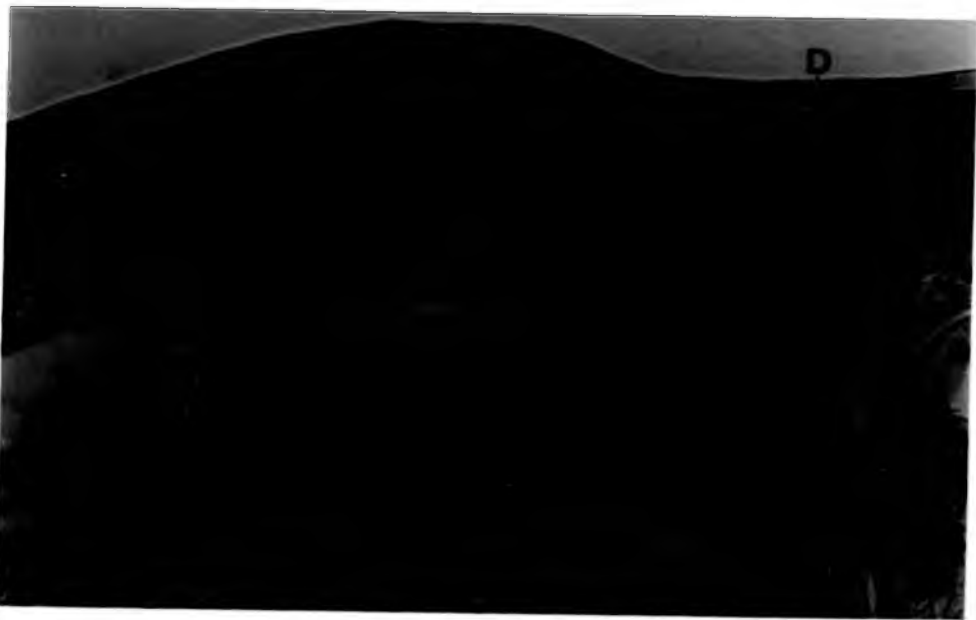
Figure 3.3.36 High power view of epidermis from clinically normal frog. Stratum corneum (Co), stratum intermedium (I), stratum germinativum (G), mucous gland (M), gland duct (D) (H&E x 604).

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Figure 3.3.37 High power view of thigh skin from septicaemic frog. In contrast to the parenchymatous organs, there was little change in the skin. This animal contains more melanocytes (Mc) than the previous normal specimen but that is part of normal variation in pigment patterns and unrelated to the disease. Stratum corneum (Co), stratum intermedium (Si), stratum germativum (Sg), mucous gland (M), granular gland (G), stratum spongiosum (Ss), stratum compactum (Sc) (H&E x 300).

3.4 DISCUSSION

The histopathological lesions described in this chapter are similar to those observed in motile aeromonad septicaemias of numerous fish species: brown trout (Thorpe and Roberts, 1972); channel catfish (De Figueiredo and Plumb, 1977; Ventura and Grizzle, 1988); largemouth bass (Huizinga *et al.*, 1979); crucian carp (Miyazaki and Kaige, 1985); ayu (Miyazaki and Jo, 1985); European eel (Esteve *et al.*, 1993). The principal features of the disease in teleosts are well summarised in various texts (Roberts, 1989, 1993; Thune *et al.*, 1993). Infections are characterised in all species by hyperaemia of capillary beds and extensive tissue damage, however the severity of involvement of the various internal organs appears to differ between the species.

Roberts (1993), in a general text on teleosts suffering from motile aeromonad septicaemia, describes severe changes in the intestine. He states that the intestinal capillary beds are highly congested, inflammatory cells infiltrate the oedematous submucosa, there is widespread necrosis of the mucosa, large areas of which slough into the gut lumen. Such severe intestinal changes have been described in brown trout and crucian carp (Thorpe and Roberts, 1972; Miyazaki and Kaige, 1985). However some fish, e.g. largemouth bass and Ayu, show pathology similar to the Au affected frogs, with little involvement of the intestinal mucosa despite widespread necrosis of other organs (Huizinga, *et al.*, 1979; Miyazaki and Jo, 1985).

Huizinga *et al.*, (1979) in a study on the histopathology of *A. hydrophila* infections of largemouth bass found a conspicuous absence of heart muscle necrosis in fish that exhibited severe changes in other organs. In contrast Ventura and Grizzle (1988), investigating natural and experimental septicaemias in channel catfish, found oedema

and necrosis in the stratum spongiosum myofibres of the heart ventricle. However no studies on fish describe the severe cardiac myopathy observed in the septicaemic frogs.

Most accounts in the literature on motile aeromonad infections of fish agree that the haemopoietic tissue is greatly affected, in particular, an extensive, severe necrosis of the spleen is described (Bach *et al.*, 1978; Ventura and Grizzle, 1988). The spleens of frogs suffering from Au septicaemias were similarly affected with massive destruction of the haemopoietic tissue producing a large amount of cellular debris and the formation of spaces in the tissue.

Motile aeromonad infections of teleosts elicit an intense inflammatory response, with infiltration of monocytic and granulocytic cells into infected tissues (Thune *et al.*, 1993). A similar response was observed in the organs of the septicaemic frogs. The inflammatory cells present in the congested blood vessels and infiltrating the damaged organs were predominantly macrophages, although some eosinophilic granular cells were observed.

Rapid bacterial growth and elaboration of extracellular products leading to physiological disturbances and death is the mechanism of pathogenesis supported by most fish pathologists. The histopathology observed in Au septicaemia indicates a similar mechanism of pathogenesis in frogs. Although the severity of changes in affected organs may differ between fish and frogs, the depletion and necrosis of the haemopoietic tissue is consistent. Haemopoietic tissue is very sensitive and highly active, and necrosis may occur readily as a result of toxins circulating from elsewhere.

Pathological change in the lungs of Au affected frogs was consistent and severe. This is of interest, as previous studies on "red-leg" in frogs have reported little change in the lungs. Russell (1898) reported no lung involvement in his experimental infections. Emerson and Norris (1905), in a study on natural "red-leg" infections, stated that the lungs were the seat of no apparent change apart from mild parasitic infections. Kulp and Borden (1942) however reported that in many spontaneous and experimental "red-leg" infections the lungs appeared highly congested, while in others no abnormal changes were observed. In the frogs suffering from Au septicaemia, the lung capillaries were highly congested and the respiratory epithelium was generally necrotic with areas sloughing into the lung cavity. The severe diffuse change is probably a generalised toxic necrosis associated with bacterial colonies in the lung blood vessels.

It is interesting that there was very little pathological change of the skeletal muscle from the thigh of the frog, although previous studies on *Aeromonas* infections in frogs have emphasised the involvement of the muscles. Russell (1898) experimentally induced *Aeromonas* septicaemia in frogs by intramuscular, intraperitoneal and dorsal lymph sac injection. He examined muscles from the thigh and observed areas of congestion, haemorrhage and degeneration; in contrast he found the lungs and kidney showed little or no change. However the infections Russell describes were experimentally induced by three different routes of injection, one of which was intramuscular. Russell did not distinguish between the three routes of injection when describing the histological change, if bacteria were introduced intramuscularly then severe changes would be expected in the muscle.

Gibbs *et al.,* (1966) studied the histological appearance of the sartorius muscle in frogs suffering from natural *Aeromonas* infections. They reported the presence oedema, necrosis and haemorrhage but did not describe in detail the extent of the changes in the muscle, nor did they examine histological changes in any other organs.

Gosling (1996) in a review of *Aeromonas* disease of amphibians, states that considerable information has accumulated regarding *Aeromonas* infection in frogs. However his description is based mainly on the gross pathology. The histopathology is confined to four sentences and emphasises the involvement of the skeletal muscle. He describes myodegeneration and myositis characterised by oedematous separation of muscle bundles and fibres, and by loss of cross striation in the fibres. This description originates from the work published in 1898 by Russell, which, as mentioned above, involved artificially induced infections and may even have resulted from intramuscular injection of the bacteria.

Attention has probably focused on the muscular changes because of the name: "red-leg" given to *Aeromonas* septicaemias in frogs. This designation first appeared in the literature in an article by Emerson and Norris in 1905. They claimed that the name "red-leg" was commonly used to describe septicaemic disease in laboratory frogs and was also used by wild frog catchers in America, who encountered the disease frequently in the Autumn months. Emerson and Norris thought the name ideal because it highlighted the circulatory congestion of the belly and legs, which they described as varying from a faint flush to deep haemorrhagic injection, and considered the most striking characteristic of the disease in frogs. Kulp and Borden (1942) also considered petechial haemorrhages on the surface of the abdomen and legs, varying from light red

to deep scarlet colour, as a pathogonomic lesion common to frogs suffering from *Aeromonas* septicaemia.

However some workers think that this may be a misnomer as "red-leg" is not always a presenting sign. Gibbs *et al.*, (1966) believe that the presenting signs of *A. hydrophila* infections are not sufficiently consistent to warrant the title "red-leg". These workers found that nearly half of the frogs reaching the point of death in their laboratory exhibited only mild clinical signs, such as being thin or lacking brilliance of skin colouration. More striking clinical signs included; slumped posture with palms turned outward, disinclination to move when prodded, tense abdomen, cutaneous haemorrhages, eroded toes and feet with bare bones exposed, eroded jaws, perforations of the skin on the dorsal surfaces (particularly the nose), rough and bleeding nictitating membranes, haemorrhages within the eyes, and numerous neurological signs.

In the present study the frogs which yielded the Au cultures, presented with a variety of signs, of which lethargy was common to all. Some did possess a faint red flush to the thighs although this never deepened to the scarlet described by Kulp and Borden (1942). It is possible that different species of frogs may have different presenting signs and that the scarlet red-leg described in *Rana pipiens* is a feature of that species.

Generalised oedema and degenerative changes in the skeletal muscle are not a feature of fish bacterial Gram negative septicaemias, although bacterial lesions in the muscles of fish are frequent. The two species most frequently associated with acute lesions are *Aeromonas salmonicida* and *Vibrio anguillarum.* In each case the initial stage of the

lesion is a severe necrotising "focal" myositis in which bacterial products such as extracellular proteases break down the myofibrillar integrity and destroy the sarcoplasm. In motile aeromonad septicaemias of fish there may be focal myonecrosis, but this is usually superficial and associated with external lesions such as ulcers (Roberts 1993). The present studies indicate that the Au septicaemia of frogs is more like the motile aeromonad infection in fish, in that the parenchymatous organs, and in particular the haemopoietic tissues, are affected and the skeletal muscle shows little involvement. The diffuse changes described elsewhere probably take place in the final stages of an acute necrotising septicaemia which has affected all the organs in the body and should not be considered a defining feature of the infection. The findings presented in this study support the opinion of Gibbs *et al.,* (1966) that "red-leg" is a misnomer for frog septicaemic disease.

Histological examination of acute Au septicaemias revealed widespread vascular congestion, severe cardiac myopathy and pulmonary, renal, hepatic and splenic necrosis. The major histopathological changes observed in the Au infected frogs were similar to those of a motile aeromonad infection in fish, although there was very little change in the mucosa of the intestine. It is tempting to speculate, given the histopathology described, that the Au bacteria gain entry to the host through the thin respiratory epithelium, rather than the intestinal mucosa. The bacteria are then rapidly transported to the heart by the pulmonary circulation. In both the lungs and heart severe, diffuse necrotic changes were observed and large focal colonies of bacteria were present. The widespread necrosis observed in all the organs indicates that the bacteria are capable of elaborating exotoxins. Possible mechanisms of pathogenesis such as toxin production are discussed in detail in chapter 7.

Chapter 4. Phenotypic Analysis

4.1 INTRODUCTION

4.1.1 GENERAL DESCRIPTION OF THE GENUS

Frogs and bacteria of the genus *Aeromonas* have been associated for over 100 years. The first record of an aeromonad is credited to Sanarelli, who isolated the bacterium *Bacillus hydrophilus* from a frog in 1891 (cited by Carnahan and Altwegg, 1996). This bacterium when reinoculated into cold and warm-blooded animals, caused septicaemia and disease (Ewing *et al.*, 1961 cited by Janda and Duffey, 1988). The genus *Aeromonas* was proposed in 1936 by Kluyver and van Niel to accommodate rod-shaped bacteria possessing the general properties of the enteric group but motile by means of polar flagella (MacInnnes *et al.*, 1979).

The aeromonads are Gram-negative rods with rounded ends 0.3-1.0 µm in diameter and 1.0-3.5µm in length. They are generally motile by a single polar flagellum (one species, *Aeromonas salmonicida*, is non-motile). They are non-spore forming, oxidase positive, facultative anaerobes and are resistant to the vibriostat 0/129. Metabolism of glucose is both oxidative and fermentative. Optimum temperature for growth is 22-28°C or 35-37°C depending upon the species, although some strains do not grow at 37°C. The guanine-plus-cytosine content of the DNA is 57 to 63 mol% (Roberts, 1993, Joseph and Carnahan, 1994, von Graevenitz and Altwegg, 1991).

4.1.2 TAXONOMY

Although the genus *Aeromonas* is currently placed within the family Vibrionaceae, phylogenetic studies by Colwell *et al.*, (1986), based on 5s ribososmal sequencing, have resulted in the proposal of a new family, Aeromonadaceae. The genus *Aeromonas* is divided into two distinct groups: the psychrophilic, non-motile organisms and the motile mesophile group. The non-motile group are an easily identified homogeneous collection designated *Aeromonas salmonicida*. The identification of the motile mesophilic aeromonads is more complex (Janda and Duffey, 1988, Joseph and Carnahan, 1994). In Bergey's Manual of Systematic Bacteriology the motile group is divided, on the basis of biochemical characteristics, into three species: *A. hydrophila, A. caviae* and *A. sobria* (Popoff 1984). However DNA-DNA hybridisation studies have revealed that each of the three species contains members of different hybridisation groups (HG) which are biochemically indistinguishable from each other (Kuijper *et al.*, 1989). Biochemically distinct species are referred to as phenospecies, whereas members of discrete DNA HGs are called genospecies or genomic species (Cascon *et al.*, 1996). In the last decade the taxonomy of the genus *Aeromonas* has undergone major revisions. The original three phenospecies now comprise fourteen DNA hybridisation groups and there are currently ten designated phenospecies: *A. caviae, A. eucrenophila, A. hydrophila, A. jandaei, A. media, A. schubertii, A. sobria, A. trota A. veronii* biovar sobria and *A. veronii* biovar veronii (Carnahan and Altwegg, 1996).

Two additional new species have been proposed recently. The first *A. allosaccharophila* was proposed by Martinez-Murcia *et al.* (1992a) as a new

mesophilic aeromonad species based on phenotypic studies, 16S rRNA gene sequence analysis and DNA-DNA hybridisation studies of three atypical strains (Martinez-Murcia *et al.* 1992a). The second *A. encheleia* was proposed by Esteve *et al.* (1995a) as a new aeromonad species isolated from European eels and based only on four isolates. These studies both used extremely small numbers of isolates and neither of the authors included DNA HG2 in their DNA-DNA hybridisation studies. The recommendations from the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics (Wayne *et al.* 1987) suggested that DNA-DNA hybridisation represents the best procedure for establishing new species at this time. Therefore until the newly proposed species have been shown to be different from DNA HG2 and larger numbers of isolates have been examined their identity cannot be confirmed.

New additions to the genus *Aeromonas* have often contributed to the taxonomic confusion. For example *Aeromonas sobria,* originally proposed by Popoff and Vernon in 1976, was found to encompass at least three separate DNA hybridisation groups (DNA groups 7, 8 and 9) with the type strain CIP 7433, residing in DNA group 7. Later DNA hybridisation studies revealed that all clinical isolates with the *A. sobria* phenotype belonged to DNA group 8. A new species, *A.veronii* (ornithine decarboxylase positive) which did not hybridise with any of the known *Aeromonas* type strains, including that of *A. sobria* (DNA group 7) was proposed as DNA group 10. However clinical *A. sobria* (DNA group 8) was found to be genetically identical to *A. veronii* (DNA group 10). Although the species *A. sobria* (DNA group 7) is a valid one, it is represented by only a small number of environmental strains including the type strain, CIP 7433. To date all clinical strains resembling *A. sobria* belong to DNA

group 8, not DNA group 7, and since DNA group 8 is genetically identical to DNA group 10, clinical strains formerly identified as *A. sobria* are now a biovar of *A. veronii* (Carnahan *et al.*, 1991a).

Further confusion exists over the phenospecies *A. enteropelogenes* and *A. ichthiosmia* which have been found to be identical to the earlier proposed *A. trota* and *A. veronii* biovar sobria (Collins *et al.*, 1993). *Aeromonas trota* and *A. veronii* biovar sobria are widely accepted in the literature however many researchers remain unaware that *A. enteropelogenes* and *A. ichthiosmia* may also be synonyms for these phenospecies (Carnahan and Altwegg, 1996).

4.1.3 AEROMONAS INFECTIONS

Aeromonas spp. are widely distributed in stagnant and flowing fresh waters, in salt waters interfacing with fresh waters, in fish tanks, in water supplies (even chlorinated ones) and in sewage (von Graevenitz and Altwegg, 1991). *Aeromonas* spp. are common, often predominant in the microflora of aquatic animals and are frequently recorded as the cause of disease in amphibians, reptiles and fish (Roberts, 1993). Until recently interest in the role of aeromonads as the cause of disease in aquaculture facilities was dominated by the non-motile *Aeromonas salmonicida* which is the widely distributed cause of septicaemia and furunculosis in fish (Joseph and Carnahan, 1994). Diseases caused by motile aeromonads were described using non-specific terms such as "red-leg" in frogs and "bacterial haemorrhagic septicaemia" or "motile aeromonad septicaemia" in fish because of the confused taxonomy of the genus. Identification of motile aeromonads isolated from disease outbreaks was

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restricted to either *A. hydrophila* or the *A. hydrophila* group (Joseph and Carnahan 1994). Investigations into diseases of aquatic animals by *Aeromonas* spp. other than *Aeromonas hydrophila* were limited, though *A. caviae* and *A. sobria* have on occasion been associated with diseases (Joseph and Carnahan, 1994, Roberts, 1993).

Outbreaks of motile aeromonad septicaemia in farmed aquatic animals can reach epizootic proportions with high rates of mortality (Joseph and Carnahan, 1994). Disease outbreaks are often associated with stressed or compromised hosts. The immediate cause of death appears to be a bacterial toxaemia. Bacterial invasion leads to generalised bacteraemia, followed by elaboration of toxins and tissue necrosis. Clinical signs of the disease are rupture of minor blood vessels causing haemorrhages which may be associated with ulcerative skin lesions (Roberts, 1993).

Despite the fact that *Aeromonas hydrophila* was recognised as a pathogen for reptiles and other poikilothermic animals by Sanarelli in 1891, it was not until the 1950s that it was recognised as a human pathogen (Wadstrom and Ljungh 1988). Since then the genus has received increasing attention from the medical community as an important pathogen of humans (Janda and Duffey, 1988). Many mesophilic aeromonads have been associated with gastrointestinal infections and other human diseases such as septicaemia, meningitis, endocarditis, corneal ulcers, wound infections and peritonitis (Martinez-Murcia *et al.*, 1992b). Initially the bacterium was thought to cause systemic disease only in immunosuppressed individuals, however members of the genus increasingly are being recognised as primary, opportunistic agents of disease in

humans (Joseph and Carnahan, 1994). The correct identification of organisms belonging to the genus is therefore an area of great concern.

Accurate identification of *Aeromonas* spp. may lead to association of particular species of aeromonads with specific disease syndromes. Altwegg *et al.,* (1990) discovered that HGs were not evenly distributed among 127 clinical isolates from humans and environmental isolates which were subjected to DNA-DNA hybridisation. Most clinical isolates resided in DNA HGs 1, 4, 5a, 5b, and 8, whereas more than half of the environmental strains resided in DNA HGs 2, 3 and 5a only. However only 13 environmental isolates were examined, as opposed to 110 clinical isolates, making it difficult to draw firm conclusions on the relative frequencies of hybridisation groups from this data. Nevertheless the difference in distribution was striking and suggests that strains from certain DNA HGs might be less virulent to humans than others. It may then be possible to discriminate between potentially pathogenic and non-pathogenic isolates in the environment, which given the ubiquitous nature of the genus in water sources would be of great advantage to medical personnel and to fish and frog farmers.

4.1.4 PHENOSPECIES IDENTIFICATION

Bacterial classification by analyses of physical properties and biochemical capabilities is slow, relatively insensitive and relies on phenotypic properties which vary with conditions of growth. However classification based on genospeciation requires technology which is not available in most diagnostic laboratories (Wilcox *et al.,* 1992). Simple and reliable speciation techniques are required if diagnostic

microbiology laboratories are to be able to identify *Aeromonas* isolates, and so provide further information on their prevalence and pathogenicity.

The classical methods of speciating bacteria are therefore still of interest and many studies have been undertaken to try to reconcile currently proposed phenotypic species with the currently recognised genospecies (Altwegg *et al.*, 1990; Abbott *et al.*, 1992; Kampfer and Altwegg, 1992; Wilcox *et al.*, 1992; Carnahan and Joseph, 1993; Hanninen, 1994). These studies have assessed large numbers of phenotypic characteristics of mostly clinical aeromonads to establish reliable identification profiles and schema. They included growth properties, morphology, biochemical tests for the detection of metabolic products or enzyme action, carbon source utilisation tests and antibiotic resistance studies. Conventional biochemical tests are however time consuming. They are not suitable for the occasional speciation of small numbers of isolates because of the limited shelf life of media, and are unwieldy if large numbers of isolates are handled. Several large studies have combined the use of conventional biochemical tests with commercial micro-methods such as the API-20E strip (API Systems, BioMeirieux, France) (Altwegg *et al.*, 1990, Wilcox *et al.*, 1992, Carnahan and Joseph 1993).

The approach developed by Carnahan *et al.*, (1991a) and designated Aerokey II has been used successfully to identify human clinical and veterinary isolates. Aerokey II was developed from a large data base of over 50 characteristics on 167 *Aeromonas* isolates. A subset of 18 tests were selected through a customised, computer software system (FLOABN) designed to select those tests which provided the greatest

specificity for differentiation between species. An identification system consisting of seven tests was then developed to identify seven of the most frequently isolated human clinical species and biovars including *A. hydrophila, A. veronii* bv sobria, *A. caviae*, *A. veronii* bv veronii, *A. schubertii, A. jandaeii* and *A. trota*. This selection of tests provides a single dichotomous key using a combination of a commercialised micro-method, the API-20E strip, and three conventional tests readily available in most clinical diagnostic laboratories.

One important factor in the biochemical taxonomy of *Aeromonas* spp. is the dependence of test results on external conditions, such as the incubation temperature and slight variations in the composition of test medium. Commercial tests such as API 20E can give false-negative results in the production of acid from D-sorbitol, rhamnose or arabinose if the incubation temperature is 37°C. In general an incubation temperature of 30°C has been shown to give reproducible results in carbon source utilisation or sugar fermentation tests (Hanninen, 1994).

Other methods of phenotypic characterisation of the aeromonads which have so far been attempted include serotyping, haemagglutination patterns, enzyme electrophoresis and whole-cell protein fingerprints. Serotyping has not proved very successful because many strains cannot be typed with currently available sera. Haemagglutination suffers from poor reproducibility. Whole-cell protein electrophoresis has been used but has the disadvantage that a large number of bands (up to 60) need to be analysed (Mulla and Millership, 1993).

Sensitivities to antibiotics, chemotherapeutics and other antimicrobials such as the vibriostatic agent 0/129, have frequently been tested in numerical taxonomic studies (Logan, 1994). Where sensitivity patterns are fairly constant they may be of value for classification and identification. However bacteria of medical or veterinary importance frequently encounter antimicrobial agents and may acquire drug resistance. Antibiotics play an important role in the intensive culture of aquatic animals and several groups of antibiotics are in use in aquaculture. These include the sulphonamides and potentiated sulphoamides, tetracyclines, quinolones, nitrofurans, macrolides, chloramphenicol, penicillins, aminoglycosides and cephalosporins. Antibiotic sensitivity patterns of isolates from aquatic animals are therefore not constant and are of little value for phenotyping isolates.

Antibiotic resistance data is presented in this chapter to complete the phenotypic description of the bacteria. However this data is not presented as an aid to characterising the isolates but is descriptive of the microflora from the farms at the time of sampling. It is included as a reference to which farm management practices may be related.

Most studies agree that a fairly large number of biochemical tests show significant differences among the three major clinical species: *A. hydrophila*, *A. veronii* bv sobria and *A. caviae* (Altwegg *et al*, 1990, Wilcox *et al*, 1992, Carnahan and Joseph, 1993). However additional tests that are probably not very suitable for routine diagnostic laboratories (e.g. multi-locus enzyme electrophoresis) are needed to separate the remaining DNA groups. Identification of *Aeromonas* isolates to the genospecies level

by phenotypic analysis therefore although possible is often impractical. A feasible alternative is to identify aeromonads to the level of the major clinical phenospecies on the basis of selected biochemical tests whilst accepting that phenospecies identification and genospecies identification do not always correlate and that failure to use a reasonable number of biochemical tests will increase the number of misidentifications.

4.1.5 OBJECTIVES

The objectives in this part of the study were;

To characterise *Aeromonas* spp. isolated from healthy and diseased cultivated frogs using classical biochemical methods and two commercial micromethods: API 20 E and Biolog^{TM}.

To establish whether species of interest in frog septicaemic disease can be phenotypically differentiated from other motile aeromonads.

4.2 MATERIALS AND METHODS

Bacterial isolates were initially distinguished on the basis of colony morphology and by growth on selective media. Suspected pathogens were identified to generic level by examination of the shape, Gram staining characteristics and motility of the cells, and by testing isolates for the presence of oxidase enzymes and the ability to metabolise glucose fermentatively or oxidatively. The procedures for these standard first stage identification tests are given in Appendix 1.

Isolates were originally classified to species level on the basis of their biochemical reactions in the API 20 E enterobacteriacae system (BioMerieux, France). Further phenospeciation was carried out following the identification system (Aerokey II) described by Carnahan *et al.*, (1991a). The Aerokey II uses a combination of an API 20E strip and three conventional tests: aesculin hydrolysis on agar, gas from glucose on a Triple Sugar Iron Agar slant (Oxoid) and resistance to cephalothin (30μg) using the disc diffusion method. Selected frog isolates and twelve type strains were also classified using the Biolog GN Microplate™. The antibiotic sensitivity patterns of all *Aeromonas* isolates from Thailand were examined.

4.2.1 BACTERIAL ISOLATES

Type strains of *Aeromonas hydrophila* (DNA 1) and *Aeromonas veronii* biovar *sobria* (DNA 8) were obtained from the National Collection of Marine and Industrial Bacteria (NCIMB), Aberdeen, Scotland. The type strain of *Aeromonas jandaei* (DNA 9) was obtained from the American Type Culture Collection (ATCC). Type strains of

Aeromonas salmonicida (DNA 3), *Aeromonas caviae* (DNA 4), *Aeromonas media* (DNA 5), *Aeromonas eucrenophila* (DNA 6), *Aeromonas sobria* (DNA 7), *Aeromonas veronii* biovar *veronii* (DNA 10), *Aeromonas schubertii* (DNA 12) and *Aeromonas trota* (DNA 13) were obtained from the Coleccion Espanola de Cultivos Tipo (CECT) Valencia, Spain. Two *Aeromonas* isolates were obtained from pond water samples; isolate 3W1 came from farm 3 and isolate 5W1 came from farm 5. All other *Aeromonas* strains were isolated from septicaemic and clinically normal cultured frogs in Thailand as described in Chapter 2.

4.2.2 CULTURE CONDITIONS AND MAINTENANCE OF BACTERIA

Bacterial isolates were maintained for short periods at room temperature on tryptone soya agar (TSA, Oxoid) slants and for the longer term at -70°C in cryo-preservative in commercially prepared glass beads (Protect, Technical Service Consultants Ltd, UK). Isolates were subsequently recovered into tryptone soya broth (TSB, Oxoid) and subcultured on TSA. Unless stated otherwise all bacterial cultures were incubated at 30°C.

4.2.3 API 20E IDENTIFICATION KIT

The API 20E strip is a rapid identification kit containing 23 conventional biochemical tests in miniaturised form. It consists of microtubes containing dehydrated media which are reconstituted when inoculated with a suspension of bacterial culture. During incubation, metabolism produces colour changes that are either direct or are revealed

by the addition of reagents. The test strips were inoculated according to the
manufacturers instructions. In brief:

- An API strip was placed in a moist incubation tray.

- Several colonies of growth were removed from an overnight TSA culture and
  suspended in 2ml sterile distilled water.

- The suspension was aliquoted into the microtubes. Those tests requiring anaerobic
  conditions were then sealed with sterile liquid paraffin.

- The strip was covered and incubated overnight at 30°C.

- The strip was examined and direct reactions were recorded. Additional reagents
  were added where required and the final results recorded.

- The results were read by referring to the manufacturers Interpretation Table in the
  API 20 E manual.

4.2.4 AESCULIN HYDROLYSIS.

A single bacterial colony from an overnight culture on TSA was streaked onto
aesculin agar (Appendix 1) and the plate incubated overnight at 30°C. Development
of a deep blue/black colour around the bacterial colonies was considered positive, no
colour change in the media was considered negative.

4.2.5 GAS FROM GLUCOSE ON TRIPLE SUGAR IRON AGAR

Triple sugar iron agar (Oxoid) was prepared following the manufacturers instructions
and dispensed into test tubes. Tubes were inoculated from an overnight TSA culture

by a single stab with a straight wire and incubated at 30°C. Production of gas bubbles along the line of inoculation after overnight culture was considered a positive reaction, no bubbles was considered negative.

4.2.6 BIOLOG

The Biolog GN Microplate™ is a standardised micromethod for identification of Gram negative bacteria. The Microplates test the ability of micro-organisms to utilise 95 different single carbon sources. Each well on a microtitre plate contains dehydrated media and tetrazolium violet, a redox dye, which colorimetrically indicates utilisation of the carbon sources. The test therefore yields a characteristic pattern of purple wells which constitutes a "metabolic fingerprint" for each inoculated isolate. The Microplates were inoculated according to the manufacturers instructions. In brief:

- Sterile 0.85% NaCl and the required number of microplates were prewarmed to 30°C thus avoiding any loss of bacterial viability due to temperature stress.

- Cells from an overnight TSA culture were suspended in the sterile saline and adjusted to give a density of approximately 3×10^8 cells/ml using the Biolog turbidimeter™.

- The cell suspension was promptly dispensed into the Microplate using a multichannel pipette. Isolates were not held in saline for longer than 10 minutes before pipetting as some strains can lose metabolic activity if held too long without nutrients. All wells were filled with precisely 150µl of cell suspension.

- The microplates were covered and incubated overnight at 30°C.

- A visual reading of the pattern of purple wells was recorded after 4 hours incubation and after overnight incubation (16-24) hours. The colour density in each well was compared to the negative control well, A-1, on each plate. All wells visually resembling the A-1 well were scored as negative and all wells with a noticeable purple colour (compared to well A-1) were scored as positive. Wells with extremely faint colour, or with small purple flecks were scored as borderline.

- Isolates were identified using the Microlog 1 software package (Version 3) which found the closest species match.

- Biolog identification criteria were used, i.e. species identification was considered acceptable if a similarity index ≥ 0.75 at 4h or ≥ 0.50 at 24h was achieved.

4.2.7 CLUSTER ANALYSIS

The Biolog characters were scored as positive (1), negative (0) or borderline (0.5). Cluster analysis was performed with the Microlog^{TM} software package, using the squared Euclidean distance coefficient and UPGMA clustering strategy (unweighted pair group method with arithmetic averages).

4.2.8 ANTIBIOTIC SENSITIVITY PROFILES

Selected *Aeromonas* isolates from Thailand were tested for sensitivity to a range of antibiotics used commonly in aquaculture facilities. Where several *Aeromonas* isolates were recovered from one animal only those showing differing biochemical properties were included in the analysis of antibiotic sensitivity. Sensitivity to Cephalothin was determined for all isolates as part of the Aerokey II profile. A

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modification of the disc diffusion method described by Acar and Goldstein (1986) was followed. Bacterial lawns were prepared from colony suspensions in sterile distilled water spread on TSA using a sterile glass spreader. Commercially prepared filter paper discs (Oxoid) impregnated with Oxytetracycline 30μg, Oxolinic acid 2μg, Amoxycillin 10μg, Sulphamethoxazole 25μg, Sulphamethoxazole/trimethoprim 25μg, Chloramphenicol 10μg and Cephalothin 30μg, were placed on the agar by an automatic disc dispenser. The plates were incubated overnight at 30°C. The diameter of the zone of inhibition around the antibiotic disc was measured and sensitivity assessed according to the following scale (modified from Inglis *et al.*, 1991):

Zone size: >18mm = sensitive, 13 - 18mm = reduced sensitivity, < 13mm = resistant.

4.3 RESULTS

4.3.1 FIRST STAGE IDENTIFICATION AND API 20E

First stage identification tests on samples from the field trips revealed the presence of many suspected aeromonads in the diseased frogs and some suspect isolates from the clinically normal frogs. Isolates which were Gram negative, motile, glucose fermentative and oxidase positive were further characterised using the API 20 E system into *Aeromonas hydrophila*-like isolates and *Aeromonas sobria*-like isolates. The API tests also identified a group of isolates which utilised a very small number of sugars and notably did not utilise sucrose. The pattern of sugar utilisation within this group was consistently different from any of the other isolates and also differed from that of the type strains tested see Table 4.3.1.1. Production of acid from sucrose is a common feature of motile aeromonads except *Aeromonas jandaei* and *Aeromonas schubertii*. However *A. jandaei* utilises melibiose, a reaction for which the Thai sucrose negative isolates were 100% negative and *A schubertii* does not produce indole and cannot utilise mannitol whereas the Thai sucrose negative isolates were 100% positive for both these reactions. The sugar reactions were remarkably consistent within this group, unlike those of the *A. hydrophila*-like isolates and *A. sobria*-like isolates where there was considerable phenotypic variation. This sucrose negative group was designated *Aeromonas* unspeciated (Au). The morphology of the group designated Au also differed from that of the other *Aeromonas* isolates. In Gram stained smears *A. hydrophila*-like and *A. sobria*-like isolates typically appeared as Gram-negative, regular, short to medium rods (Figure 4.3.1.1), whereas the Gram negative Au isolates exhibited a strong pleiomorphism with bacterial shape ranging

from coccobacilli to rods and sometimes filamentous forms could be identified (Figure 4.3.1.2).

Table 4.3.1.1 Percentage of isolates giving positive reactions on API 20 E tests

Test	A. u n = 23	A. s n = 26	A. h n = 13	ref. A s. ncimb 37	ref. A h. ncimb 89	ref. A. j atcc 49568	ref. A. sch* atcc 43700
β-galactosidase	100	100	100	+	+	+	+
Arginine dihydrolase	100	100	100	+	+	+	+
Lysine decarboxylase	95.7	92.6	21.4	-	-	+	+
Ornithine decarboxylase	0	3.7	0	-	-	-	-
Citrate utilisation	78.3	88.9	92.9	+	+	+	+
Indole production	100	96.3	85.7	+	+	+	-
Voges-Proskauer	54.5	100	92.3	+	+	+	-
Gelatin hydrolysis	100	100	100	+	+	+	+
Acid from:							
Glucose	100	100	100	+	+	+	+
Mannitol	100	100	100	+	+	+	-
Rhamnose	0	0	35.7	-	+	-	-
Sucrose	0	100	100	+	+	-	-
Melibiose	0	7.4	0	-	-	+	-
Amygdalin	0	29.6	92.9	-	+	-	NA
Arabinose	0	0	100	+	+	-	-

Au = *Aeromonas* unspeciated, As = *Aeromonas veronii* biovar *sobria*, Ah = *Aeromonas hydrophila*, Aj = *Aeromonas jandaei*, A. sch = *A.schubertii*, ref. = reference strains, * data from Hickman-Brenner *et al.*, (1988), NA = not available. (The tests for; H_2S production, urease, tryptophane desaminase, acid from inositol, acid from sorbitol were negative for all isolates and are therefore not included in the table.)

Figure 4.3.1.1 Gram's stain of *Aeromonas hydrophila*.

Figure 4.3.1.2 Gram's stain of *Aeromonas* unspeciated.

Aeromonas unspeciated was isolated from the internal organs of frogs from all five of the regions visited. It was isolated from one skin sample and one ulcer but not from any intestinal samples. In contrast *A. sobria* and *A. hydrophila* were isolated from the skin and intestine of post-metamorphic diseased and clinically normal frogs. No isolates were obtained from the internal organs of post metamorphic frogs. However two isolates of *A. sobria* and three isolates of *A. hydrophila* were obtained from the internal organs of septicaemic metamorphosing tadpoles. The distribution of the *Aeromonas* isolates is summarised in Table 4.3.1.2.

Table 4.3.1.2 Number of bacterial isolates from internal and external organs

	A. unspeciated	*A. sobria*	*A. hydrophila*
Liver	9		2*
Heart	7		
Spleen	9		
Kidney	8	1*	1*
Muscle	3	1*	
Pericardial fluid	1		
Peritoneal fluid	1		
Total internal isolates	**38**	**2***	**3***
Intestine		9	4
Skin	1	13	6
Ulcer	1		
Total external isolates	**2**	**22**	**10**

* isolated from tadpole

4.3.2 AEROKEY II

Further identification tests using the Aerokey II allowed division of the initial two *A. hydrophila* and *A. sobria* like groups into six species. There was however considerable variation of reactions within the species, indeed the Aerokey II includes variants of *A. hydrophila* which have different arabinose utilisation patterns. The group of sucrose negative isolates identified by the first set of tests were again clearly and consistently different (Table 4.3.2) however following the criteria of Aerokey II these isolates should be classified as *A. hydrophila*.

Table 4.3.2 Percentage of isolates giving positive reactions in Aerokey II

Test	Au n=10	Ah n=12	Avbvs n=11	Ac n=5	Avbvv n=3	A sch n=1	ref As ncimb37	ref Ah ncimb89	ref Aj atcc49568	ref A sch* atcc43700
AES	100	100	0	100	100	-	-	+	-	-
TSI	90	100	82	0	100	+	+	+	+	-
ARA	0	83	0	60	0	-	+	+	-	-
CEPH	100	83	9	100	0	-	-	+	+	-
IND	100	92	100	100	100	-	+	+	+	-
VP	80	92	100	80	100	-	+	+	+	-
SAC	0	100	100	100	100	+	+	+	-	-

AES = aesculin hydrolysis, TSI = production of gas from glucose on triple sugar iron agar slant, ARA = acid from arabinose, CEPH = resistance to cephalothin, IND = indole production, VP = Voges-Proskauer, SAC = acid from sucrose.

Au = *Aeromonas* unknown, Ah = *Aeromonas hydrophila*, Avbvv = *Aeromonas veronii* biovar veronii, Avbvs = *Aeromonas veronii* biovar sobria, Ac = *Aeromonas caviae*, Asch = *Aeromonas schubertii*, ref. = reference strain *Data from Hickman-Brenner *et al.*, (1988).

4.3.3 BIOLOG IDENTIFICATION

Readings were taken from the Biolog plates initially at 4 hours and 24 hours following the manufacturers instructions. However at 4 hours the colour changes were too few on the plates inoculated with Au to give an identification. The plates inoculated with type strains had many more positive reactions at this stage including the *A. hydrophila* type strain. However the similarity index was usually under 0.75 and therefore unacceptable at that time interval. Further work with the technique indicated that an incubation period of 60 hours at 30°C gave consistent and easily readable results for Au. Incubation periods under that gave variable results with often unacceptable similarity indices (Table 4.3.3.1).

Table 4.3.3.1 Biolog species identification of Au isolates after 24 hours and 60 hours incubation

Isolate code	24 hours	Similarity Index	60 hours	Similarity Index
1D12	*Pasteurella caballi*	0.640	*A. schubertii*	0.611
2D1	*P. caballi*	0.722	*A. schubertii*	0.747
2D8	*A. schubertii*	0.709	*A. schubertii*	0.820
2D16	*A. schubertii*	0.792	*A. schubertii*	0.848
2D25	*Vibrio mimicus*	0.464	*A. schubertii*	0.603
3D29	*P. caballi*	0.566	*A. schubertii*	0.859
4D3	*V. mimicus*	0.179	*A. schubertii*	0.523
5N21	*Pasteurella* spp. taxon 13	0.199	*A. schubertii*	0.435

After 60 hours incubation the Microlog computer program identified all 10 of the Au isolates as *A. schubertii*, with only one isolate (5N21) given an unacceptable (<0.5) similarity index.

The type strains were readable at 24 hours and their patterns did not change on further incubation. However the system failed to accurately identify 5 of the 11 type strains screened (Table 4.3.3.2). The system accurately identified the following six type strains: *A. hydrophila* DNA 1, *A. veronii* biovar *sobria* DNA 8, *A. trota* DNA 13, *A. jandaei* DNA 9, *A. veronii* DNA 10 and *A. eucrenophila* DNA 6.

Table 4.3.3.2 Type strains wrongly identified by Biolog system

Type strain	Biolog ID	Similarity Index
A. schubertii DNA 12	*Kingella kingae*	0.443
A. caviae DNA 4	*A. media*-like DNA group 5A	0.476
A. sobria DNA 7	*A. schubertii* DNA 12	0.804
A. media DNA 5	*Vibrio cholerae*	0.896
A. salmonicida DNA 3	*Aeromonas* group 11	0.789

The system confirmed the findings of the previous tests that the Au isolates were very consistent in their biochemical reactions. The dendrogram (Figure 4.3.3) generated from this data indicates that the Au isolates form a cluster distinct from the motile *Aeromonas* type strains tested.

Figure 4.3.3 Cluster analysis of Biolog data constructed using unweighted pair-group average and Euclidean distances, showing phenotypic similarity of Au isolates.
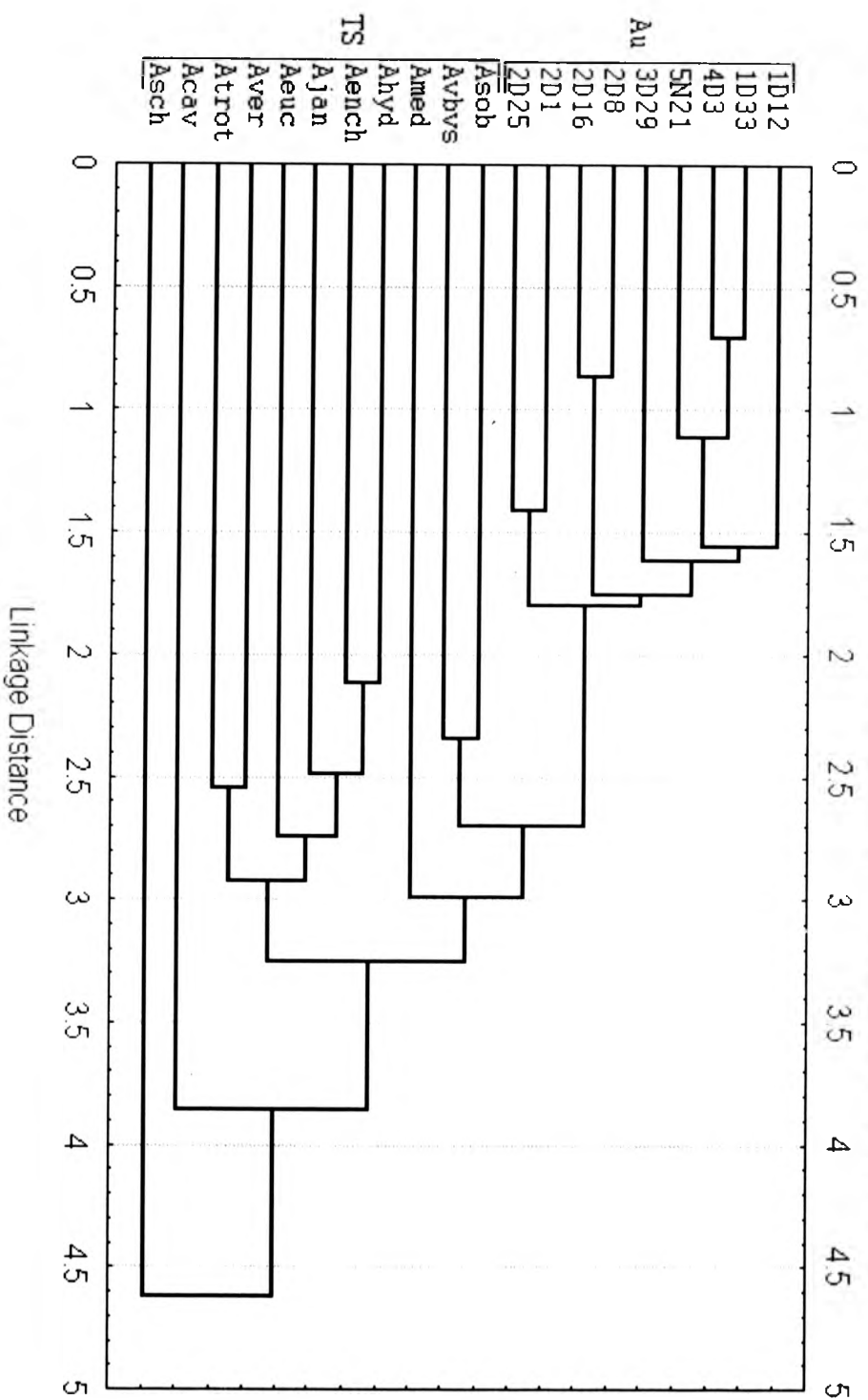
Au = unspeciated Aeromonad
TS = type strains listed in section 4.2.1

4.3.4 ANTIBIOTIC SENSITIVITY PROFILES

The antibiotic sensitivity profiles of all the Thai isolates are summarised in Figures 4.3.4.1, 4.3.4.2 and 4.3.4.3. All isolates were fully resistant to amoxycillin and sulphamethoxazole therefore this data is not included in the graphs. Resistance to cephalothin is presented as part of the Aerokey II profile on page 124.

Resistant strains could be identified for all antibiotics examined. The majority of both Au and *A. hydrophila* isolates were sensitive to oxytetracycline whereas 100% of *A. sobria* isolates were resistant to this antibiotic. A similar pattern was noted for oxolinic acid resistance; under 20% of Au and *A. hydrophila* isolates were resistant to this antibiotic whereas over 70% of *A. sobria* showed resistance. Least resistance existed to chloramphenicol although this was present in several isolates, in particular isolates of *A caviae*.

The patterns of multiple resistance were compared, for each species (Table 4.3.4.1), for each farm (Table 4.3.4.2) For the purposes of these tables, isolates showing reduced sensitivity were considered resistant as the numbers were small and in addition antibiotic treatments would not be recommended in these cases.

Multiple resistance was widespread, all isolates were resistant to three or more antibiotics while 28% were resistant to five antibiotics and 14% were resistant to all 6 antibiotics tested.

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Figure 4.3.4.1 *Aeromonas* isolates (%) resistant to antibiotics
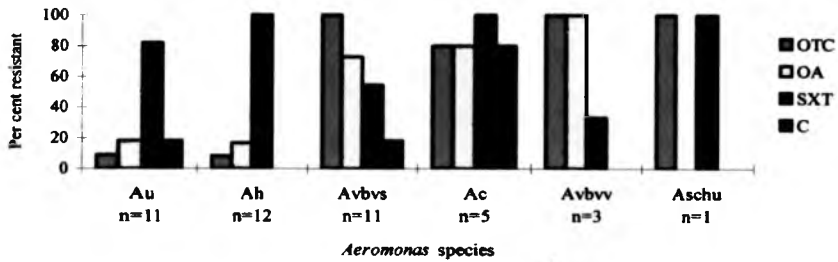
Figure 4.3.4.2 *Aeromonas* isolates (%) with reduced sensitivity to antibiotics
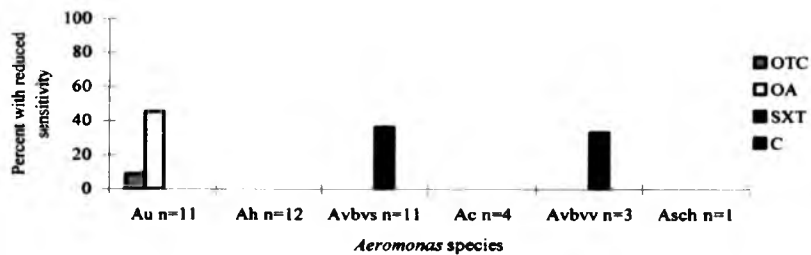
Figure 4.3.4.3 *Aeromonas* isolates (%) sensitive to antibiotics
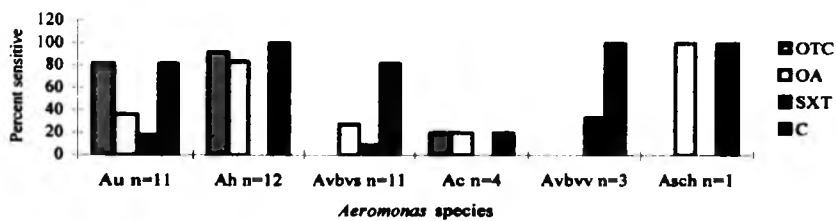
Table 4.3.4.1 Antibiotic resistance patterns of motile *Aeromonas* isolates from

Thailand

Resistance to antibiotic	Au n = 11	Ah n = 12	Avbvs n = 11	Ac n = 5	Avbvv n = 3	Asch n = 1	Total n =43
SXT, RL, AML	4 (36.4)	10 (83.3)		1 (20)			15 (34.9)
OA, RL, AML	2 (18.2)						2 (4.7)
OA, SXT, RL, AML	2 (18.2)	1 (8.3)				1 (100)	3 (7.0)
OTC, SXT, RL, AML			3 (27.3)				4 (9.3)
OT, OA, RL, AML					1 (33.3)		1 (2.3)
OTC, OA, SXT, RL, AML	1 (9.1)	1 (8.3)	6 (54.5)		2 (66.7)		10 (23.3)
OA, SXT, C, RL, AML	1 (9.1)						1 (2.3)
OTC, OA, C, RL, AML			1 (9.1)				1 (2.3)
OTC, OA, SXT, C, RL, AML	1 (9.1)		1 (9.1)	4 (80)			6 (14)

Table 4.3.4.2 Geographic distribution of antibiotic resistance patterns

Resistance to antibiotic	Farm 1 n = 8	Farm 2 n = 10	Farm 3 n = 17	Farm 4 n = 5	Farm 5 n = 3	Total n =43
SXT, RL, AML	4 (50)	1 (10)	9 (52.9)		1 (33.3)	15 (34.9)
OA, RL, AML		2 (20)				2 (4.7)
OA, SXT, RL, AML		3 (30)				3 (7.0)
OTC, SXT, RL, AML				3 (60)	1 (33.3)	4 (9.3)
OT, OA, RL, AML			1(5.9)			1 (2.3)
OTC, OA, SXT, RL, AML	1 (12.5)	3 (30)	4 (23.5)	2 (40)		10 (23.3)
OA, SXT, C, RL, AML					1 (33.3)	1 (2.3)
OTC, OA, C, RL, AML			1 (5.9)			1 (2.3)
OTC, OA, SXT, C, RL, AML	3 (37.5)	1 (10)	2 (11.8)			6 (14)

n = number of isolates

figures in brackets are percentages of number of isolates

Nine distinct antibiotic resistance patterns were observed, none of which were

common to all isolates. Similarities in distribution were again observed between Au

and *A. hydrophila* in that 54.4% of Au and 83.3% of *A. hydrophila* were resistant to

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just 3 antibiotics whereas 100% of *A. sobria* isolates were resistant to four or more

antibiotics. The pattern of antibiotic resistance did not appear to be related to

geographic origin of the isolates.

3.4 DISCUSSION

It is important to identify *Aeromonas* isolates as closely as possible, to determine if different species are associated with certain disease processes (Joseph and Carnahan, 1994). However most clinical laboratories do not have the facilities necessary to identify isolates to the level of genospecies. Many commercial micromethods do not even allow identification to the level of the three phenotypically separable species *A. hydrophila*, *A. caviae* and *A. veronii* bv sobria. In addition strains that possess atypical biochemical properties for a given genospecies are commonly encountered in the clinical laboratory. Such strains do not lend themselves to accurate speciation using either a limited number of phenotypic properties or a dichotomous scheme (Abbott *et al.*, 1992).

The unspeciated *Aeromonas* isolated in this study is unusual in that it appears easily recognisable at an early stage in the process of identification using a standard commercial micromethod. The biochemical homogeneity demonstrated by the Au isolates is uncommon among geographically diverse isolates of motile aeromonads and suggests that they are at least very closely related.

Ninety-five per cent of the Au isolates were obtained from the frog's internal organs. Only two external Au isolates were found; one from the skin and one from an ulcer. These two external Au isolates came from animals which were severely septicaemic and yielded heavy pure cultures of Au from all the internal organs. Au was not isolated from any intestinal samples, nor was it isolated from any internal or external

samples of clinically normal animals. This indicates that Au is not part of the bacterial flora of clinically normal frogs.

In an investigation into the origin of aerobic, Gram negative bacteria in *Rana pipiens,* Van der Waaij *et al.,* (1974) concluded that potentially pathogenic bacteria do not belong to an indigenous frog microflora but originate from external sources such as food supply. In their survey numerous bacterial species and biotypes were isolated from non-hibernating animals. Many biotypes identical to those in the frogs were identified in the insects that were fed as live feed. Almost no Gram-negative species and biotypes were isolated from hibernating frogs which were not fed. The authors therefore deduced that potentially pathogenic bacteria were not indigenous to frogs and infections with these organisms originate exogenously, with the digestive tract as the most likely port of entry.

Although Au was not isolated as part of the normal flora of the gut this does not preclude the gut as a portal of entry for pathogenic bacteria. Many of the frogs with skin ulceration often produced no growth from internal organs or light mixed growth. Therefore entry through skin and in particular damaged skin may not be as significant a portal of entry as previously thought. Indeed the frog skin, although it is thin and permeable and therefore vulnerable to bacterial attack, is equipped with special leucocytopoietic centres which fight such infections. These are associated with large veins or are between the skin and the muscle. Hosts of phagocytic cells can be mobilised from these to attack bacterial and protozoal invaders coming in with water diffusing through the skin. (Nigam, 1977).

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Aeromonas hydrophila and *A. sobria* were found on the skin and in the gut of both healthy and diseased animals and were also isolated from water samples. Although these species are most frequently reported as the cause of disease, they did not appear to be the cause of septicaemia in adult frogs.

Hird at al., (1981) isolated *A. hydrophila* from healthy wild caught frogs and tadpoles (*Rana pipiens*) in Minnesota. The majority of the isolates (80% in adult frogs and 79% in tadpoles) were recovered from the intestine. However 2% and 6% of isolates were recovered from frog and tadpole muscle respectively and 4% of the frog isolates were recovered from frog heart blood. The authors rarely observed dead or obviously ill frogs and tadpoles in the field and yet *A. hydrophila* was frequently isolated. The present studies agree with these findings that *A. hydrophila* and *A. sobria* are part of the normal flora of both the skin and gut of clinically healthy and diseased animals.

Sugita *et al.*, (1985) investigated the microflora in the contents of intestinal tract of the bull frog *R. catesbeiana* at different stages of its development and discovered that the microflora of tadpoles is similar to that of freshwater fishes whereas that of the adult bull frog resembles that of soft-shelled turtles and other higher animals and differs significantly from that of freshwater fish.

It is interesting that different species of motile aeromonads appear to be pathogenic for different life stages of the frogs. *Aeromonas hydrophila* and *A. sobria* were isolated from the internal organs of septicaemic tadpoles while Au was only isolated

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from septicaemic adults. Somsiri *et al,* (1997) reported that *A. hydrophila* and *A. sobria* were pathogenic to tadpoles in laboratory challenges. It is possible that, like the microflora of the intestine, the bacteria which are pathogenic to various frog life stages reflect the differing milieu of the host animals. The tadpole isolates resemble typical aquatic opportunists, as observed in most fish infections. Au, however, may be a special biovar of *A. hydrophila* or a previously unspeciated motile *Aeromonas* which is adapted to infect the terrestrial stage of the frog life cycle.

Despite the fact that many authors (Altwegg *et al.,* 1990, Abbott *et al.,* 1992, Millership and Want, 1993) think that standard commercial micromethods are insufficient to identify the majority of motile aeromonad genospecies and even phenospecies, the API strip was sufficient to distinguish Au from the *A. hydrophila* and *A. sobria* isolates. Therefore this micromethod with first stage identification tests proved an adequate screening for the motile aeromonads from the frogs.

The attempt to further speciate the isolates with the Aerokey II was not satisfactory and illustrates the limitations of dichotomous keys. One of the problems with a scheme such as Aerokey II is that atypical results in a single test, particularly at the top of the hierarchy, will lead to major errors in identification (Millership, 1996). The use of a dichotomous key entails progression along branching characterisation pathways, depending upon clear-cut "yes" (positive) or "no" (negative) test results at each stage, to finally reach an identification. Thus the initial identification characteristics are very heavily weighted. Tables have an advantage in that a more comprehensive range of

characteristics is considered, individual characteristics are more evenly weighted and allowance is made for aberrant reactions. Misidentification is therefore less likely.

Using Aerokey II all the Au isolates were identified as *A. hydrophila*. It can however be seen from Table 4.3.1.1 that Au is consistently different to *A. hydrophila* in several of the sugar fermentation reactions. However these tests received very little weighting in the key. In particular all the Au isolates were negative for sucrose fermentation whereas the type strains of *A. hydrophila* are positive for this reaction and in previous surveys (Altwegg *et al.*, 1990, Abbott *et al.*, 1992) 100% of *A. hydrophila* isolates utilised sucrose. The ability to utilise sucrose was selected by Abbott *et al.* (1992) as one of nine standard biochemical tests which could be used as a starting point to separate DNA HGs. In this biochemical format *A. hydrophila* isolates are 100% positive for sucrose utilisation. The Aerokey however did not include this reaction on the right hand side of the key, therefore the Au isolates, although sucrose negative, were identified as *A. hydrophila*.

The Aerokey was developed for clinical isolates of motile aeromonads and the majority of isolates (131) on which the key is based were human clinical isolates. Only 8 veterinary and 13 environmental strains were used (Carnahan *et al.*, 1991a). The weighting of the key therefore reflects this and is probably not appropriate for the speciation of aquatic animal pathogens.

The results from the Biolog trials were similarly inconclusive. It is worrying that Biolog failed to accurately identify 5 of the 11 *Aeromonas* type strains screened, as fingerprints for these bacteria form part of the Biolog data base. It is possible that reader error contributed to the misidentifications, the plates were read visually and some reactions were marked as border line which may have been positive. Knight *et al.*, 1993) reported that while Biolog had a high success rate identifying *Acinetobacter* at the level of genus (82.8%), it did not give results which compared well with earlier identification of genospecies. In addition when 11 type strains were run through the Biolog system, 1 type strain was misidentified, two were not identified and 1 was identified but with a low ID value, the remaining 7 were successfully identified. Earlier studies also report a similar error rate. This has been attributed to visual reading of plates (Miller and Rhoden, 1991) but Knight *et al.*, (1993) do not consider that this explained totally their results as with practice plate reading is not difficult. The authors concluded that the data base although recently upgraded may not contain sufficient strains of *Acinetobacter*.

The Biolog system indicated that under laboratory conditions the Au isolates were utilising metabolic pathways closer to *A. schubertii* than *A. hydrophila*. This is of interest because current evidence suggests that *A. schubertii* is a clinically significant human pathogen (Joseph and Carnahan 1994). Although relatively few strains of *A. schubertii* have been recovered, these have invariably been isolated from extra-intestinal sites such as abscesses, wounds, blood and pleural fluid unlike other aeromonads, a large number of which have been isolated from gastrointestinal specimens (Janda 1991). This distribution reflects the distribution of the unspeciated

aeromonad in frogs and lends credence to the suggestion that, rather than being an opportunistic aquatic pathogen, Au may be adapted to infect the terrestrial stage of the frog life cycle. However, although the Biolog data base identified Au as *A. schubertii,* when the type strain of *A. schubertii* was grown on a Biolog plate the results obtained were the furthest removed from the Au cluster and the *A. schubertii* type strain was not identified by the system.

It is probable that the data base does not contain sufficient environmental and aquatic isolates, and as with the Aerokey II, caution should be exercised when applying systems designed for other uses. The Biolog identifications use a data base which is heavily skewed towards human clinical isolates. The *Aeromonas* information came from over one hundred strains consisting of type strains and a collection supplied by Dr Altwegg of the University of Zurich (Barry Bochner pers. comm.). However the studies Dr Altwegg has published are mostly on human faecal isolates (Altwegg *et al.,* 1988, 1990, Martinetti-Lucchini and Altwegg, 1992, Kampfer and Altwegg, 1992). This data base therefore may not be applicable to aquatic animal isolates, and its use could lead to misidentifications. The Biolog data base is considered excellent for most human related microbiological studies but is probably capable of identifying only 35 to 40% of environmental isolates (Barry Bochner pers. comm.). The data base needs to be extended and protocols possibly modified to improve usefulness for aquatic isolates.

If their limitations are recognised, human based systems can be adapted for use with aquatic isolates. The API 20E system was originally developed for medically

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important isolates of the family Enterobacteriacae which grew at 37°C within 48 hours. For non-human isolates lower incubation temperatures have been used and the system successfully applied in fish diagnostic laboratories for many years (Austin *et al.*, 1995).

Biolog can be used for aquatic and environmental isolates to give phenotypic fingerprints and to recognise certain isolates again when they occur. The system has recently been extended to recognise *Aeromonas* hybridisation groups (A. Hamilton unpublished data). The dendrogram generated by cluster analysis of the Biolog oxidation profiles demonstrated that the Au isolates were all phenotypically very closely related and formed a clearly delineated cluster within the genus see Figure 4.3.4.

While the Biolog data was not completely satisfactory it did show that the Au isolates, distinguished by standard phenotypic tests from different geographical areas in Thailand, were more closely related to each other than to any of the *Aeromonas* type strains tested.

The patterns of antibiotic resistance indicated some phenotypic differences between the *Aeromonas* species isolated, although these characteristics can vary under selection pressure. All isolates were resistant to amoxycillin and sulphamethazole. Amoxycillin is a synthetic derivative of penicillin-G, closely related to ampicillin. *Aeromonas* species are generally resistant to ampicillin, this resistance is thought to be

chromosomal in nature (Carnahan *et al.*, 1991c). *Aeromonas* selective media incorporates 10μg/ml ampicillin to reduce background microbiota, however there are isolates of *Aeromonas* which are susceptible to ampicillin e.g. *Aeromonas trota* (Carnahan *et al.*, 1991c). This fact suggests that the use of such media may result in a negative selection against sensitive isolates. Awareness of ampicillin susceptibility is important not only for *A. trota* but also for other aeromonads e.g. a subset of ampicillin susceptible *A. caviae* strains exists (Carnahan *et al.*, 1991c). The majority of the ampicillin susceptible isolates of *A. trota* originated in Southern or South East Asia, it was therefore felt appropriate to include a synthetic pencillin in the antibiogram particularly as amoxycillin has been used on frog farms in Thailand. However all the Thai motile aeromonads proved resistant to amoxycillin.

The uniform resistance to the sulphonamide drug sulphamethoxazole was also not unexpected. Resistance to sulphonamide drugs is common among bacterial isolates from aquaculture facilities where the drugs have been in use for many years (Snieszko and Bullock 1957). When sulphonamides are used with a potentiating agent such as trimethoprim the therapeutic index is increased and bacteria develop resistance less readily. Both a potentiated sulphonamide and the unpotentiated sulphamethazole were included in the antibiogram as both are in use in Thai aquaculture.

Chloramphenicol is not permitted for treatment of food producing animals in the UK, however its use was known to occur on the farms in Thailand and resistant strains of bacteria were isolated. Pettibone *et al.*, (1996) examined 74 strains of Aeromonas isolated from the skin, intestine, kidney and liver of 16 brown bullhead trout taken

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from Buffalo River NY. This river is highly polluted with discharges from industrial sources and sewage outfalls as well as seepage from inactive hazardous wastes sites. Pollution levels may influence the genetic make-up of resident bacteria and higher incidences of antibiotic-resistant bacteria have been reported from polluted aquatic environments than from less polluted sites. However these authors found that although all bacterial strains demonstrated multiple antibiotic resistance, none of the strains tested were resistant to chloramphenicol. Development of resistance in the Thai isolates to this antibiotic is worrying as it implies undiscriminating use of chloramphenicol.

Multiple antibiotic resistance was present in isolates from all the farms visited. Farms 1, 2 and 3 all had isolates that were resistant to all the antibiotics tested. These farms had reported that red-leg was a frequent problem. On farm 1, 37.5 % of Aeromonas isolates were resistant to the six antibiotics tested. This confirms work carried out by AAHRI scientists in 1993 who isolated bacteria on farm 1 which were resistant to all antibiotics used in Thai aquaculture. Motile aeromonads are potential pathogens of humans causing gastroenteritis and wound septicaemias. The presence of multiple antibiotic resistant strains of this bacteria in a food animal is therefore of public health concern due to the possibility of contaminated meat and also from the point of view of the health of the farm workers.

The incidence of resistance is almost certainly linked to antibiotic usage. It was found in Japan (Aoki *et al.*, 1983) that drug resistant strains of *A. salmonicida* isolated from farmed fish in Japan had increased in direct proportion to the enhanced uncontrolled

use of antimicrobial substances. The presence of multiple resistant bacterial strains on frog farms emphasises the need for properly regulated health control strategies to prevent farmers from relying on blanket antibiotic cover.

The ability to identify potentially pathogenic bacteria present on the farm is fundamental to any health management scheme. Phenotypic tests alone could not speciate Au however the results indicate that a group of motile aeromonads which may be of clinical significance in frog septicaemic disease can be distinguished at an early stage using standard commercial strips.

Further analysis was necessary to establish whether Au was a virulent biovar of *A. hydrophila,* the species described in the literature as the main cause of "red-leg" in frogs; or whether Au, like *A. trota,* was a previously unidentified species of *Aeromonas* arising in South East Asia. Study of the bacterial genome was therefore undertaken to establish the relationships of Au within the genus *Aeromonas*. This work is described in detail in Chapter 5.

Chapter 5. Genotypic Analysis

5.1 INTRODUCTION

The inadequate phenotypic taxonomy of *Aeromonas* species has hampered assessment

of their clinical significance as well as understanding of the epidemiological aspects

of infections. Fortunately the rise in interest in the motile aeromonads has coincided

with increasing technical skills in molecular biology. Investigations are now possible

into the composition of bacterial nucleic acids, which are universal in distribution,

contain vast amounts of information and are unaffected by most changes in

environment (Logan, 1994).

A wide range of techniques exist for analysing variation in DNA sequences. These

employ general principles such as molecular hybridisation, restriction endonuclease

analysis and polymerase chain reaction (PCR) analysis, each of which has

characteristic strengths and limitations (Huys *et al.*, 1996; Moritz and Hillis, 1996).

5.1.1 DNA-DNA HYBRIDISATION

Hybridisation reactions take advantage of the organisation of nucleic acid molecules

into double strands with nucelotides on opposing sides held together by hydrogen

bonds. When double stranded DNA is heated to 100°C, the hydrogen bonds between

complementary base pairs are broken. Subsequent cooling of the solution allows

annealing of complementary strands. DNA from two different species can be

combined, denatured and then allowed to reassociate. The specificity (stringency) and

speed of such a hybridisation reaction can be controlled by manipulating the

reassociation conditions (e.g. salt concentration, temperature, viscosity, pH, DNA

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concentration, fragment size). The greater the stringency, the greater the specificity, or "match", that is required for successful hybridisation to occur. At progressively lower stringency conditions, increased mismatch is tolerated up to a point where random reassociation occurs (Towner and Cockayne, 1993, Werman *et al.*, 1996). A genospecies or hybridisation group is composed of strains whose DNAs have relative binding ratios of 70% under optimal conditions (60°C) and 55% under stringent conditions (70°C) with 5% or less divergence (Altwegg *et al.*, 1990).

The main disadvantages of hybridisation reactions are the laborious procedures, the use of radiolabelling and the relatively large amounts of DNA required in comparison to other methods of nucleic acid analysis. In addition discrete character data are not recovered, the differences among molecules are measured by a single variable (DNA hybridisation) which does not resolve the nature of the sequence variation (Werman *et al.*, 1996, Towner and Cockayne, 1993).

5.1.2 RESTRICTION FRAGMENT LENGTH POLYMORPHISMS (RFLPs)

Variation in nucleotide sequence can be examined indirectly using restriction endonucleases to create DNA fragments. Restriction endonucleases are enzymes, isolated from bacteria, which cut DNA at a constant position within a specific nucleotide recognition sequence, usually 4-6 base pairs (bp) long. There are thousands of restriction endonucleases each of which acts at a characteristic site (Dowling *et al.*, 1996). The number and location of these sites are unique for each genome. Thus when a sample of DNA is digested with a selection of restriction endonucleases and the fragments are separated by gel electrophoresis, the pattern created is unique to that sample. Different endonucleases will give different, reproducible patterns, the

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variations in fragment patterns are referred to as restriction fragment length
polymorphisms (RFLPs) (Logan, 1994, Dowling *et al.,* 1996).

Restriction endonuclease analysis of total chromosomal DNA is a highly
discriminatory tool. However, the complexity of some RFLP patterns can make it very
difficult to obtain useful typing information. Fragments can be so numerous that it is
impossible to read their sizes accurately or reproducibly making the technique tedious,
time consuming and inaccurate when comparison of gel patterns is done by eye.
Automated analysis of photographs using a laser scanning densitometer overcomes
this problem and the data may then be subjected to computer analysis (Altwegg *et al.,*
1988; Logan, 1994)

Alternatively the patterns can be simplified by transferring restriction fragments
separated by gel electrophoresis to a membrane and hybridising with a suitable
labelled probe. Only the fragments that generate a positive hybridisation signal are
then visualised. Use of probes can greatly reduce the number of bands in the original
fingerprint and thereby facilitate comparison between strains. (Towner and Cockayne
1993, Myers *et al.,* 1988). The main disadvantage of this approach is that information
is provided only about those specific regions within the total genome that happen to
hybridise with the particular probe being used.

The technique of "ribotyping" is based on this method. A probe consisting of
ribosomal RNA (rRNA) is labelled with either radioactive or non-radioactive reporter
molecules and hybridised with restriction fragments from genomic DNA. A major
advantage of this approach is that, because of the conserved nature of parts of rRNA

sequences, such a probe is "universal" and therefore able to hybridise with different DNA fragments from a wide variety of bacterial species (Towner and Cockayne, 1993). Unfortunately ribotyping can still be time consuming and inaccurate if automated analysis is not available and band patterns are compared by eye (Huys *et al.*, 1996).

An alternative method for simplifying RFLP patterns has recently been developed. This technique, known as AFLP, detects DNA polymorphisms by selective amplification of restriction fragments (Carnahan and Altwegg, 1996). Essentially the AFLP method combines the principle of restriction fragment polymorphism analysis with highly specific PCR amplification (see section below). Chromosomal DNA is digested with selected restriction enzymes and the resulting fragments are used as templates for primers designed to amplify a range of PCR products. The products are separated by gel electrophoresis, visualised on an autoradiograph which is then analysed automatically (Huys *et al*, 1996).

Although fragment analysis offers less resolution than actual nucleotide sequencing, the techniques are simple, rapid, require very little DNA and are thus useful and cost effective where large numbers of individuals or large segments of a genome are being screened (Dowling *et al.*, 1996).

5.1.3 THE POLYMERASE CHAIN REACTION

This procedure allows specific regions within a sample of genomic DNA to be amplified by as much as a million-fold. The procedure is simple in concept and execution and takes advantage of the normal behaviour of DNA polymerases in synthesising genetic material (Saiki *et al.*, 1988). DNA polymerases act anywhere there is a double/single strand junction in DNA. Polymerases bind to single stranded DNA and catalyse a reaction that attaches deoxynucleotide triphosphates (dNTPs) to form a second complementary DNA strand.

In vitro it is possible to synthesise DNA by adding DNA polymerase to a solution of single stranded DNA template and giving it the appropriate signal to act. This signal is a short stretch of single stranded DNA (oligonucleotide primer) designed to hybridise to target sequences on the template DNA. In PCR amplification two oligonucleotide primers are designed to anneal to specific sites on target DNA. DNA polymerases can only synthesise new DNA in the 5' to 3' direction. The two primers are therefore designed to face each other in a 5' to 3' direction on opposing strands of DNA. Heat denaturation of the DNA produces single stranded templates. Upon cooling single stranded DNA reassociates, the oligonucleotides therefore anneal to template DNA, and extension with DNA polymerase forms two new complementary strands of DNA. This cycle of heat denaturation/annealing/primer extension can be repeated. The extension products are also capable of binding the primers, therefore successive cycles of amplification continue to double the amount of DNA synthesised in the previous cycle. The result is an exponential accumulation of the specific target fragment (Figure 5.1.3).
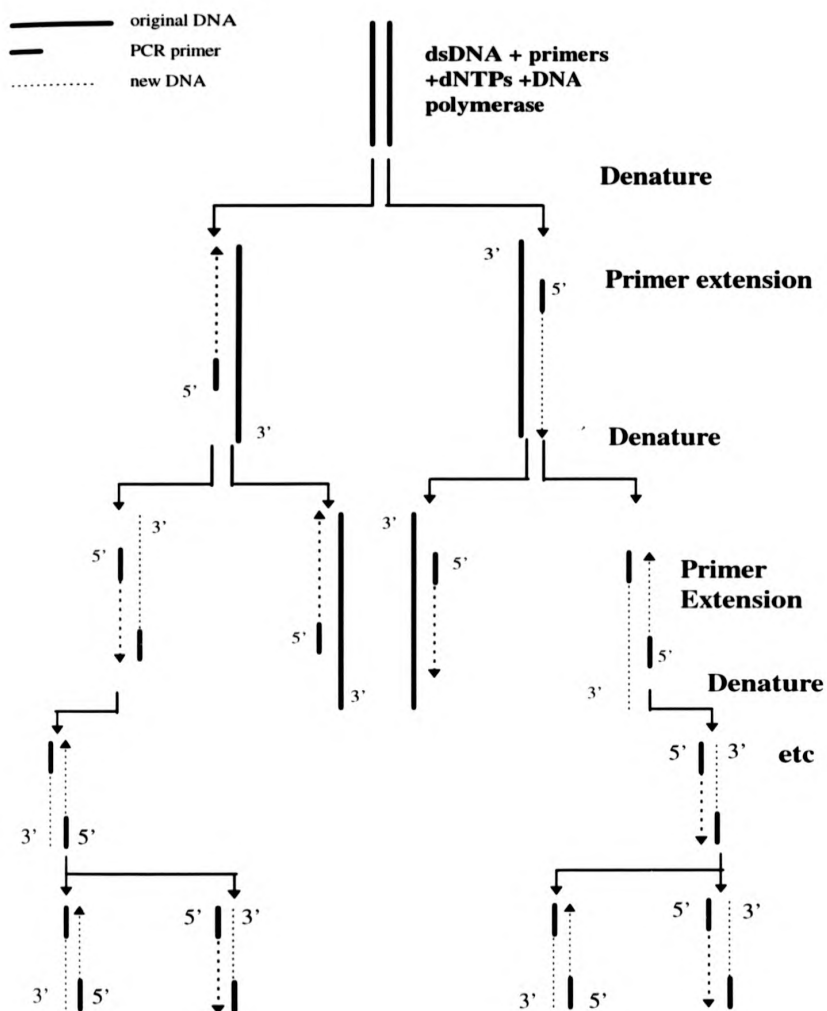
Figure 5.1.3. The polymerase chain reaction (adapted from Saiki *et al.*, 1988). Only the first two reactions are shown completely. The long primer extensions from the original template can only increase additively with each cycle. However the short copies of the DNA bridging the two primers, which first appear in the third cycle, double during successive cycles and rapidly come to dominate the mixture.

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However heat treatment of double stranded DNA to form the single stranded template also destroys most enzyme activity. Originally new polymerase had to be added after every denaturation step making the procedure very laborious, moreover the temperatures at which most DNA polymerases act allow too much non-specific annealing of primers to template. The use of a thermostable DNA polymerase isolated from the bacterium *Thermus aquaticus* (*Taq*) makes PCR practical. *Taq* polymerase retains its activity after heat denaturation of the DNA, making possible continual, uninterrupted cycles of denaturation/annealing/extension. In addition to simplifying the reaction, the high temperature optimum of the enzyme (70-75°C) significantly increases the specificity, yield and length of targets that can be amplified. (Saiki *et al.*, 1988, Myers *et al.*, 1988, Palumbi, 1996).

The polymerase chain reaction plays an important part in the following two methods of DNA analysis; 16s rDNA sequencing and randomly amplified polymorphic DNA analysis.

5.1.4 16s rDNA SEQUENCING

Although nucleic acid sequencing is a relatively new approach in phylogenetic analysis it has gained wide acceptance. During the past few years, sequencing studies have accounted for about one-quarter of phylogenetic studies in general (Hillis *et al.*, 1996). This widespread use is a direct result of the high information content relative to effort needed to collect sequence data compared to some other techniques. The technique is popular because the character data generated (nucleotides) are the basic units of information encoded in organisms and permit quantitative calculation of

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relationships. In addition the information gained is cumulative, as new sequence information can be added to the data set (Hillis *et al*, 1996, Lane *et al.*, 1985).

Of the macromolecules used for phylogenetic analysis, the ribosomal RNAs, particularly 16s RNA have proven most useful because of their high information content, conservative nature and universal distribution (Lane *et al.*, 1985). The extent and nature of the differences among a set of homologous sequences can be used to construct phylogenetic trees. A wealth of comparative sequence data now exist on the small-subunit rRNA genes (rDNA) of the Eubacteria, providing considerable insight into their relationships (Hillis *et al.*, 1996).

All sequencing methods follow the same basic principles. A target sequence must first be defined which contains significant variation within the group of individuals to be compared. The target sequence from each individual must be copied to produce sufficient amounts of DNA for analysis and the copies must be isolated and purified. Amplification of target DNA sequences by the polymerase chain reaction has become the most widely used approach for comparative studies. Cloning using recombinant DNA technology is also a common method but requires more time and effort.

The purified DNA is then sequenced. This step relies on the production of labelled DNA fragments from the amplified DNA. There are two methods currently in use: base specific chemical cleavage of labelled DNA strands (chemical degradation) or controlled interruption of enzymatic DNA replication using dideoxynucleotides (chain termination) (Sambrook *et al.*, 1989).

Most commonly used sequencing protocols are based on the dideoxynucleotide chain

termination method (Rawlinson and Barrel, 1997). Dideoxynucleotides (ddNTPs)

differ from conventional dNTPs in that they lack a hydroxyl residue at the 3' position

of deoxyribose. They can be incorporated by DNA polymerases into a growing DNA

chain through their 5' triphosphate groups. However the absence of a 3'-hydroxyl

residue blocks further DNA extension (Hillis *et al.,* 1996). In dideoxy sequencing

reactions a primer known to be complementary to a segment on the target DNA is

annealed to the target sample. A DNA polymerase is used to produce complementary

copies of the DNA, which incorporate a label that can be visualised in some way.

Although labelling of the nascent template is possible, the efficiency of the reaction

can be greatly increased by using end labelled primers. In fluorescent dye-primer

sequencing and radioactive primer sequencing, the label is incorporated on the 5' end

of the primer. A reaction mixture for DNA synthesis is made up combining a small

amount of one ddNTP with the four conventional dNTPs. There is competition

between extension of the chain and infrequent but specific termination. By using four

different ddNTPs in four separate enzymatic reactions, populations of labelled DNA

fragments are generated that terminate at positions occupied by every A, C, G or T in

the template strand. The labelled fragments can be separated on a polyacrylamide gel

and the nucleotide sequence examined. In cycle sequencing a thermostable enzyme

such as Thermo Sequenase DNA polymerase™ is used to allow repeated cycles of

thermal denaturation, annealing and extension/termination. This increases the amount

of product and signal levels and therefore decreases the amount of template required.

(Hillis *et al.,* 1996; Rawlinson and Barrell, 1997, Sambrook *et al.,* 1989).

Non-automated sequencing is a laborious procedure involving the separation of radioactively labelled DNA fragments on polyacrylamide gel which is exposed to X-ray film to produce an autoradiograph. The sequence of the DNA sample is read by eye directly from the autoradiograph.

Automated sequencing requires costly equipment but eliminates the need for tedious and inaccurate analysis of autoradiographs by eye. Most automatic sequencers use dideoxy-mediated chain termination with fluorescently labelled (rather than radioactively labelled) DNA fragments. The fragments are detected during electrophoresis with the use of a laser. The laser is static with respect to the electrophoresis apparatus and fragments are recorded as they pass a single point. The sequence is recorded directly into a computer and may be interpreted, by computer software or visually, into a DNA sequence (Hillis *et al.*, 1996).

Once determined, homologous DNA sequences must be aligned, examined and relatedness values determined (Hillis *et al.*, 1996). A number of different software packages are available for handling and analysing sequence data (Rawlinson and Barrell, 1997).

In the past decade rRNA gene sequencing has emerged as an immensely powerful tool for determining the interrelationships of organisms (Collins *et al.*, 1993). There are however disadvantages to the technique. If molecular sequences are used to study interrelationships then homologous sequences must already be available from reference organisms. The target sequences must be carefully selected to provide a reliable means of distinguishing among individuals and indicating their relatedness.

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Molecular sequencing is not always a cost or time effective method for obtaining relevant data, especially if many individuals are to be examined. All sequencing work requires highly purified molecular fragments, a high degree of cleanliness is essential, as is strict standardisation of the complicated procedures to avoid erroneous results (Hillis *et al.,* 1996).

5.1.5 RAPD ANALYSIS

The techniques mentioned above are very labour intensive and require specialist skills and equipment often out of reach of the normal diagnostic laboratory. Other studies have focused on finding easy, rapid and accurate ways of analysing the bacterial genome. Several workers have studied the technique of randomly amplified polymorphic DNA (RAPD) as a swift method of nucleic acid analysis without a necessary previous knowledge of the sequence of the bacterial genome. With this technique a single randomly designed primer anneals to complementary sequences on template DNA. A number of arbitrary DNA fragments may then be generated from the bacterial genome by the polymerase chain reaction. These fragments can be separated according to size by agarose gel electrophoresis to form a fingerprint unique to the bacterial genome.

A great advantage of RAPDs is that no prior knowledge of the target DNA sequence is required. The technique is entirely random therefore the entire complement of genomic DNA is targeted. If a sufficient number of primers are used a section of every chromosome is likely to be amplified (Palumbi, 1996).

There are however many disadvantages to the technique. RAPD is not always a

precise process that gives exactly the same results each time. Minor variations in

reaction conditions such as: template quantity or quality, buffer concentration,

polymerase enzymes, primer/template ratio and annealing temperature can lead to

differing results (Inglis *et al.*, 1996). The random nature of the technique means that

artefact due to contamination of samples with extraneous DNA can be difficult to

distinguish from genuine amplification products, therefore stringent hygiene practices

are necessary.

5.1.6 GENOTYPIC ANALYSIS OF MOTILE AEROMONADS

Various investigations into the genome of the aeromonads have been undertaken.

These have included DNA-DNA hybridisation studies, RFLP analysis and ribotyping,

sequencing of the 16s ribosomal DNA, AFLP fingerprinting and RAPD-PCR analysis

(Kuijper *et al* 1989; Martinez-Murcia *et al* 1992a; Moyer *et al.*, 1992; Martinetti-

Lucchini and Altwegg, 1992; Huys *et al* 1996; Oakey *et al.*, 1996).

Popoff *et al.* (1981) studied 55 *Aeromonas* strains and proposed three species of

mesophilic aeromonads *Aeromonas hydrophila, Aeromonas sobria* and *Aeromonas

caviae,* containing 7-8 DNA hybridisation groups (HGs). Fanning *et al.* (1985)

analysed a large group of reference, environmental and clinical strains. They

confirmed the 7-8 genospecies of Popoff *et al.* (1981) and extended the number of

hybridisation groups to at least 10. Over the next six years a number of new species

were proposed and the DNA hybridisation groups of *Aeromonas* were extended to

fourteen (Hickman-Brenner *et al.*, 1987; Hickman-Brenner *et al.*, 1988, Carnahan *et*

al., 1991b; Carnahan *et al.*, 1991c). Table 5.1.6 lists the currently recognised DNA

HGs of *Aeromonas* and their phenospecies identification.

DNA-DNA hybridisation studies have clarified genetic relationships within the genus

Aeromonas however there are still serious problems with *Aeromonas* species

identification because of poor correlations between genotypes and phenotypes. The

lack of reliable phenotypic traits for species discrimination and delineation has led to

continued molecular investigations.

Table 5.1.6 Current taxonomic status of the genus *Aeromonas*

DNA hybridisation group	Genospecies	Phenospecies
1	*A. hydrophila*	*A. hydrophila*
2	Unnamed	*A. hydrophila*
3	*A. salmonicida*	*A. salmonicida*
4	*A. caviae*	*A. caviae*
5A	*A. media*	*A. caviae*
5B	*A. media*	*A. media*
6	*A. eucrenophila*	*A. eucrenophila*
7	*A. sobria*	*A. sobria*
8/10	*A. veronii*	*A. veronii* biovar sobria
9	*A. jandaei*	*A jandaei*
10/8	*A. veronii*	*A. veronii* biovar veronii
11	Unnamed	*Aeromonas* sp.
12	*A. schubertii*	*A. schubertii*
13	Unnamed	*Aeromonas* Group 501
14	*A. trota*	*A. trota*

Adapted from Carnahan and Altwegg (1996)

In a study of different typing methods, Altwegg *et al.*, (1988) assessed the ability of

restriction endonuclease analysis and ribotyping to distinguish between 58

epidemiologically unrelated *Aeromonas* isolates. The authors found that both methods

were highly discriminatory, but concluded that restriction endonuclease analysis was

slightly over-sensitive.

Moyer *et al.*, (1992) investigated the usefulness of ribotyping for the differentiation of clinical and environmental isolates of aeromonads. Results showed that the clinical isolates were all unique but environmental isolates with ribotypes identical to those of the clinical strains were found in water samples. The authors concluded that ribotyping could effectively differentiate otherwise indistinguishable strains of bacteria and that the method could be a powerful tool for epidemiological investigations.

Lucchini-Martinetti *et al.*, (1992) ribotyped restricted genomic DNAs from different *Aeromonas* hybridisation groups. The authors were able to discriminate 12 HGs by qualitative evaluation of band patterns and concluded that this molecular method could identify most *Aeromonas* strains to the level of genospecies. The authors reported that the method was easier than DNA-DNA hybridisation because only minimal amounts of genomic DNA were needed and several strains were analysed on a single gel .

Huys *et al.*, (1996) investigated the ability of AFLP to differentiate the 14 currently defined DNA hybridisation groups. They included a total of 98 *Aeromonas* type and reference strains. Numerical analysis of the band patterns produced by AFLP revealed 13 AFLP clusters which in general supported the current *Aeromonas* taxonomy derived from DNA homology data.

Martinez-Murcia *et al.*, (1992) a undertook a systematic investigation into the interspecific relationships of aeromonads by 16s rDNA sequence analysis. The rDNA sequences were aligned and a phylogenetic tree was produced.
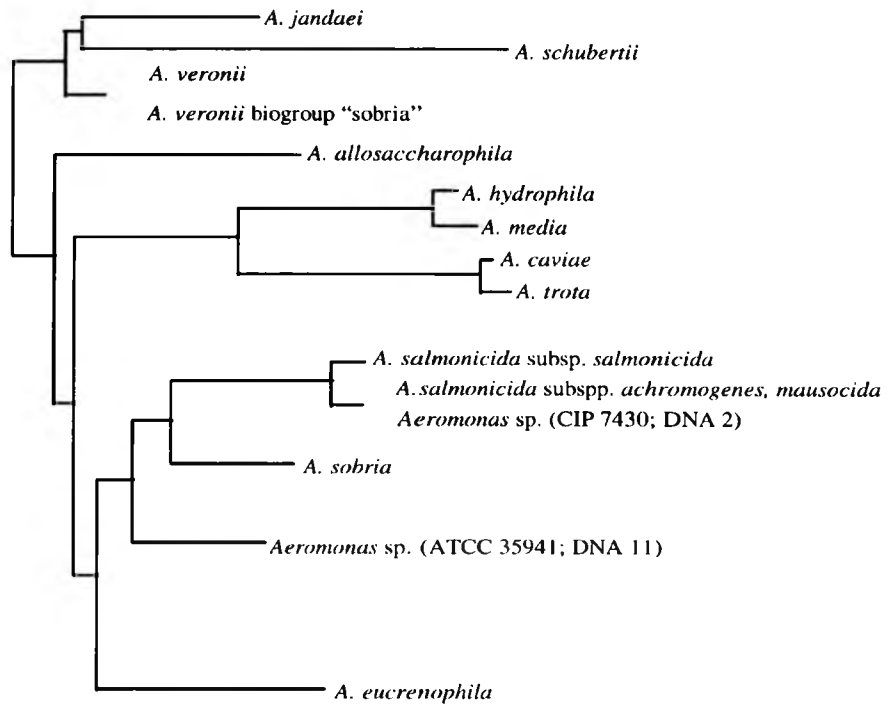
Figure 5.1.6 Phylogenetic interrelationships in the genus *Aeromonas* as determined by 16s rDNA sequence comparison (not to scale). Adapted from Martinez-Murcia *et al.*, (1992b).

The authors showed that species of *Aeromonas* exhibited very high levels of overall sequence similarity with each other. However there were characteristic regions within the 16s rDNA which could be used to differentiate *Aeromonas* species. These hypervariable "signature areas" correspond to the regions around 160 and 460 using *E. coli* numbering systems.

The relationships between *Aeromonas* species derived by 16s rDNA sequencing generally correlated well with the results of DNA-DNA hybridisation, although several discrepancies were noted. The rDNA sequences of *A. caviae* (HG 4) and *A. trota* (HG14) differed by only a single nucleotide in the signature area, although these species are biochemically distinct and share a DNA homology of only 30%. Likewise the 16s rDNA sequence of *A. hydrophila* (HG 1) revealed only three nucleotide differences from the sequence of *A. media* (HG5). In contrast *A. sobria* (HG7) and *A. veronii* biovar sobria (HG 8) which are relatively closely related on the basis of DNA hybridisation data (60 to 65%) form well separated 16s rDNA phylogenetic lines.

Martinez-Murcia *et al.*, (1992b) examined the 16S rRNA gene sequence of some atypical aeromonad strains of uncertain taxonomic position and established that these strains represented a hitherto unknown genetic line within the genus *Aeromonas* for which the authors proposed the name *Aeromonas allosaccharophila* sp. nov. The authors concluded that 16s rRNA sequencing was the most precise method for determining bacterial phylogeny.

In a later paper Collins *et al.*, (1993) used 16s rRNA gene sequence analysis to show that *Aeromonas enteropelogenes* and *Aeromonas ichthiosmia* were identical to *A. trota* and *A. veronii* bv veronii respectively. However in this paper, although the authors recognised that rRNA sequencing is an immensely powerful tool for determining the interrelationships of organisms, they stated that the method failed sometimes to discriminate between closely related species.

Various studies using RAPD-PCR have been undertaken on motile aeromonads (Miyata *et al.*, 1995, Inglis *et al.*, 1996, Oakey *et al.*, 1996). Oakey *et al.*, (1996) noted that the production of a multi-banded fingerprint was dependent on the primer used but found that at least one 10-mer primer will form different fingerprints of multiple bands for all hybridisation groups thus suggesting that RAPD may have potential as a discriminatory tool for aeromonads. Inglis *et al.*, (1996) noted that while RAPD may be of use to identify species in one laboratory, considerable inter laboratory variation occurred. However the authors showed that RAPD analysis can demonstrate DNA similarity and recognise DNA diversity and concluded that the technique may be useful in epidemiological studies for rapid identification of bacteria where a reference source of DNA is available and also in preliminary investigations of relatedness within groups (Inglis *et al.*, 1996).

All of the techniques discussed above have been applied successfully to the typing of aeromonads. No technique is best under all circumstances. Each approach provides interesting and important insights into DNA relatedness of isolates. The choice of which molecular techniques are appropriate to a particular problem is difficult. Currently the definition of bacterial species is based upon chromosomal DNA-DNA relatedness ($\geq 70\%$ homology with a ΔT_m of 0 to 5°C) (Wayne *et al.*, 1987). However DNA-DNA hybridisation requires large quantities of DNA, the procedure is laborious, most methods involve the use of radio-isotopes and only distance data are produced.

In many cases techniques should be used in combination. Most studies of population genetics require examination of large numbers of individuals over large numbers of loci. DNA sequencing is applicable at this level but, despite the introduction of PCR

and autosequencing, large numbers of samples cannot be processed easily. Sequencing

and fragment analyses can be combined with excellent results. Studies that combine

such approaches can maximise effectiveness by combining high resolution with broad

coverage of individuals.

5.1.7 OBJECTIVES

The objective of this part of the study was to establish the phylogenetic

interrelationships of aeromonads pathogenic to frogs and other *Aeromonas* species

using the following broad screening and high resolution methods:

RAPD fragment analysis to compare genomic DNA fingerprints from a large number

of motile aeromonads isolated in Thailand plus type strains of *Aeromonas* HGs.

16s rRNA gene sequencing of 10 Au isolates to provide detailed character data on

their genomic DNA and, compare with previously published *Aeromonas* sequence

data.

5.2 MATERIALS AND METHODS

5.2.1 BACTERIAL STRAINS AND CULTURE CONDITIONS

All the bacterial strains used in this part of the study are listed in Table 5.2.1. Fifteen

reference strains of *Aeromonas* were obtained from the Spanish Type Culture

Collection (CECT). All other *Aeromonas* strains were isolated from septicaemic and

clinically normal cultured frogs in Thailand as described in Chapter 2. Bacterial

strains were grown on Heart Infusion agar or in Heart Infusion broth (Difco) at 30°C

except for *A. salmonicida* which was incubated at 25°C and *A. veronii, A. jandaei and

A. trota* which were incubated at 37°C.

5.2.2 EXTRACTION OF BACTERIAL GENOMIC DNA

Chromosomal DNA was prepared following a modification of the miniprep method

outlined by Ausubel and Frederick (1989).

- Cultures were grown to late log phase in 5mls of heart infusion broth at the

  appropriate temperature. 1.5 ml of each culture were centrifuged for 2 minutes at

  4000g to pellet the cells. The supernatant was discarded and the cells resuspended

  in TE buffer (Appendix 2).

- The bacterial cells were lysed with 30µl of 10% SDS (Appendix 2) and 3µl of 20

  mg/ml proteinase K to give a final concentration of 100µg/ml proteinase K in 0.5%

  SDS. The solution was mixed thoroughly, incubated for 30 minutes at 55°C, re-

  mixed and incubated again until the mixture cleared (usually another 30 minutes).

Table 5.2.1 Bacterial isolates used for genotypic analysis

Code	Phenospecies	Hybridisation group	Source
1D12	Au	ND	liver
1D33	Au	ND	liver
1D54	Au	ND	muscle
2D1	Au	ND	liver
2D8	Au	ND	heart
2D16	Au	ND	liver
2D25	Au	ND	liver
3D29	Au	ND	liver
4D3	Au	ND	spleen
5N21	Au	ND	muscle
2D12	*A. veronii* biovar sobria	ND	intestine
4N15	*A. veronii* biovar sobria	ND	intestine
1D75	*A. hydrophila*	ND	intestine
2D20	*A. hydrophila*	ND	intestine
2N36	*A.veronii* biovar sobria	ND	skin
4D11	*A.veronii* biovar sobria	ND	skin
3D14	*A. hydrophila*	ND	skin
3D37L	*A. hydrophila*	ND	skin
1N15L	*A. caviae*	ND	skin
2N14	*A. caviae*	ND	skin
5N11	*A. schubertii*	ND	skin
2N1	*A. veronii* biovar veronii	ND	intestine
3D37S	*A. veronii* biovar veronii	ND	skin
5W1	*A. hydrophila*	ND	water
3W1	*A. veronii* biovar sobria	ND	water
CECT 839 T	*A. hydrophila*	1	
CECT 894 T	*A. salmonicida ssp. salmonicida*	3	
CECT 838 T	*A. caviae*	4	
CECT 4232 T	*A. media*	5	
CECT 4224 T	*A. eucrenophila*	6	
CECT 837 T	*A. sobria*	7	
CECT 4246	*A. veronii* biovar sobria	8	
CECT 4228 T	*A.jandaei*	9	
CECT 4257 T	*A.veronii* biovar veronii	10	
CECT 4253	*Aeromonas* sp.	11	
CECT 4240 T	*A. schuberti*	12	
CECT 4254	*Aeromonas* Group 501	13	
CECT 4255	*A. trota*	14	
CECT 4199	*A. allosaccharophila*	15	
CECT 4342 T	*A. encheleia*	ND	

- The salt concentration of the solution was raised by the addition of 100µl of 5M NaCl, this was followed by 80µl of CTAB/NaCl solution (Appendix 2), and incubated for 10 minutes at 65°C. It is important that the salt concentration of the solution does not fall below 0.5M at room temperature, as a CTAB-nucleic acid precipitate will form. This part of the protocol removes cell wall debris, denatured protein and polysaccharides complexed to CTAB, while retaining the nucleic acids in solution.

- The CTAB-protein/polysaccharide complexes were extracted by the addition of an approximately equal volume (0.7 to 0.8ml) of chloroform/isoamyl alcohol (24:1) and spinning at 7000g for 5 minutes. A white interface was then visible. The aqueous supernatent was removed to a fresh microcentrifuge tube leaving the interface behind. Any remaining CTAB precipitate was then extracted by the addition of an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and centrifugation at 7000g for 5 minutes. The supernatant was transferred to a new tube and this step was repeated if the solution was not clear.

- Total genomic DNA was precipitated with 0.6 volumes of isopropanol. The DNA was pelleted by centrifugation at 7000g for 1 minute, washed with 500µl 75% ethanol and vacuum dried for 3 minutes.

- The DNA was redissolved in 100µl of TE buffer and treated with 3µl of 10mg/ml RNAase for 30 minutes at 37°C. The solution was then made up to 300µl with TE and extracted once with phenol/chloroform/isoamyl alcohol and once with diethyl

ether. The supernatant was discarded and 0.1 volume of 3M sodium acetate pH5.2 was added.

- The DNA was precipitated in 2 volumes 100% chilled ethanol to make approximately 1.5ml total volume and the tube was mixed gently as the DNA strands formed. The DNA was pelleted and washed with 500µl of 75% chilled ethanol. Finally the DNA was vacuum dried and redissolved in nano pure sterile water.

- The concentration of the DNA was determined from absorbance of solutions at 260 nm (GeneQuant, Pharmacia) (1µl DNA solution in 9µl dH$_2$0). The purity was assessed using the 280:260 nm absorbance ratio. The molecular weight and integrity of the 10µl sample were then checked by electrophoresis on an 0.8% agarose gel containing ethidium bromide. All extracts were stored at -20°C.

5.2.3 RAPD ANALYSIS

Protocols in this section were established by Prof. T. Aoki (pers. comm.) and published by Miyata *et al.,* (1995).

5.2.3.1 Oligonucleotide primers

A randomly designed 12-mer oligonucleotide set was obtained from Wako Pure Chemical Industries Ltd, Japan. The amplification products of seven primers arbitrarily selected from the kit were assessed and the four primers giving the clearest patterns were selected for further analysis. Primers sequences were:

A41 GTG ACC GAT CCA

A43 AAG TGG TGG TAT

A45 GGT CAG GCA CCA

A50 CCT TTC CGA CGT

5.2.3.2 RAPD-PCR

The PCR reaction mixtures (25μl) contained 2.5μl of 10x *Ex Taq* PCR reaction buffer
(TaKaRa Biomedicals, Japan), 2μl (0.2mM each) of deoxyribonucleotide mixture, 1μl
(0.4μM) single primer, 25 -50 ng of template DNA diluted in 10μl sterile distilled
water, 0.15μl (0.75U) of *Ex Taq* DNA polymerase (TaKaRa Biomedicals, Japan) and
sterile distilled water to bring the final volume to 25μl. The reaction mix was
overlayed with mineral oil and DNA amplification was performed in the temperature
control system PC-100 (Astec Co.Ltd Japan). After a hot start of 94°C for 3 minutes
reactions were subjected to 40 cycles of: denaturation at 94°C for 30 seconds,
annealment at 30°C for 30 seconds, and extension at 72 °C for 1 minute. The samples
were then post heated at 72°C for 5 minutes.

5.2.3.3 Assessment of PCR products

Amplified DNA fragments were examined on 1.5% agarose gel prestained with
ethidium bromide (0.5mg/l). Gels were run for 30 minutes in 1% TE electrophoresis
buffer then visualised with UV light.

5.2.3.4 Data analysis

RAPD analysis was performed as described by Lilley (1997). The presence or absence of bands in each RAPD fingerprint was marked by hand and scored as positive (1) or negative (0). The data were analysed in the statistics package SPSS for windows release 7.5 using the "Dice" binary matching coefficient and the UPGMA hierarchial clustering method (Dice, 1945; SPSS, 1988).

5.2.4 16s rDNA SEQUENCING

Protocols in this section were adapted from Sambrook *et al.,* (1989).

5.2.4.1 Oligonucleotide primers 16s PCR

Two primers derived from highly conserved areas of the 16s rRNA were selected for the PCR reaction. Primers were selected on the basis of the nucleotide sequence of 16s rRNA gene from *A. hydrophila* published by GenBank (Appendix 3). The sequences of the two primers of 26 and 30 bases respectively were:

5' - CAGACTCNTACGGGAGGCAGCAGTGG - 3'

5' - TGTTGCGGTGAATAGGTTCCCGGGCCTTGT - 3'

5.2.4.2 16s PCR reaction mix

The PCR reaction mixtures (50µl) contained 5µl of 10x *Ex Taq* buffer (TaKaRa Biomedicals, Japan), 4µl of deoxyribonucleotide mixture, 0.5µl (1µM) of each primer, 5µl of template DNA to give between 20 and 200ng of DNA per reaction, 0.5µl (2.5U) of Taq enzyme and sterile distilled water to bring the final volume to 50µl. The reaction mix was overlayed with mineral oil and DNA amplification was performed in

the temperature control system PC-100 (Astec Co. Ltd Japan). The PCR temperature profile included a hot start at 95°C and then thirty cycles of: 1 minute denaturation at 95°C, 1 minute annealing at 55°C, 1 minute primer extension at 72°C. After completion of 30 cycles the reaction mix was held at 72°C for 5 minutes to allow full extension of all amplified products. The PCR products were assessed using agarose (0.8%) gel electrophoresis.

5.2.4.3 Ligation of PCR products with plasmid vector

The 16s rDNA PCR products were cloned into a plasmid vector using the pMOS Blue T-vector kit (Amersham, UK). The plasmid DNA contains sites for two vector directed universal primers which are inexpensive and readily available. Insertion of the PCR products into vector plasmid DNA therefore eliminates the requirement for expensive, purpose-designed, labelled primers in the sequencing reaction. The PCR products were ligated with the pMOS blue vector following the manufacturers instructions.

Residual DNA polymerase activity was removed from the PCR products by extracting each sample with 1 volume of chloroform:isoamyl alcohol (24:1). Two μl of the PCR product (insert solution) were then added to the following ligation mix: 1μl 10x ligation buffer, 0.5μl 100mM Dithiothreitol (DTT), 0.5μl 10mM Adenosine triphospate (ATP), 1μl 50ng/ml T vector, 0.5μl T4 DNA ligase (2-3 weiss units), 49.5μl distilled water. The mix was incubated at 16°C for 2 hours.

5.2.4.4 Transformation

1µl of the ligase mix containing the recombinant plasmid was then added to 20µl of MOS blue competent cells (*E. coli*). The transformation mix was chilled on ice for 30 minutes. The cells were heat shocked for exactly 40s in a 42°C water bath, and placed on ice for a further 2 minutes. Eighty µl of room temperature SOC medium (Appendix 2) was added to the cell solution and the tubes were shaken at 37°C for 1 hour. Fifty µl of each transformation mix was then spread on L-agar antibiotic plate supplemented with ampicillin, tetracycline, X-gal and IPTG (Appendix 2). Plates were incubated at 37°C overnight and then kept at 4°C to increase blue colouration of untransformed colonies.

5.2.4.5 Screening of recombinants

The plasmid carries a gene for antibiotic resistance therefore transformed bacteria can be grown on plates containing the appropriate antibiotics (ampicillin and tetracycline). However, cells with both recombinant as well as non-recombinant plasmids will grow under these conditions, so a second screening condition is imposed. The structure of the pMOS Blue vector allows blue-white screening of recombinant colonies. The plasmid carries a β-galactosidase gene that bridges the cloning site. When the appropriate substrates are added to the media (X-gal and IPTG), colonies carrying the plasmid with the insert, which have non-functional β-galactosidase are white, colonies carrying untransformed plasmid are blue. White colonies were selected from the culture plates, streaked in a straight line onto L-agar plates and incubated for 12 hours at 37°C. Broth cultures using the resultant streaks were set up in 1.5ml of 2x YT

medium (Appendix 2) supplemented with ampicillin to give a concentration of 50µg/ml. Broths were incubated overnight at 37°C.

5.2.4.6 Isolation of plasmid DNA

- One and a half ml of the overnight culture in YT medium were centrifuged at 4000g for 2 minutes. The supernatant was removed by aspiration to leave the bacterial pellet as dry as possible.

- The bacterial pellet was resuspended in 100µl of Solution I (Appendix 2) by vigorous vortexing. 100 µl each of 2%SDS and 0.4N NaOH (Solution II) were added and the tubes were mixed gently until the solution cleared. 150µl of Solution III (Appendix 2) were added and the tubes thoroughly mixed to disperse the solution through the viscous bacterial lysate.

- The tubes were placed on ice for 5 minutes. 10µl of chloroform were then added and the tubes were spun at 7000g for 5 minutes at 4°C. The supernatant was transferred to a fresh tube and extracted with an equal volume of phenol/chloro/isoamyl alcohol. The supernatant was transferred to a fresh tube.

- Two volumes of chilled 95% ethanol were added, mixed and the tubes were left to stand at room temperature for ten minutes. The tubes were spun at 7000g for 5 minutes at 4°C and the supernatant was removed and discarded. The DNA pellets were vacuum dried and the DNA then resuspended in 50µl TE buffer.

- Three µl of a 2mg/ml RNAase A solution were added, mixed and the tubes incubated at 37°C for 1 hour. A 0.6 volume of 20% polyethylene glycol 6000 in 2.5MNaCl was added and the tube gently mixed until the solution cleared. The tubes were placed on ice for 30-60 minutes and then spun at 7000g for 5 minutes at 4°C.

- The supernatant was discarded and 500µl of chilled 75% ethanol were added, the tubes were spun again at 7000g rpm for 5 minutes at 4°C. The supernatant was discarded and the tubes were vacuum dried. The DNA was resuspended in 20µl sterile distilled water. At this stage samples were checked for the presence of the insert in the plasmid DNA (see below).

- Samples were made up to 300µl with sterile distilled water and extracted once with an equal volume of phenol/chloroform/isoamyl and once with an equal volume of diethylether. A 0.1 volume of 3M sodium acetate pH5.2 was added.

- The DNA was then precipitated in 2 volumes 100% chilled ethanol to make approximately 1.5ml total volume and the tube was mixed gently as the DNA strands formed. The DNA was pelleted and washed with 500µl of 75% chilled ethanol. Finally the DNA was vacuum dried and redissolved in nano pure sterile water. The molecular weight and integrity of a 2µl sample were then checked by electrophoresis on an 0.8% agarose gel containing ethidium bromide. All extracts were stored at -20°C.

5.2.4.7 Double Digestion of Plasmid DNA

Plasmid DNA was digested with restriction enzymes: Eco R1 and Hind III (double digestion), to determine presence and size of the insert DNA. The site of action of the two restriction enzymes used for the digest lie on either side of the cloning site. A sample of the PCR product and the uncut plasmid DNA were run as controls with lambda Hind III as a marker. The digest was run on a standard 0.8% agarose gel stained with ethidium bromide.

5.2.4.8 Sequencing primers and sequencing reactions

The nucleotide sequences of the insert were determined by thermal cycle sequencing, based on the dideoxynucleotide chain termination method. Reagents were obtained from a commercial kit, "Thermo sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP" (Amersham Life Science, Bucks, England). The sequencing primers were fluorescently labelled vector directed primers:T7 promotor primer and U-19 universal primer, obtained from Amersham Life Science as related kit products.

T7 promoter (foward) primer:

5' - TAATACGACTCACTATAGGG - 3'

U-19 universal (reverse) primer:

5' - CAAAAGGGTCAGTGCTGCA - 3'

The kit provided prepared nucleotide termination mixes containing: buffer, dNTPs, the appropriate ddNTP and enzyme Thermo Sequenase DNA polymerase. Eight tubes were prepared for each DNA sample, one tube for each nucleotide termination mix with each primer. 1 μl of the appropriate nucleotide termination mix was added to each tube. A premix was prepared for each primer reaction, containing 1-2μg of

template DNA made up to 11.5 µl in ddH$_2$O and 0.5µl of either the forward or reverse primer. 3µl of the premix was then added to each G, A, T and C tube for that primer. The reaction mixes were overlayed with a drop of mineral oil and placed in a thermal cycler (TaKaRa Biomedicals, Japan).

The PCR temperature profile included a hot start for five minutes at 95°C and then thirty cycles of: 30 seconds denaturation at 95°C, 30 seconds annealing at 55°C, 1 minute primer extension at 70°C. After the thermocycling was completed 2 µl of formamide loading dye (for fluorescent samples) (Amersham Life Sciences) were added to each tube and samples were loaded onto the gel in the fluorescent sequencer.

5.2.4.9 Preparation of sequence gel.

The sequencing gel was prepared using a proprietary acrylamide solution, Long Ranger™ (AT Biochem) Gel Solution.

4.8ml of Long Ranger gel plus 7.2ml 10xTBE (Appendix 2) were added to 25.2g urea and made up to 60ml with dH$_2$O. The solution was covered and stirred for 30 minutes until dissolved. The solution was then filtered and 400µl 10% ammonium persulphate and 32µl TEMED (*N,N,N',N'*-tetramethylethylenediamine) were added just before the gel was poured.

The basic units of the gel apparatus were two long glass plates. These were prepared by scrubbing in detergent, rinsing in distilled water and ethanol and then scrubbing with ethanol. The plates were clamped together, separated only by teflon spacers. The

head of the glass plates was raised slightly and the gel mixture poured in slowly whilst the plate was tapped to ensure even distribution. A toothless plastic template for the comb was placed at the top of the gel, both ends of the gel plates were sealed with cling film and the gel was allowed to set overnight. Once the gel was set, the cling film and plastic template were removed. A sharks tooth comb was inserted at the top of the gel so that the teeth just touched the gel surface. The plates were clamped into place in the automated sequencer (LI-COR Model 400, USA) and the reservoirs were filled with 1xTBE buffer (Appendix 2). The nucleotide fragments generated in the chain elongation reactions were then loaded into the wells created by the spaces between the teeth of comb, and the gel was run on the automated DNA sequencer.

5.2.4.10 Analysis of sequence data

The sequences were determined by software on the automated DNA sequencer. The products of the forward and reverse primers i.e. both strands of DNA were sequenced. Any discrepancies between the two strands were checked manually by visual interpretation of the chromatograph produced on the automated sequencer. The resultant amino acid sequences were aligned using Genetyx-Mac computer programme (SDC software, Japan) and similarity searches were carried out by matching the sequences to known *Aeromonas* data in the GenBank nucleic acid sequence database published on the World Wide Web (http://www.ncbi.nlm.nih.gov//).

5.3 RESULTS

5.3.1 RAPD ANALYSIS

The primer profiles for the Au isolates gave rise to highly similar but not identical fingerprints for all Au isolates. The Au group consistently shared common dominant bands with each primer, though there were slight variations within the minor bands. In contrast the selection of speciated aeromonads from Thailand and the type strains showed scattered profiles with some common bands but these were not consistent from primer to primer. All primers revealed at least one common dominant band between the Au isolates and *A hydrophila* type strain. The RAPD fingerprints are displayed in Figures 5.3.1.1 to 5.3.1.12.

The pooled data from the four primers produced a total of 105 bands for Au and the type strains, and a total of 88 bands for Au and the selection of Thai aeromonads. These data were analysed using the "Dice" binary proximity measure to generate similarity coefficients for each isolate (Figures 5.3.1.13 and 5.3.1.14). Coefficients of variation (standard deviation x100/average similarity coefficient) were then calculated between the isolates. The coefficient of variation (c) for all the Au isolates was 7.1, whereas for Au and the type strains c was 76.5 and for the type strains alone c was 52.2. The coefficient of variation for Au and the selection of other Thai motile aeromonads was 55 and for the speciated aeromonads alone c was 32.3. These results indicate that Au is genetically distinct from both the type strains and the other Thai motile aeromonads.
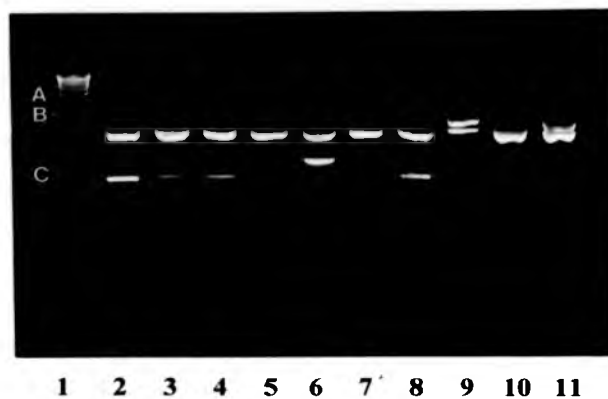
Figure 5.3.1.1 RAPD profiles of Au with primer 41. Lanes (1) *Hind*III cut lambda DNA marker, A= 6557 bp, B = 2027 bp, C = 564 bp; (2) 1D12; (3) 1D33; (4) 1D54; (5) 2D1; (6) 2D8; (7) 2D16; (8) 2D25; (9) 3D29; (10) 4D3; (11) 5N21.
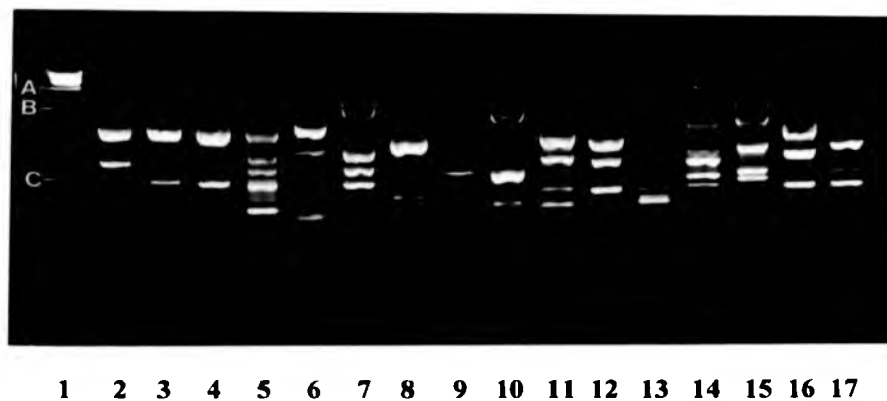
Figure 5.3.1.2 RAPD profiles of Au and type strains with primer 41. Lanes (1) *Hind*III cut lambda DNA marker, A = 6557 bp, B = 2027 bp, C = 564 bp; (2) Au 2D8; (3) Au 2D25; (4) Au 1D12; (5) *A. hydrophila* HG1; (6) *A. salmonicida* HG3; (7) *A. caviae* HG4; (8) *A. media* HG5; (9) *A. eucrenophila* HG6; (10) *A. sobria* HG7; (11) *A. veronii* bv sobria HG8; (12) *A. veronii* bv veronii HG10; (13) *A. jandaei* HG 9; (14) *A. schubertii* HG12; *A. trota* HG13; (16) *A. allosaccharophila* ND; (17) *A. encheleia* ND.

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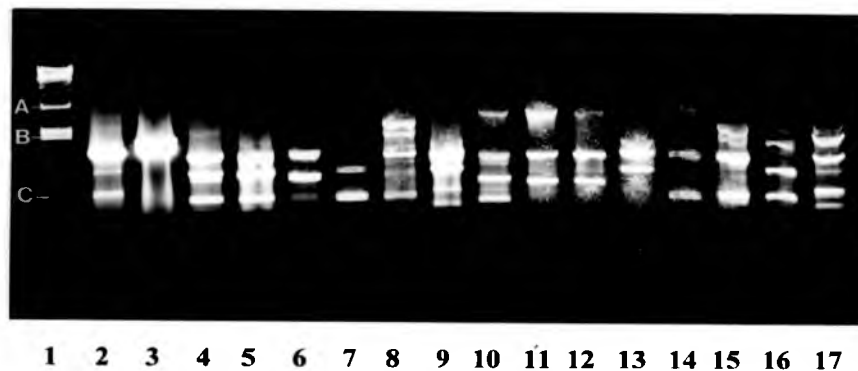
Figure 5.3.1.3 RAPD profiles of Au and speciated Thai aeromonads with primer 41.
Lanes (1) *Hind*III cut lambda DNA marker, A = 6557 bp, B = 2027 bp, C = 564 bp; (2)
Au 1D33; (3) Au 3D29; (4) *A. veronii* bv sobria 2D12; (5) *A. veronii* bv sobria 4N15; (6)
A. hydrophila 1D75; (7) *A. hydrophila* 2D20; (8) *A. veronii* bv sobria 2N36; (9) *A.
veronii* bv sobria 4D11; (10) *A. hydrophila* 3D37L; (11) *A. caviae* 1N15L; (12) *A. caviae*
2N14; (13) *A. schubertii* 5N11; (14) *A. veronii* bv veronii 2N1; (15) *A. veronii* bv veronii
3D37S; (16) *A. hydrophila* 5W1; (17) *A. veronii* bv sobria 3W1.
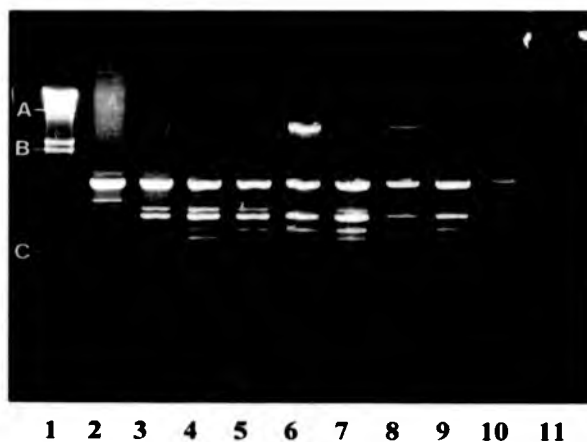
Figure 5.3.1.4 RAPD profiles of Au with primer 43. Lanes (1) *Hind*III cut lambda DNA
marker, A = 6557 bp, B = 2027 bp, C = 564 bp; (2) 1D12; (3) 1D33; (4) 1D54; (5) 2D1;
(6) 2D8; (7) 2D16; (8) 2D25; (9) 3D29; (10) 4D3; (11) 5N21.

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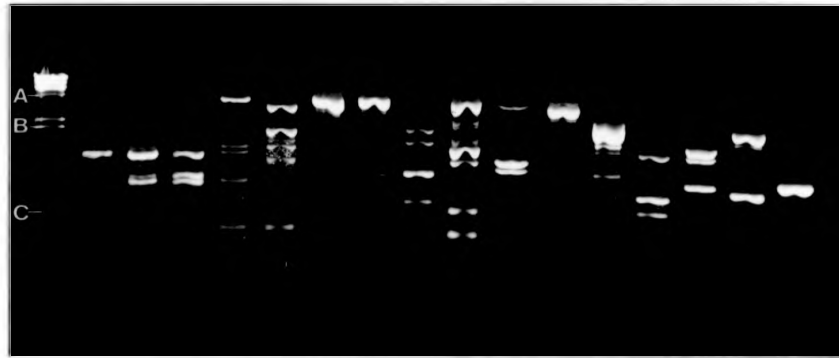
Figure 5.3.1.5 RAPD profiles of Au and type strains with primer 43. Lanes (1) *Hind*III cut lambda DNA marker, A = 6557 bp, B = 2027 bp, C = 564 bp; (2) Au 1D12; (3) Au 1D33; (4) Au 5N21; (5) *A. hydrophila* HG1; (6) *A. salmonicida* HG3; (7) *A. caviae* HG4; (8) *A. media* HG5; (9) *A. eucrenophila* HG6; (10) *A. sobria* HG7; (11) *A. veronii* bv sobria HG8; (12) *A. veronii* bv veronii HG10; (13) *A. jandaei* HG 9; (14) *A. schubertii* HG12; *A. trota* HG13; (16) *A. allosaccharophila* ND; (17) *A. encheleia* ND.
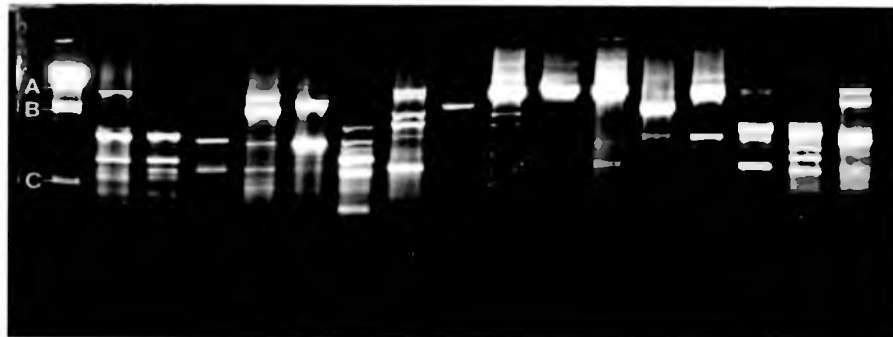
Figure 5.3.1.6 RAPD profiles of Au and speciated Thai aeromonads with primer 43. Lanes (1) *Hind*III cut lambda DNA marker, A = 6557 bp, B = 2027 bp, C = 564 bp; (2) Au 2D25; (3) Au 3D29; (4) *A. veronii* bv sobria 2D12; (5) *A. veronii* bv sobria 4N15; (6) *A. hydrophila* 1D75; (7) *A. hydrophila* 2D20; (8) *A. veronii* bv sobria 2N36; (9) *A. veronii* bv sobria 4D11; (10) *A. hydrophila* 3D37L; (11) *A. caviae* 1N15L; (12) *A. caviae* 2N14; (13) *A. schubertii* 5N11; (14) *A.veronii* bv veronii 2N1; (15) *A. veronii* bv veronii 3D37S; (16) *A. hydrophila* 5W1; (17) *A. veronii* bv sobria 3W1.

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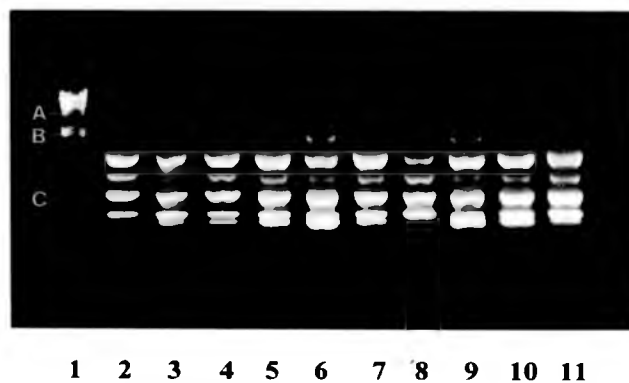
Figure 5.3.1.7 RAPD profiles of Au with primer 45. Lanes (1) *Hind*III cut lambda DNA marker, A = 6557 bp, B = 2027 bp, C = 564 bp; (2) 1D12; (3) 1D33; (4) 1D54; (5) 2D1; (6) 2D8; (7) 2D16; (8) 2D25; (9) 3D29; (10) 4D3; (11) 5N21.
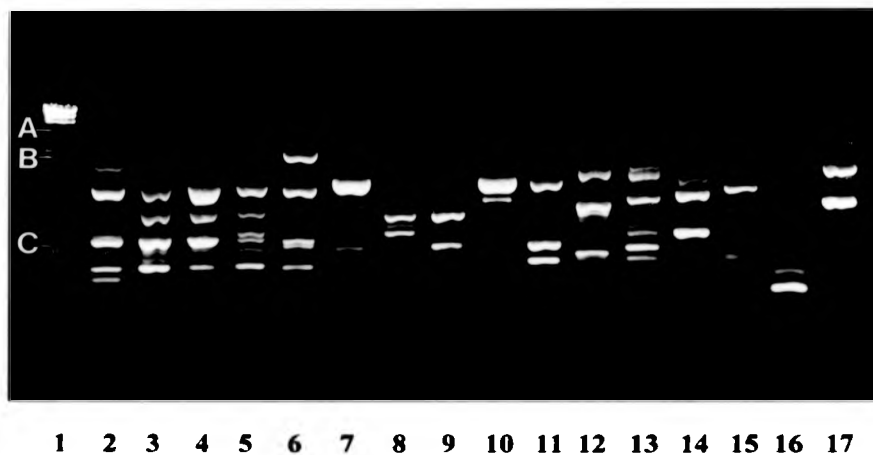
Figure 5.3.1.8 RAPD profiles of Au and type strains with primer 45. Lanes (1) *Hind*III cut lambda DNA marker, A = 6557 bp, B = 2027 bp, C = 564 bp; (2) Au 2D8; (3) Au 2D25; (4) Au 1D12; (5) *A. hydrophila* HG1; (6) *A. salmonicida* HG3; (7) *A. caviae* HG4; (8) *A. media* HG5; (9) *A. eucrenophila* HG6; (10) *A. sobria* HG7; (11) *A. veronii* bv sobria HG8; (12) *A. veronii* bv veronii HG10; (13) *A. jandaei* HG 9; (14) *A. schubertii* HG12; *A. trota* HG13; (16) *A. allosaccharophila* ND; (17) *A. encheleia* ND.

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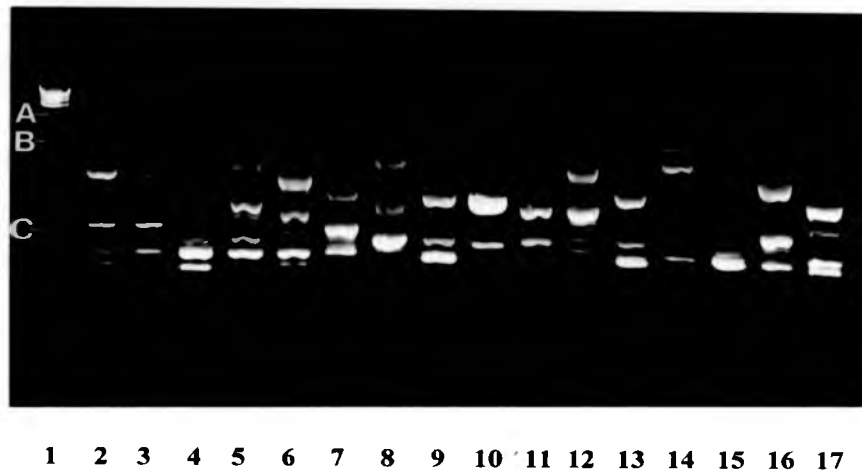
Figure 5.3.1.9 RAPD profiles of Au and speciated Thai aeromonads with primer 45. Lanes (1) *Hind*III cut lambda DNA marker, A = 6557 bp, B = 2027 bp, C = 564 bp; (2) Au 1D33; (3) Au 3D29; (4) *A. veronii* bv sobria 2D12; (5) *A. veronii* bv sobria 4N15; (6) *A. hydrophila* 1D75; (7) *A. hydrophila* 2D20; (8) *A. veronii* bv sobria 2N36; (9) *A. veronii* bv sobria 4D11; (10) *A. hydrophila* 3D37L; (11) *A. caviae* 1N15L; (12) *A. caviae* 2N14; (13) *A. schubertii* 5N11; (14) *A. veronii* bv veronii 2N1; (15) *A. veronii* bv veronii 3D37S; (16) *A. hydrophila* 5W1; (17) *A. veronii* bv sobria 3W1.
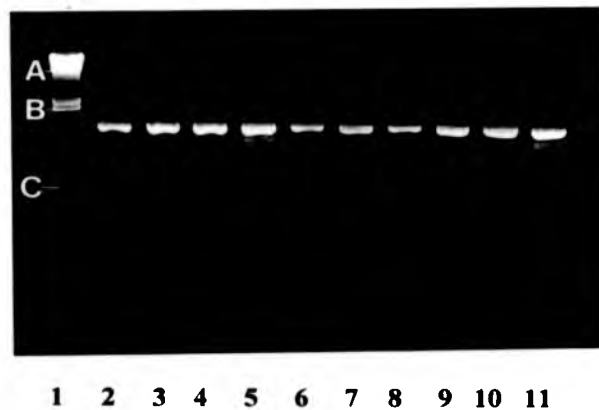
Figure 5.3.1.10 RAPD profiles of Au with primer 50. Lanes (1) *Hind*III cut lambda DNA marker, A = 6557 bp, B = 2027 bp, C = 564 bp; (2) 1D12; (3) 1D33; (4) 1D54; (5) 2D1; (6) 2D8; (7) 2D16; (8) 2D25; (9) 3D29; (10) 4D3; (11) 5N21.

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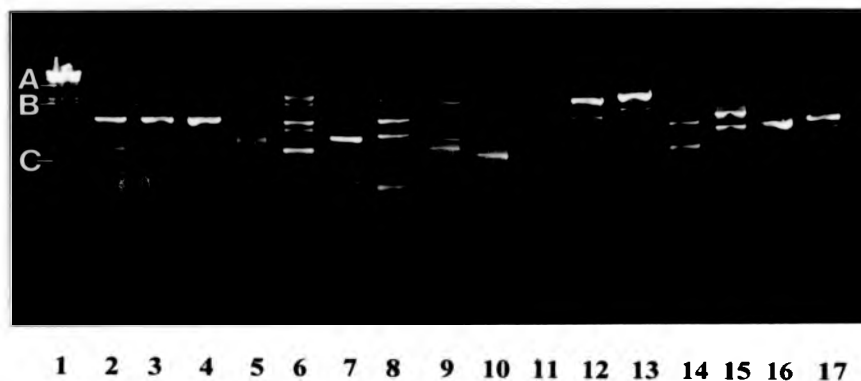
Figure 5.3.1.11 RAPD profiles of Au and type strains with primer 50. Lanes (1) *Hind*III cut lambda DNA marker, A = 6557 bp, B = 2027 bp, C = 564 bp; (2) Au 1D12; (3) Au 1D33; (4) *A. hydrophila* HG1; (5) *A. salmonicida* HG3; (6) *A. caviae* HG4; (7) *A. media* HG5; (8) *A. eucrenophila* HG6; (9) *A. sobria* HG7; (10) *A. veronii* bv sobria HG8; (11) *A. veronii* bv veronii HG10; (12) *A. jandaei* HG 9; (13) *A. schubertii* HG12; (14) *A. trota* HG13; (15) *A. allosaccharophila* ND; (16) *A. encheleia* ND; (17) Au 2D25,

Figure 5.3.1.12 RAPD profiles of Au and speciated Thai aeromonads with primer 50. Lanes (1) *Hind*III cut lambda DNA marker, A = 6557 bp, B = 2027 bp, C = 564 bp; (2) Au 1D54; (3) Au 2D25; (4) *A. veronii* bv sobria 2D12; (5) *A. veronii* bv sobria 4N15; (6) *A. hydrophila* 1D75; (7) *A. hydrophila* 2D20; (8) *A. veronii* bv sobria 2N36; (9) *A. veronii* bv sobria 4D11; (10) *A. hydrophila* 3D37L; (11) *A. caviae* 1N15L; (12) *A. caviae* 2N14; (13) *A. schubertii* 5N11; (14) *A.veronii* bv veronii 2N1; (15) *A. veronii* bv veronii 3D37S; (16) *A. hydrophila* 5W1; (17) *A. veronii* bv sobria 3W1.

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Figure 5.3.1.13 Similarity Matrix for Au and *Aeromonas* type strains

	ID12	ID33	ID54	2D1	2D8	2D16	2D25	3D29	4D3	5N21	CECT 839	CECT 894	CECT 838	CECT 4232	CECT 837	CECT 837	CECT 4246	CECT 4257	CECT 4228	CECT 4240	CECT 4255	CECT 4199
ID12																						
ID33	0.88																					
ID54	0.80	0.93																				
2D1	0.86	0.93	0.91																			
2D8	0.78	0.82	0.82	0.89																		
2D16	0.82	0.91	0.88	0.88	0.89																	
2D25	0.92	0.90	0.82	0.93	0.93	0.86																
3D29	0.76	0.80	0.82	0.83	0.88	0.80	0.84															
4D3	0.86	0.85	0.82	0.83	0.88	0.85	0.85	0.90														
5N21	0.81	0.85	0.85	0.82	0.88	0.83	0.88	0.84	0.89													
CECT 839	0.57	0.53	0.50	0.52	0.49	0.70	0.51	0.79	0.72	0.44												
CECT 894	0.19	0.18	0.18	0.17	0.18	0.19	0.16	0.54	0.54	0.22	0.78											
CECT 838	0.22	0.20	0.10	0.20	0.15	0.18	0.19	0.20	0.11	0.22	0.19	0.10										
CECT 4232	0.24	0.22	0.23	0.38	0.28	0.29	0.25	0.22	0.25	0.34	0.20	0.16	0.36									
CECT 837	0.42	0.39	0.40	0.20	0.27	0.28	0.29	0.25	0.17	0.30	0.24	0.16	0.13	0.13								
CECT 4224	0.22	0.21	0.11	0.38	0.21	0.22	0.23	0.23	0.25	0.26	0.20	0.25	0.12	0.24	0.27							
CECT 837	0.22	0.11	0.20	0.20	0.29	0.41	0.38	0.31	0.32	0.38	0.34	0.15	0.28	0.17	0.05	0.48						
CECT 4246	0.36	0.33	0.29	0.33	0.29	0.30	0.37	0.32	0.20	0.31	0.22	0.13	0.23	0.28	0.16	0.24	0.37					
CECT 4257	0.24	0.23	0.23	0.27	0.18	0.24	0.25	0.25	0.15	0.26	0.27	0.23	0.30	0.40	0.13	0.17	0.27	0.17				
CECT 4228	0.25	0.23	0.29	0.27	0.23	0.20	0.26	0.25	0.06	0.21	0.14	0.04	0.21	0.28	0.18	0.17	0.11	0.05	0.17			
CECT 4240	0.12	0.11	0.11	0.11	0.05	0.11	0.06	0.06	0.41	0.20	0.16	0.00	0.12	0.07	0.13	0.24	0.17	0.18	0.07			
CECT 4255	0.46	0.42	0.38	0.46	0.47	0.44	0.41	0.41	0.41	0.06	0.31	0.05	0.07	0.15	0.18	0.07	0.24	0.11	0.05	0.07		
CECT 4199	0.25	0.34	0.29	0.33	0.34	0.36	0.26	0.26	0.45	0.26	0.17	0.28	0.15	0.09	0.13	0.13	0.17	0.17	0.18	0.07	0.07	
CECT 4342	0.15	0.13	0.07	0.19	0.20	0.14	0.15	0.15	0.15	0.08	0.19	0.00	0.08	0.09	0.24	0.07	0.07	0.14	0.00	0.33	0.19	

Details of bacterial isolates are given in Table 5.2.1

Figure 5.3.1.14 Similarity matrix for Au and other motile aeromonads from Thailand

	ID12	ID33	ID54	2D1	2D8	2D16	2D25	3D29	4D3	5N21	2D12	4N15	ID75	2D20	2N36	4D11	3D14	IN15L	2N14	5N11	2N1	3D37S	5W1
ID12	0.88																						
ID33	0.80	0.93																					
ID54	0.88	0.96	0.93																				
2D1	0.80	0.84	0.81	0.93																			
2D8	0.82	0.80	0.88	0.88	0.88																		
2D16	0.92	0.90	0.88	0.95	0.90	0.88																	
2D25	0.76	0.80	0.82	0.90	0.87	0.77	0.84																
3D29	0.87	0.85	0.82	0.85	0.82	0.90	0.72	0.78															
4D3	0.81	0.81	0.85	0.90	0.87	0.84	0.89	0.72	0.89														
5N21	0.16	0.25	0.21	0.25	0.26	0.79	0.89	0.72	0.89	0.28													
2D12	0.31	0.38	0.34	0.42	0.38	0.17	0.28	0.17	0.28	0.36	0.59												
4N15	0.27	0.35	0.31	0.30	0.31	0.32	0.44	0.32	0.41	0.33	0.39	0.46											
ID75	0.37	0.39	0.35	0.34	0.35	0.32	0.28	0.33	0.41	0.28	0.27	0.38	0.38										
2D20	0.29	0.32	0.28	0.36	0.37	0.31	0.32	0.28	0.27	0.30	0.35	0.63	0.30	0.24									
2N36	0.32	0.35	0.31	0.31	0.26	0.38	0.30	0.35	0.40	0.16	0.33	0.40	0.33	0.50	0.50								
4D11	0.21	0.24	0.20	0.20	0.20	0.33	0.22	0.33	0.22	0.33	0.17	0.36	0.33	0.54	0.49	0.49							
3D14	0.32	0.29	0.24	0.33	0.26	0.21	0.28	0.22	0.22	0.32	0.27	0.49	0.27	0.29	0.26	0.39	0.39						
IN15L	0.36	0.33	0.29	0.29	0.34	0.31	0.37	0.33	0.33	0.27	0.32	0.35	0.37	0.29	0.20	0.36	0.36	0.63					
2N14	0.18	0.17	0.11	0.17	0.17	0.35	0.19	0.37	0.37	0.32	0.33	0.35	0.19	0.27	0.29	0.29	0.20	0.15	0.24				
5N11	0.43	0.45	0.46	0.45	0.46	0.18	0.39	0.13	0.19	0.19	0.28	0.46	0.44	0.06	0.50	0.69	0.43	0.33	0.37	0.41			
2N1	0.40	0.37	0.27	0.37	0.32	0.47	0.41	0.50	0.50	0.50	0.29	0.48	0.29	0.32	0.50	0.26	0.34	0.21	0.33	0.53	0.49		
3D37S	0.33	0.31	0.32	0.31	0.32	0.39	0.34	0.29	0.29	0.41	0.17	0.33	0.23	0.23	0.36	0.47	0.17	0.14	0.22	0.32	0.40	0.49	
5W1	0.20	0.18	0.14	0.18	0.19	0.32	0.20	0.29	0.29	0.34	0.20	0.32	0.25	0.10	0.36	0.35	0.24	0.18	0.24	0.39	0.25	0.37	0.41
3W1	0.20	0.18	0.14	0.18	0.19	0.19	0.20	0.20	0.20	0.15	0.20	0.33	0.25	0.10	0.36	0.35	0.24	0.18	0.24	0.39	0.25	0.37	0.41

Details of bacterial isolates are given in Table 5.2.1

To facilitate comparisons between isolates, dendograms were constructed from the similarity values using the unweighted pair method with arithmetic averages (UPGMA) (Figs 5.3.1.15 and 5.3.1.16). The dendogram (Fig 5.3.1.15) demonstrates that Au isolates can be grouped into one distinct cluster. The Au cluster branches from the *A. hydrophila* type strain at a similarity index of 0.513, and from the other Aeromonad type strains at a similarity index of 0.417.

The Au isolates also formed a separate and quite distinct cluster from the selection of other motile aeromonads from Thailand (Figure 5.3.1.16). The two isolates more closely related to Au than the other Thai aeromonads were both phenotypically identified as *A. hydrophila*. The Au cluster branched from these two *A. hydrophila* isolates at a similarity index of 0.326 while it branched from the other Thai Aeromonads (including two other *A. hydrophila* phenospecies) at a similarity index of 0.297.

5.3.2 16s RIBOSOMAL DNA SEQUENCING

The 16s rDNA sequences of 10 isolates of Au were determined, the sequences are shown in Appendix 4 and comprise a stretch of 1057 nucleotides (ranging from positions 336 to 1393 in the *E. coli* numbering system).

The Au 16s rDNA sequences were aligned and compared with the sequences of nine *Aeromonas* type strains, the percentage of sequence similarities are shown in Table 5.3.2. High levels of sequence similarity, from 98.1-99.8% were observed between Au

Figure 5.3.1.15 Dendrogram based on RAPD results using UPGMA, showing similarity of Au isolates compared to *Aeromonas* type strains.
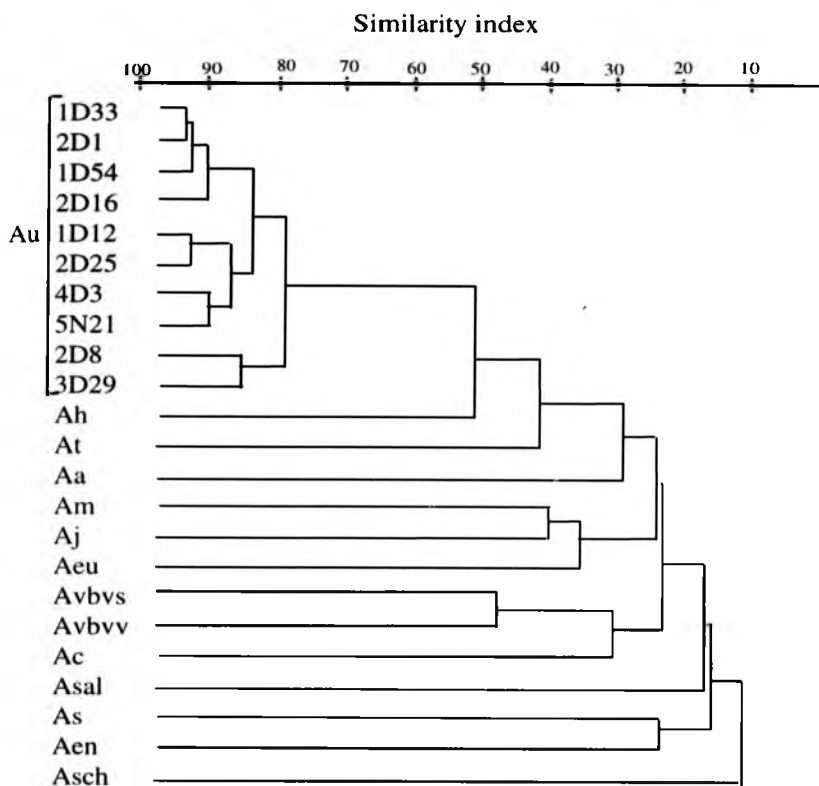
Au	= *Aeromonas* unspeciated	ND
Ah	= *A. hydrophila*	HG1
At	= *A. trota*	HG14
Aa	= *A. allosacharophila*	ND
Am	= *A. media*	HG5
Aj	= *A. jandaei*	HG9
Aeu	= *A. eucrenophila*	HG6
Avbvs	= *A. veronii* biovar sobria	HG8
Avbvv	= *A. veronii* biovar veronii	HG10
Ac	= *A. caviae*	HG4
Asal	= *A. salmonicida*	HG3
As	= *A. sobria*	HG7
Aen	= *A. encheleia*	ND
Asch	= *A. schubertii*	HG12

HG = hybridization group
ND = not determined

Figure 5.3.1.16 Dendrogram based on RAPD results using UPGMA, showing similarity of Au isolates compared to other motile aeromonads from Thailand.
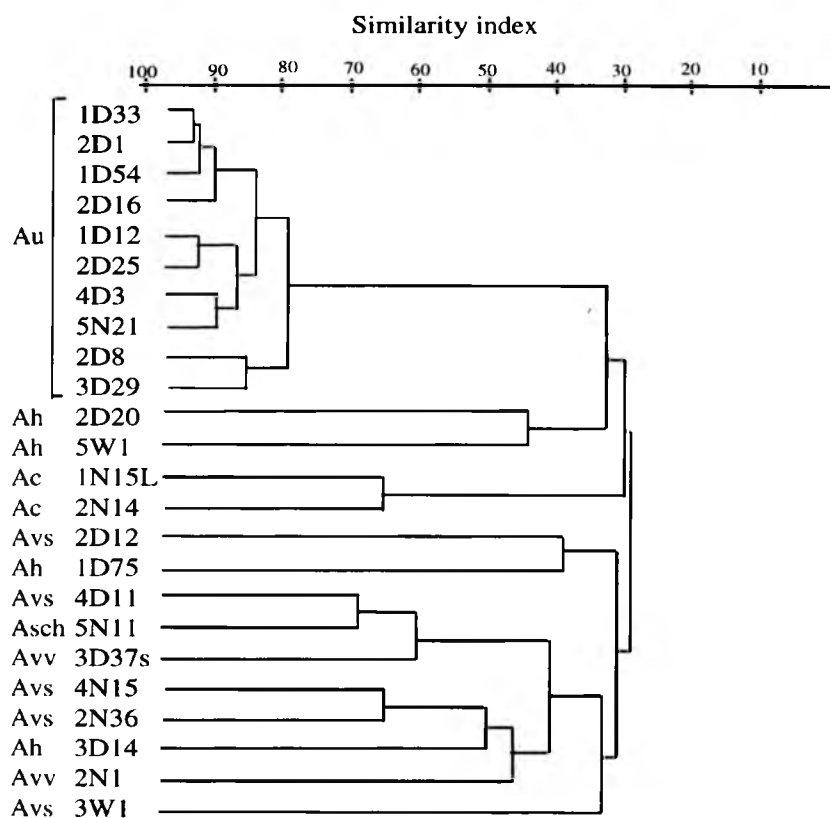
Ah   = *A. hydrophila*
Ac   = *A. caviae*
Avs = *A.veroni* biovar sobria
Asch = *A. schubertii*
Avv = *A. veroni* biovar veroni

and all the *Aeromonas* type strains. The highest similarity was observed between *A.*

hydrophila and Au with an average percentage sequence similarity of 99.57 ±0.14.

Table 5.3.2 Similarity values for 16s rDNA of ten Au isolates and nine sequences of

Aeromonas type strains published in GenBank, USA.

Au isolate	A. h	A. sal	A. c	A. m	A. eu	A. vbvs	A. vbvv	A. j	A. t
1D12	99.6	99.0	98.5	99.4	99.2	98.9	98.9	98.7	98.4
1D33	99.7	99.1	98.6	99.5	99.2	99.0	99.0	98.8	98.5
1D54	99.4	98.8	98.3	99.2	99.0	98.8	98.7	98.5	98.2
2D1	99.6	99.3	98.5	99.4	99.5	99.1	99.2	98.7	98.4
2D8	99.5	99.2	98.4	99.3	99.4	99.0	99.2	98.6	98.3
2D26	99.3	98.7	98.2	99.2	98.9	98.6	98.6	98.4	98.1
2D25	99.6	99.0	98.5	99.4	99.2	98.9	98.9	98.7	98.4
3D29	99.6	99.0	98.5	99.4	99.2	98.9	98.9	98.7	98.4
4D3	99.6	99.0	98.5	99.4	99.2	98.9	98.9	98.7	98.4
5N21	99.8	99.2	98.7	99.6	99.3	99.1	99.1	98.9	98.6

A.h = *A. hydrophila*, A. sal = *A. salmonicida*, A. c = *A. caviae*, A. m = *A. media*, A. eu = *A. eucrenophila*, A. v bv s = *A. veronii* biovar sobria, A. v bv v = *A. veronii* biovar veronii, A. j = *A. jandaei*.

An inspection of the rDNA sequence alignments revealed that, in variable region V3

(the most diagnostic region for delineating *Aeromonas* genospecies) at positions 457

to 476 (*E. coli* numbering), all the Au isolates were identical and differed from all

previously published *Aeromonas* sequences (Fig 5.3.2.1). The Au isolates therefore

have a "specific signature". The Au signature differs from the *A. hydrophila* signature

by a single base pair, this difference is however consistent for all 10 Au isolates

sequenced and the three published *A. hydrophila* sequences.

Figure 5.3.2.1 Characteristic aeromonad signature area V3 in approximately 460

region of rDNA.

	DNA group	457 | 5'TGATACCTAATACGTATCAA3'	476 | M
Au			
A. hydrophila	1	TGAT*G*CCTAATACGTATCAA	1
A. salmonicida	3	TGG*CG*CCTAATACGT*G*TCAA	3
A. caviae	4	*CAG*TAGCTAATA*TCTGCTGG*	11
A. media	5	TGAT*G*CCTAATACG*C*ATCA*G*	3
A. eucrenophila	6	TGAT*G*CCTAATACG*C*ATCA*G*	3
A. sobria	7	TG*GCAG*CTAATA*TCTG*TCA*G*	7
A. veronii	8/10	TG*G*TAGCTAATA*AC*TG*C*CA*G*	7
A. jandaei	9	*CAG*TAGCTAATA*TCTGCTGG*	11
Aeromonas spp.	11	TG*G*T*CG*CTAATA*A*CG*G*C*C*AA	8
A. schubertii	12	TG*G*T*GGT*TAATAC*C*TG*C*CA*G*	8
A. trota	14	*CAG*TAGCTAATA*TCTGCTGG*	11
A. allosaccharophila	15	TG*G*TA*G*C*G*AATA*AC*TG*C*CA*G*	8

Nucleotides in bold face italics indicate mismatches with the Au sequence. M

represents the number of nucleotide differences in this stretch between Au and other

Aeromonas species.

5.4 DISCUSSION

The results of the RAPD analysis indicate a lack of genetic diversity between the Au isolates which correlates with the phenotypic homogeneity previously demonstrated, and is unusual for geographically diverse isolates of motile aeromonads. The scattered profiles generated for the type strains and other motile aeromonads from Thailand agree with the findings of earlier RAPD studies which demonstrated genomic heterogeneity among motile aeromonads (Miyata *et al.*, 1995, Inglis *et al.*, 1996, Oakey *et al.*, 1996).

The homogeneous profiles produced for the Au isolates resemble the profiles generated for *A. salmonicida* by Miyata *et al.*, (1995), and Inglis *et al.*, (1996). Miyata *et al.*, (1995) analysed the genetic differentiation of 13 strains of *A. salmonicida* subspecies *salmonicida* and 7 species of *A. hydrophila*. The RAPD profiles of all the non-motile aeromonads, *A. salmonicida* subspecies *salmonicida* were exactly the same. However profiles of the motile aeromonads, *A. hydrophila*, differed for each isolate. These findings indicate the genomic homogeneity of *A. salmonicida* subspecies *salmonicida* and the genetic variety of the motile aeromonads.

Inglis *et al.*, (1996) continued this work in Scotland with a range of *A. salmonicida* from the UK and a diverse collection of motile aeromonads from S.E Asia and found similar profiles. All of the subspecies *salmonicida* yielded remarkably uniform fingerprints. The scattered RAPD profiles of *A. hydrophila* consistently demonstrated the genomic diversity of the motile aeromonads.

The homogeneous fingerprints produced for Au may point towards the existence of a species of motile aeromonad pathogenic to frogs in SE Asia which has a remarkably conserved genome, paralleling that of *A. salmonicida* subspecies *salmonicida* in salmonids in Northern Atlantic and Europe.

Oakey *et al.* (1996) assessed the ability of RAPD to differentiate genospecies of *Aeromonas,* and, as in the other studies found, that the aeromonads are genetically quite diverse. The authors analysed type strains of 13 genospecies and a further 67 clinical and environmental isolates. They found that RAPDs had little value as a typing tool for unknown isolates, since almost every isolate had a unique RAPD fingerprint. The authors similarly concluded that RAPD analysis would not be useful for phylogenetic separation of the genus *Aeromonas,* as such studies rely on the analysis of homologous characters, and the high level of genetic diversity detected gave no guarantee that RAPD fragments of the same molecular weight would have the same nucleotide sequence.

Although aeromonads could not be typed using RAPD profiles, Oakey *et al.,* (1996) did detect distinct differences in banding patterns between the type strains of previously determined hybridisation groups. Moreover similarities were observed between those hybridisation groups thought to be more closely related. The profiles of HG1, HG2, and HG3 (all previously designated as *A. hydrophila*) contained common bands with one primer. In addition HG1 and HG2 produced common bands with five of the six primers used, suggesting that these two were more closely related to each other than HG3. The finding of common dominant bands between Au and *A.*

hydrophila (HG1) indicates that they are closely related but may, as for HG1 and HG2, form separate genetic lines.

Although the RAPD fingerprints for the Au isolates were not all identical, most of the variation was in the minor bands. This illustrates one of the disadvantages with the RAPD technique. PCR is not always a precise process that gives exactly the same results each time. The occurrence of faint products is not always caused by genomic differentiation but can depend on the purity of the template DNA. Sometimes minor differences in template quality or abundance cause artefactual differences in whether a particular product is seen from individual to individual (Palumbi, 1996).

It is interesting that type strains of HG8 and HG10, which are phenotypically distinguishable but are said to be genomically identical, produced differing fingerprints with the primers used in this study. Primers 43 and 45 produced very different profiles for the two HGs, the fingerprints from primer 50 contained some common bands but were still distinct and the fingerprints for primer 41 although almost identical differed in two dominant bands.

DNA-DNA hybridisation studies, analysis of the 16s rRNA and AFLP fingerprinting (Hickman-Brenner *et al.*, 1987, Martinez-Murcia *et al.*, 1992a, Huys *et al.*, 1996) have indicated that HG8 and HG10 are a single genospecies, despite being phenotypically distinct. However the RAPD results of Oakey, *et al.*, (1996) are similar to those presented here, in that the patterns produced for HG8 and HG10 differed for each of the six primers examined although there were some similarities with respect to

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fragment sizes. It may be that this inconsistency with previous genomic studies illustrates again the problem with RAPD analyses in distinguishing PCR artefact from actual polymorphisms. On the other hand this inconsistency may illustrate the advantage of the RAPD technique, which potentially analyses the whole genome and is therefore highly discriminatory. The striking phenotypic diversity within HG8/10 has led to the conclusion that *A. veronii* should be split into two biovars corresponding to HG8 and HG10 (Huys *et al.*, 1996). RAPD analysis appears able to distinguish between these two biovars.

Despite the disadvantages of the technique, the RAPD profiles fulfilled their intended role in this project. The technique was used as a broad screening method to assess a large collection of individuals. The RAPD fingerprints adequately demonstrated the likeness of the Au isolates, the fact that they differed from the other motile aeromonads isolated on the same farms and from all the type strains tested but appeared to be closely related to *A. hydrophila*. The findings presented here support the work of Inglis *et al.*, (1996) and Oakey *et al.*, (1996) that with RAPD analysis where there is DNA similarity it can be recognised and where there is diversity, differentiation can be made. More detailed analysis using rDNA sequencing was undertaken to further describe the interrelationships of Au with other *Aeromonas* genospecies.

The history of microbial taxonomy is littered with tales of misconceptions and disillusionment. Kluyver and van Niel (1936) proposed that a phylogenetic system of microbial classification could be reliably based on morphological characteristics such as the shape of bacterial cells. Bacterial evolution was seen as a progression from the

simplest forms (cocci) to the most complex (e.g. spore formers). However

microbiologists found that new organisms and new characteristics did not fit into

existing categories and over the following decades became disillusioned with the

natural system of classification. Many felt that the development of formal taxonomic

systems for bacteria would not be possible (Stanier *et al.*, 1970, Stanier and van Niel,

1962) and that it was more important to concentrate on defining the submicroscopic

and molecular organisation of bacterial cells. However even as microbiologists

became less focused on microbial evolution, techniques were being developed which

could provide enormous amounts of phylogenetic information using molecules as

documents of evolutionary history (Woese 1991).

Ribosomal RNA is universal in distribution and highly conserved. Microbiologists

therefore expected these molecules to most precisely reflect bacterial phylogeny

(Carnahan and Altwegg, 1996). Woese (1991) states that "there can be little doubt that

the small subunit rRNA is as reliable a molecular measure of phylogenetic

relationships among organisms as is now available".

Therefore much work was undertaken to define the 16sDNA sequences of the

aeromonads. Martinez-Murcia *et al.*, (1992b) determined the respective 16s rDNA

sequences for 13 of the *Aeromonas* genospecies then recognised. The various species

exhibited very high levels of overall sequence similarity (98-100%) to each other and

formed a distinct evolutionary line within the gamma subclass of the *Proteobacteria*.

The Au 16s rDNA sequences showed a similar level of homology with the *Aeromonas*

type strains. Levels of sequence similarity from ranged 98.1% to 99.8% indicating that

these isolates are part of that evolutionary line.

Martinez-Murcia *et al.*, (1992b) found that differences in the nucleotide sequences of 16srDNA were not evenly distributed over the entire molecule, but clustered in two "hypervariable" regions corresponding to the regions around positions 160 and 460 respectively of the *E. coli* sequence. Our amplified portions of the Au 16s rDNA gene did not contain the 160 region, however in the hypervariable region around the 460 region all the Au isolates were identical and differed from all previously published *Aeromonas* sequences. This 16s rDNA data indicates that all 10 Au isolates are the same species and that this species differs from previously recognised species.

However, as in the case of the early morphological attempts to classify bacteria, the information supplied by the analyses of 16s rDNA was found to be less definitive than at first hoped. Several of the relationships derived by Martinez-Murcia *et al.*, (1992b) from the 16s rDNA sequences were in marked disagreement with previously published DNA-DNA hybridisation results.

Ribosomal sequences of HG4 (*A. caviae*) and HG14 (*A. trota*) differ by only one single nucleotide, although these species are quite different biochemically and share a DNA-DNA hybridisation homology of only 30%. Likewise HG1 (*A. hydrophila*) and HG 5 (*A. media*) are biochemically different, but their rDNA sequences differ by only three nucleotides. In contrast *A. sobria* (HG 7) and *A. veronii* bv sobria (HG 8) which on 16s rDNA analysis appeared only distantly related to each other, had a DNA-DNA homology of 60-65% as originally determined by Popoff and colleagues under optimal reassociation conditions.

Martinez-Murcia *et al.* (1992a) in describing *A. allosaccharophila* stated that 16s rDNA sequencing was accepted as the most precise method for determining bacterial phylogeny. However the authors stated that a simple empirical species definition such as that provided by hybridisation data (bacterial genospecies exhibit 70% or greater chromosomal DNA relatedness under optimal hybridisation conditions) was not possible with rDNA data due to different rates of sequence divergence.

In a paper published the following year Collins *et al.*, (1993) stated that although rDNA sequencing was an immensely powerful tool for determining the interrelationships of organisms above species level, it was apparent that the method often failed to discriminate between closely related species. They reiterated that the development of a species definition based on rDNA sequences (e.g. percentage relatedness or number of nucleotide changes) would probably prove impossible because of the high overall sequence conservation of the rRNA molecule, its relatively low information content and different rates of sequence change between lineages. Thus earlier opinions on the usefulness of rDNA data for species classification were reversed, reflecting the similar disillusionment with morphological classification systems earlier this century.

Nevertheless rDNA sequencing continues to be invaluable in the construction of stable genealogically based classifications above the species level. Almost all that is known about microbial phylogeny above the genus level has been determined within the last 15 years and comes from analysis of small subunit rDNA sequences (Woese 1991).

The disadvantages perceived with the technique are the inability to distinguish between closely related species. Ribosomal RNA is universal in distribution; its function was established at an early stage in the evolutionary process and appears extremely constant and highly constrained; it is not affected by most changes in an organism's environment (except for changes in basic physical parameters such as intracellular pH and temperature) (Woese, 1991). The conservation of rDNA sequences reduces their discriminative powers, but conversely when differences in rDNA are noted they are significant, even the single base pair differences such as those between *A. caviae* and *A. trota* and Au and *A. hydrophila*. The fact that the 16s rDNA 460 signature areas of the Au isolates were all identical to each other and differed from all other published sequences is a very strong indication that they are an as yet unspeciated taxon within the genus.

It is difficult to accurately define bacterial species as this necessitates classifying a dynamic process where bacteria constantly diverge to occupy different niches. The arbitrary definition of 70% or greater chromosomal DNA homology using hybridisation studies may in the future be considered unsatisfactory. The best option for the microbiologist is to build up a data base containing as much phenotypic and molecular information as possible. It is critical that any new species should be composed of a number of similar strains that have been extensively phenotyped and genotyped against all known species type strains and definition strains for all known HGs (Carnahan and Altwegg, 1996).

Although it is not possible at present to determine that Au is a distinct species without DNA-DNA hybridisation data, the following criteria lend support to the genotypic distinctiveness of the unknown aeromonad strains:

1) The Au isolates were unusually homogeneous in their biochemical reactions for a geographically diverse group of motile aeromonads

2) All the Au isolates were unable to utilise sucrose, a reaction selected as a primary identification test for aeromonad genospeciation by Abbott *et al.* (1992). Molecular analysis indicates that Au is most closely related to HG1 however 100% of HG1 isolates utilise sucrose.

3) RAPD fingerprints which can be used as a productive screening method to recognise DNA similarity or diversity produced very homogenous patterns for the Au isolates whilst producing scattered profiles for type strains and other Thai aeromonads. The RAPD profiles showed that the Au isolates were all closely related to *A. hydrophila* (HG1) but were more closely related to each other.

4) The unknown strains have a "specific signature" in variable region V3 of the 16srDNA sequence which serves to distinguish Au from other described *Aeromonas* species.

Thus Au can be distinguished both phenotypically and genotypically from *A. hydrophila* HG 1 indicating that Au is a previously unidentified species, recently diverged from and very closely related to *A. hydrophila*. DNA-DNA hybridisation studies are clearly necessary in order to determine the genospecific relationship between Au and *A hydrophila*.

However whether Au is a recently diverged species of *Aeromonas* or a phenotypically distinguishable biovar of *A. hydrophila* is of less importance to the aquaculturist than the fact that a group of potentially pathogenic bacteria can be quickly and easily distinguished from the majority of other motile aeromonads on the basis of a few standard biochemical tests available to even the smallest laboratory.

It is important to distinguish between non-pathogenic and pathogenic isolates in order to warn farmers of potential problems. The motile aeromonads are ubiquitous in the environment and it is difficult to predict which isolates may be virulent for farmed aquatic animals. In this study we were able to distinguish both phenotypically and genotypically a group of isolates from septicaemic animals in different geographical locations. We therefore have the opportunity to compare the factors which confer virulence on these isolates with those of type strains and other motile aeromonads from the same facilities. This work is discussed in detail in chapter 6.

Chapter 6. Virulence Properties

6.1 INTRODUCTION

Many studies have examined environmental and clinical aeromonads in an attempt to

correlate the production of potential virulence determinants with the pathogenicity of

the isolates (Hsu *et al.*, 1981, Santos *et al.*, 1988, Pin *et al.*, 1994, Vadivelu *et al.*,

1995, Kirov *et al.*, 1994, Majeed and MacRae, 1994, Esteve *et al.*, 1995b). The ability

of *A. hydrophila* to give rise to a wide range of infections in both aquatic and

terrestrial vertebrates, indicates that complex pathogenic mechanisms employing

several virulence factors may be responsible (Vadivelu *et al.*, 1991). The production

of endotoxins, extracellular enterotoxins, haemolysins, cytotoxins and proteinases, the

ability to adhere to cells and the possession of certain surface proteins have all been

reported to contribute to pathogenicity of motile aeromonads (Cahill, 1990, Kirov

1993, Esteve *et al.*, 1995, Pin *et al.*, 1994).

6.1.1 EXTRACELLULAR FACTORS

Unlike most other Gram-negative organisms *Aeromonas* spp. produce a wide range of

extracellular toxins and enzymes. In recent years studies have focused on the role of

these ECPs in the pathogenesis of *Aeromonas* septicaemias. Production of protease

and haemolysin in particular have been implicated as key extracellular activities

associated with pathogenicity of infections in both animals and humans (Cahill, 1990,

Janda, 1991, Janda *et al.*, 1994). However *Aeromonas* culture supernatants have

multiple biological activities which few studies have successfully separated out,

therefore understanding the role of individual extracellular toxins in the pathogenesis

of septicaemic disease is difficult.

6.1.1.1 Haemolysins

One of the most striking cultural features displayed by many *Aeromonas* strains is their ability to haemolyse erythrocytes. This characteristic is principally associated with certain strains belonging to the phenospecies *A. hydrophila* and *A. sobria* and is linked to the elaboration of extracellular heamolysins. Haemolysins are cytolytic proteins which insert into the lipid bilayer of cell membranes thus forming pores and causing leakage of cytoplasmic contents (Cahill 1990, Janda 1991).

Aeromonas strains cause two types of haemolysis; β haemolysis and α haemolysis. The *Aeromonas* β- haemolysin produces clear zones of haemolysis on blood agar and was first described by Bernheimer and Avigad (1974). They reported the partial characterisation of an extracellular, heat labile haemolysin which they termed aerolysin. The α haemolysin produces an opaque zone of incomplete haemolysis on blood agar. It is elaborated during the stationary phase and is not expressed when temperatures exceed 30°C.

One reason for the difficulty in understanding the role that various extracellular enzymes play in pathogenesis concerns their multifunctional nature. Aerolysin which has been demonstrated to be haemolytic, cytolytic and also enterotoxigenic is a prime example of this problem (Janda, 1991). Asao *et al.*, (1984) found that purified haemolysin caused fluid accumulation in both the suckling mouse test and rabbit ileal loop test; was toxic to Vero cells; and caused death of mice within one minute of intra venous injection. Thus one purified fraction had haemolytic, cytotoxic and enterotoxic activities; similar functions for the α haemolysin of aeromonads have not yet been

described. The β haemolysin has also been implicated in systemic disease. Isogenic mutants from which the aerolysin gene had been specifically deleted showed reduced lethal capabilities in mice as assessed by LD50s (Chakraborty *et al.*, 1987).

The confusion surrounding the activities of the various potential virulence factors will not be resolved until each of the claimed toxins has been separated by cloning and studied in highly purified form. Aerolysin is by far the best characterised of the *Aeromonas* cytotoxic proteins. The aerolysin gene from *A. hydrophila* was cloned and sequenced by Howard *et al.*, (1987). Since then it has been cloned from a number of other aeromonads including *A. trota* (previously *A. sobria*) and *A. salmonicida* (Husslein *et al.*, 1988, Hirono and Aoki 1993).

Workers in Japan have demonstrated that various haemolysin genes with different structures are present in aeromonads. Two haemolysin genes designated AHH-1 and AHH-2 were cloned from a single *A. hydrophila* isolate and showed low homology values with each other and with the aerolysin gene sequence (Aoki and Hirono, 1991). Further studies characterised three genes from *A. hydrophila* (AHH3, AHH4, AHH5), two genes from *A. salmonicida* (ASH3, ASH4) plus one gene (ASA1) from *A. sobria*, which showed a significant degree of homology (>90%) with the aerolysin sequence, indicating that aerolysin-like haemolysins are widely distributed in aeromonads (Hirono and Aoki, 1992, Hirono *et al.*, 1992, Hirono and Aoki, 1993). However a third gene cloned from an isolate of *A. salmonicida* and designated ASH1 proved very different. A search in the DNA data bank failed to reveal any homologous sequences for the ASH1 gene. Direct comparison of this amino acid sequence with those of other bacterial haemolytic proteins revealed no significant identity. A DNA probe formed

from the ASH1 gene did not react with any of 104 *A. salmonicida* strains tested except

ATCC14174 which was the source of the gene. Whereas ASH3 and ASH4 DNA

probes reacted with all 104 strains of *A. salmonicida* isolated from three geographic

regions, indicating that homologous sequences of ASH3 and ASH4 were widely

distributed. The authors concluded that the ASH1 haemolysin gene probably did not

originate with *A. salmonicida* but may have been acquired from another genus or

family of bacteria.

In summary it appears that the possession of haemolysin genes is universal amongst

aeromonads and that several different *Aeromonas* haemolysins exist. However their

exact role in the pathogenesis of *Aeromonas* septicaemias has yet to be elucidated.

6.1.1.2 Proteases

Proteases may contribute to pathogenicity by causing direct damage or enhanced

invasiveness (Cahill, 1990). A number of proteases have been described in the culture

supernatants of *Aeromonas* spp. however for most of these there is little or no

evidence of direct involvement in pathogenicity. Some of them may aid the organisms

in overcoming initial host defences and they probably provide amino acids for cell

proliferation (Howard *et al.*, 1996). Proteases may also have an indirect effect by

bringing about proteolytic activation of toxins such as aerolysin which are excreted in

an inactive state.

The ability to degrade host molecules such as elastin may be important in relation to

disease producing potential. Elastin is a major structural protein of the lung and

comprises a significant proportion of other tissues such as blood vessel endothelium

(arteries) and ligaments. Proteases with elastolytic activity may therefore enhance invasiveness of isolates.

Some studies have aimed to correlate *in vitro* toxin production with *in vivo* pathogenicity. Hsu *et al.*, (1981) examined a group of 127 strains of *A. hydrophila* to correlate the production of selected extracellular enzymes with virulence in channel catfish. Two discrete groups of *A. hydrophila* were found when elastin activity was studied. The authors found that cells from elastase positive strains produced lesions and mortalities when injected into channel catfish. The authors concluded that motile *Aeromonas* with a high elastase activity were usually highly virulent strains. Janda (1991) stated that elastase production at 30°C is a unique property of the *A. hydrophila* phenospecies. Hanninen (1994) suggested that including this test for the identification of aeromonads decreased the possible misidentification of *A. hydrophila* as *A. caviae* or *A. veronii*.

Esteve *et al.*, (1995b) found that elastase activity displayed by aeromonads isolated from eels was mostly expressed by virulent strains, reinforcing the correlation between virulence in fish and elastase production reported by other authors (Hsu *et al.*, 1981, Santos *et al.*, 1988). The authors also found that ECP from elastase-positive species induced tissue liquefaction of the abdominal wall in challenged elvers, whereas ECP from elastase-negative strains were less destructive and only caused a furuncle at the site of injection. The authors conclude that elastases probably act as invasive factors as well as providing nutrients for bacterial growth. In their study a positive relationship was observed between strain virulence and several biological activities in *Aeromonas* spp. including: fibrinolysins, mucinases, proteases,

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haemolytic and cytopathic effects. The authors concluded that elastases and
haemolysins may play a role in spread of the disease and thermolabile toxins could be
responsible for the final death of the eel.

Some workers have made use of the commercially available API ZYM system to
determine the enzymatic capabilities of bacterial isolates, although few studies on the
genus *Aeromonas* have been undertaken with this technique. The API ZYM system
allows rapid detection of 19 preformed enzymes including esterases, aminopeptidases,
proteases, phosphatases and glycosidases.

Studies on virulent and non-virulent isolates of *Pasteurella* and *Vibrios* (Magarinos *et
al.*, 1992, Fouz *et al.,* 1993, Biosca and Amaro 1996) found that isolates produced
very similar enzymatic patterns regardless of their virulence potential. Most API-ZYM
studies on aeromonads have concentrated on using the system as a tool for rapid
discrimination of isolates rather than attempting to correlate enzyme production with
potential virulence of isolates (Noterdaeme *et al.*, 1991, Magee *et al.*, 1993, Sakai, *et
al.*, 1993). Sakai *et al.*, (1993) assessed the usefulness of the API-ZYM system in
identifying important pathogenic bacteria in aquaculture and found marked variation
in the aeromonad profiles. The authors noted nine profiles in the 11 *Aeromonas*
strains isolated from fish. Only the main enzymatic activities caprylate esterase,
leucine arylamidase acid phosphatase, phosphoamidase and *N*-acetyl-glucosaminidase
were consistently strongly positive. Whether these patterns can be linked to the
pathogenic potential of isolates or are a function of the genetic heterogeneity of the
motile aeromonads has yet to be determined.

6.1.2 CELL SURFACE FEATURES

Additional protein layers, O antigens, fimbriae and other non-specific adherence

mechanisms seem to be related to the pathogenic capacity of aeromonads (Paniagua *et*

al. 1990). Mittal *et al.,* (1980) reported that a group of *A. hydrophila* virulent in fish,

exhibited a number of unique phenotypic properties that were thought to be cell-

surface associated, including autoagglutination during growth in static broth culture

and resistance to the bactericidal activity of normal serum.

The attachment of a pathogen to the epithelial tissues of its host is often the first and

most important step in the disease process. The haemagglutination assay (HA) is the

commonly used procedure to determine the ability of micro-organisms to adhere to

eukaryotic cells. Haemagglutinins are the surface features on bacterial cells which

permit their attachment to receptor groups on erythrocytes (Cahill 1990). Further

information on the nature and specificity of the receptors on the eukaryotic cell

surface can be obtained by testing various sugars for their ability to inhibit the HA.

Majeed and MacRae (1994) examined the haemagglutinating properties of 114

Aeromonas strains. Haemagglutination (HA) activity was found frequently in motile

aeromonads irrespective of phenospecies; it was present in 50% of *A. sobria* strains,

51% of *A. hydrophila* strains and 48% of *A. caviae* strains. HA was inhibited by

fucose (F), galactose (G) and mannose (M) at low concentration and in most cases two

or three of these sugars were inhibitory. A significant association was found between

certain HA-inhibition patterns and the production of cytotoxin by *Aeromonas* spp.

Results showed a strong association (100%) between cytotoxin production by

Aeromonas strains and HA inhibition in groups F-G-M+ and F-G+M+ (+ =HA

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inhibition, - = no inhibition). Only 39% of *Aeromonas* strains in group F+G+M+ were
cytotoxin producers.

6.1.3 PATHOGENICITY

Identifying strains of high and low virulence potential and comparing extracellular
and surface features associated with those strains should eventually lead to the
recognition of virulence determinants and gene products involved in microbial
pathogenicity.

Janda and Kokka (1991) examined the relative pathogenicity in mouse lethality
studies of 80 *Aeromonas* strains typed by biochemical (phenospecies) and genetic
(genospecies) methods. The authors found that aeromonads could be categorised into
three virulence groups (low, moderate and high). The relative virulence of individual
groups in decreasing order were as follows: HG9 *A.* jandaei> HG1 *A. hydrophila* and
HG12 *A.* schubertii> HG10 *A. veronii* biovar veronii> HG8 *A. veronii* biovar sobria>
HG11 unnamed> HG3 *A. salmonicida*> HG4 *A. caviae* and HG6 *A.* eucrenophila>
HG5 *A. media*> HG7 *A. sobria.*

Janda *et al.,* (1994) assessed a number of clinical and laboratory characteristics
associated with 53 strains of *Aeromonas* isolated from human septicaemias. They
found only four species to be associated with cases of human septicaemia; *A.
hydrophila, A. veronii, A. caviae* and *A. jandaei.* Of these strains, *A. hydrophila*
septicaemia was associated with the highest mortality suggesting a more pathogenic
role for this species in humans.

It is at present unclear what markers or virulence characteristics are associated with increased pathogenicity. Consensus findings indicate that toxin production (cytotoxin and enterotoxin) is more frequently expressed *in vitro* by phenospecies *A. hydrophila* and *A. sobria* than by *A. caviae*. Janda *et al.*, (1994) evaluated twenty strains of *Aeromonas* recovered from human blood for a number of *in vitro* markers potentially associated with pathogenicity and found that the least virulent isolates (*A. caviae*) had decreased production of protease and haemolysin. Wilcox *et al* (1994) found that cytotoxin production was common in *A. hydrophila* (60%) and *A. sobria* (79%) but absent in *A. caviae*.

However *in vitro* tests have a fundamental limitation in that they cannot account for all the physical and chemical properties that contribute to the ultimate virulence of an isolate. *Aeromonas* spp. display a remarkable ability to respond to changes in their surroundings and it is known that expression of most virulence factors is regulated by environmental conditions (Mateos and Paniagua, 1995). The genetic basis for virulence is therefore only expressed completely during growth *in vivo*. Consequently, *in vitro* studies of the virulence factors of bacteria isolated from clinical infections but subcultured in conventional laboratory media may not accurately reflect the *in vivo* situation. Likewise studies on comparative pathogenicity of bacterial species e.g. mouse lethality trials (Janda and Kokka, 1991) depend greatly on the cultural history of the organisms i.e. how often they have undergone laboratory passage; but these details are rarely included in papers.

Whilst these reservations on *in vitro* testing are acknowledged, the results of such studies are still useful. Certain factors such as haemolytic and cytotoxic abilities, and

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the production of elastin have been repeatedly associated with virulent *Aeromonas*

isolates. The structural and functional properties of single proteins purified from the

extracellular products of motile aeromonads are beginning to be clarified. The

relationships of such proteins with one another have been investigated by the cloning

and sequencing of their respective genes and produced valuable data which is not

affected by cultural history. Once their virulence determinants are recognised,

potentially pathogenic aeromonads can be rapidly and accurately identified and the

epidemiology of *Aeromonas* infections further elucidated.

6.1.4 AIMS OF THE STUDY

The aims of this part of the study were therefore to study the pathogenic determinants

of aeromonads isolated from farmed frogs.

To look particularly at the haemolytic, cytolytic and proteolytic properties of the

extracellular products and determine whether these could be linked to the origin of

bacterial isolates or the species identified.

To examine whether there is a link between haemagglutination (HA) and

haemagglutination inhibition (HAI) patterns and toxin production or isolate origin.

To examine the genes encoding for one possible virulence factor (β-haemolysis) by

DNA hybridisation analysis of four haemolysin genes cloned from the genus

Aeromonas.

6.2 MATERIALS AND METHODS

6.2.1 BACTERIAL ISOLATES AND CULTURE CONDITIONS

Unless otherwise stated (see section 6.2.3) the bacterial isolates and culture conditions

used in this part of the study were as described in section 4.2.1 and 4.2.2. Due to

freezer failure Au isolate 1D54, used in the early part of this study, was lost. This was

replaced by Au isolate T7 from an outbreak of septicaemic disease which occurred in

a newly imported group of frogs in the Stirling aquarium.

6.2.2 HAEMOLYSIN ASSAY

This assay was adapted from the method described by Burke *et al.*, (1982).

6.2.2.1 Preparation of extracellular products (ECPs)

Five mls of TSB were inoculated with one bacterial colony and incubated for 24 hours

at 30°C. The bacterial suspension was then centrifuged at 1000g for 10 minutes at

4°C. The supernatant was removed and filtered through a 0.45µm filter. The filtered

supernatant was stored at 4°C for no more than 24 hours.

6.2.2.2 Preparation of blood cell suspensions

Healthy Chinese bullfrogs (*Rana rugulosa*), African clawed toads (*Xenopus laevis*)

and rainbow trout (*Oncorhynchus mykiss*) were anaesthetised with 4 mls of 2.5%

benzocaine per 10l of water. Blood was withdrawn by cardiac puncture from the

amphibians and from the caudal vein of the fish into a heparin coated syringe. Blood

was placed in a solution of 0.5 ml heparin with 9.5 ml sterile physiological saline and

centrifuged at 1000g for 15 minutes. The supernatant was discarded and the blood

cells were resuspended at a range of concentrations ranging from 1 in 100 to 1 in 1600

with sterile physiological saline. Commercial suspensions of horse and sheep blood

obtained from Sigma UK were centrifuged and resuspended in the same way.

6.2.2.3 Haemolysin assay

Volumes (100µl) of prepared blood cell suspension were added to doubling dilutions

of 100µl of ECPs in 100µls of sterile saline on a microtitre plate (U-bottomed wells).

Each supernatant was tested in duplicate on each plate. 100µl of blood suspension was

added to 100µl of saline to act as a control. Plates were incubated for 1 hour at 37°C

and then for 12 hours at 4°C. Haemolytic titres were expressed as the reciprocal of the

highest dilution of crude ECP needed to produce partial haemolysis of erythrocytes.

6.2.3 DETECTION OF HAEMOLYSIN GENES BY COLONY HYBRIDISATION

Ten Au isolates, a selection of speciated Thai aeromonads and 13 *Aeromonas* type

strains were screened for homologous sequences to cloned haemolysin genes AHH1,

AHH4, ASA1 and ASH1 following the colony hybridisation method described by

Sambrook *et al.,* (1989).

6.2.3.1 Bacterial strains and culture conditions

The bacterial strains used in this part of the study are listed in Table 5.2.1 and a

description of the culture conditions is given in Section 5.2.1. In addition four

transformed *E. coli* containing haemolysin gene inserts were kindly supplied by Dr

Hirono of Tokyo University of Fisheries. These isolates were incubated at 37°C on

sheep blood agar.

6.2.3.2 Preparation of haemolysin gene probes: Restriction enzyme digest of plasmid DNA to release haemolysin gene

Four samples of plasmid DNA containing *Aeromonas* haemolysin genes: AHH1, AHH4, ASA1 and ASH1 respectively, were kindly supplied by Dr Hirono, Tokyo University of Fisheries. The DNA containing genes AHH1, AHH4 and ASA1 was digested with the restriction endonucleases *Eco*RI and *Hin*d III, whilst that containing ASH1 was digested with *Eco*RI and X*ba* I.

Reaction solutions of 300µl were prepared containing 50 µl of plasmid DNA, 30µl universal buffer (supplied by the enzyme manufacturer, TaKaRa Biomedicals, Japan), 1µl of each enzyme and 220µl of distilled water.

The reaction mix was incubated at 37°C for 1 hour. Samples were then extracted once with an equal volume of phenol/chloroform/isoamyl alcohol. The DNA was precipitated in 2 volumes 100% chilled ethanol with 0.1 volume of 3M sodium acetate pH 5.2, pelleted by centrifugation and washed with 500µl of 75% chilled ethanol. Finally the DNA was vacuum dried and redissolved in 15µl of TE buffer.

The entire restricted plasmid DNA sample was examined by electrophoresis on a 0.8% low melting point agarose L gel containing 0.5µg/ml ethidium bromide.

6.2.3.3 Preparation of haemolysin gene probes: Purification of haemolysin DNA from gel

The fingerprint of the restricted DNA was compared against a size standard (Lambda DNA-*Hin*d III digest) and the haemolysin gene DNA was identified by the size of the fragment. This area was excised from the rest of the gel and placed in an Eppendorf tube containing 300µl of TE buffer.

Gel and buffer were heated to 65-68°C for 10-30 minutes. The heated solution was extracted with 500µl of TE/phenol. The supernatant was removed, centrifuged again at 7000g at room temperature and then extracted once with an equal volume of phenol/chloroform/isoamyl alcohol and once with an equal volume of diethylether.

The DNA was precipitated in two volumes of 100% chilled ethanol with 0.1 volume of 3M sodium acetate pH 5.2. The samples were held at -80°C for 10 minutes before the DNA was pelleted and washed in 75% chilled ethanol. The DNA was then vacuum dried and redissolved in 12µl of TE buffer.

The concentration of the DNA was determined from absorbance of solutions at 260 nm (GeneQuant, Pharmacia) (1µl DNA in 9µl dH_2O). The molecular weight and integrity of a 2µl sample was checked by electrophoresis on an 0.8% agarose gel containing ethidium bromide. All extracts were stored at -20°C.

6.2.3.4 Preparation of haemolysin gene probe: Random primer labelling of haemolysin DNA with α^{32}P.

The four haemolysin gene probes were labelled with α^{32}P- dCTP (>3000Ci/ml, Du Pont-NEN Corp., Boston, MA) using a commercial random-primer labelling kit (TaKaRa Random Primer DNA Labelling Kit, Version 2, TaKaRa Biomedicals, Japan). 2µl of random primer solution were added to 25 ng of haemolysin gene DNA dissolved in TE and the volume made up to 16.5µl with distilled water. The solution was heated to 95°C for 3 minutes to denature the DNA and then chilled on ice for 5 minutes.

2.5µl 10x buffer (supplied by manufacturer), 2.5µl dNTP, 2.5µl α^{32}PdCTP and 1µl Klenow fragment (large peptide from *E. coli* DNA polymerase) were then added to the reaction mix and the whole incubated at 37°C for a minimum of two hours.

The reaction mix was heated for 3 minutes at 95°C to denature the amplified DNA and for a further 5 minutes at 65°C to denature the polymerase. The mix was placed on ice to chill for 3 minutes and 10µl of the labelled probe were then immediately placed in a polythene bag containing 5 ml of hybridisation buffer and the colony membrane (see following section).

6.2.3.5 Colony blotting of aeromonads onto Hybond^{TM}-N$^+$ nylon membrane.

A portion of Hybond™-N$^+$; positively charged nylon membrane, (Version 2.0, Amersham Life Science, Amersham International plc, UK) was grid marked with ink, to ensure correct orientation of colonies and then sterilised. The membrane was placed

on a sterile agar dish and two individual colonies from an overnight agar culture of

each test bacteria were transferred with sterile tooth picks directly onto the membrane.

Positive controls of transformed *E. coli* colonies containing haemolysin gene inserts,

were also placed on the membrane.

The membrane was then placed colony side up in a drying oven and incubated for a

minimum of two hours at 42°C. The membrane was removed from the oven and

placed colony side up on a pad of absorbent filter paper soaked in 20 mls of

denaturing solution (Appendix 2) and left for 7 minutes. It was then transferred to a

pad of absorbent filter paper soaked in 20 ml of neutralising solution (Appendix 2) for

3 minutes. This neutralising step was repeated with a fresh pad and the membrane was

then washed in 2x SSC (Appendix 2) while the bacterial debris was gently scraped

from the surface with gloved fingers. The membrane was blotted dry and washed

again in fresh 2x SSC, blotted dry and oven baked for a further 2 hours.

6.2.3.6 Hybridisation of probe with colony blots on membrane.

The membranes (one for each gene probe) were sealed in individual polythene bags

with 10μl of gene probe and 5 ml of buffer (Appendix 2). The individual bags were

then sealed in a larger bag and placed in a water bath with shaker for a minimum of 12

hours at 42°C. Hybridisation proceeded under highly stringent conditions due to the

presence of formamide in the buffer. After hybridisation, membranes were washed at

60°C for 10-15 minutes each, in 2x SSC - 0.1% SDS, in 0.5x SSC - 0.1% SDS, then

in 0.1x SSC - 1% SDS. The washed membranes were exposed to Fuji X-ray film (Fuji

Photo Film Co., Kanagawa, Japan) at -80°C for 24 hours.

6.2.4 CYTOTOXICITY STUDIES

Cytotoxicity assays were carried out following a modification of the method described

by Wilcox *et al.,* (1994) on Vero (African green monkey kidney), RTG-2 (rainbow

trout gonad), SSN-1 (snake head) and HCT (catfish) cell lines.

6.2.4.1 Preparation of extracellular products

Extracellular products were prepared as described in section 6.2.1.1 with one

additional step. Before centrifugation cultures were mixed thoroughly and the

turbidity of each suspension was adjusted with TSB using McFarland standard

equivalences (McFarland 5, API Systems). Viable counts (Appendix 5) were carried

out on three cultures from every experimental batch to verify that the McFarland

standards produced consistent results and achieved the required concentration of cells

(1×10^8 cfu/ml).

6.2.4.2 Maintenance of cell cultures

Vero cells were grown in 25cm^2 flasks in Dulbecco's Modified Eagle's Medium

(DMEM) supplemented with 10% foetal bovine serum (FBS), 2mM L-Glutamine and

1µg/ml Amphotericin, and maintained at 37°C in an atmosphere enriched with

approximately 2% carbon dioxide.

RTG-2, SSN-1 and HCT cells were grown in 25 cm^2 flasks containing Leibovitz L-15

medium supplemented with 10% FBS. In addition the HCT cells were supplemented

with 0.5µg/ml Mycoplasma Removal Agent (MRA). The RTG-2 cells were incubated

at 22°C and the SSN-1 and HCT cells were incubated at 28°C.

The cell monolayers were subcultured when they became confluent to produce a 1:3 split ratio, the cell sheet was first washed twice with 5 ml of PBS and then trypsinised with 1ml 0.05% trypsin/0.02% EDTA in Modified Puck's Saline A. The detached cell were resuspended in 6 mls of the appropriate media and distributed between 3 new flasks each containing 5 mls of media, giving a total volume of 7 ml cell suspension per flask.

6.2.4.3 Cytotoxicity assay

Doubling dilutions of 100μl of ECPs in 100μls of PBS were prepared in flat bottomed 96 well microtitre plates, each supernatant was tested in duplicate on each plate. Cell suspensions (100μl) containing approximately 1×10^6 cells/ml were added to each well. As a negative control, cell suspensions were added to doubling dilutions of the TSB in which the bacteria had grown to ensure that the broth itself had no toxic effects on the cells. The microtitre plates were incubated at the temperatures appropriate for the cell lines described above.

Cytotoxicity was assessed by visual observation at x 40 with an Olympus IMT-2 phase-contrast inverted microscope. Cytotoxicity was defined as $\geq$ 50% cells lysed within a 24 hour period for the Vero cells and within 3 days for the fish cell lines. Toxicity titres were expressed as the reciprocal of the highest dilution of crude ECP able to produce a cytotoxic response. Cytotoxin positivity was defined as a reciprocal titre of $\geq$ 4.

6.2.5 ELASTASE ASSAY

This work was carried out as part of an MSc project (Miranda, 1997) subsidiary to the present study and supervised by M. Pearson.

The elastinolytic activity of the *Aeromonas* isolates was evaluated using a quantitative plate assay technique described by Hsu *et al.,* (1981). Ten mls of TSB were inoculated with a single colony of test bacteria from an overnight culture on TSA and incubated at 30°C for 24 hours. Elastin modified agar plates (Appendix 1) were spot inoculated using a multipoint inoculator (Denley) to give 1µl of each bacterial suspension containing approximately 10^6 cfu/ml. Each bacterial suspension was tested in duplicate with four inocula per plate.

The inoculated plates were incubated at 30°C for 72 hours and the size of the zones of clearing, indicating elastinolytic activity was measured. A zone ratio was obtained by dividing the diameter of the zone of reaction by the diameter of its colonial growth. The elastinolytic activity was defined from the zone ratio values as negative, intermediate or positive according to a ratio of 0, between 0 and 3, and higher than 3 respectively.

6.2.6 API-ZYM

The general enzymatic activities of the *Aeromonas* isolates were evaluated using the API-ZYM system (BioMerieux sa, France). The assays were carried out according to the manufacturers instructions. API-ZYM strips were inoculated with overnight bacterial cultures, incubated for 4 hours at 37°C and reagents A and B were added to

each cupule as directed. Colour reactions were read after 5 minutes. The presence and

degree of enzymatic activity were scored as 0, 1, 2, 3, 4 and 5 by comparing the colour

intensity of the reaction with the manufacturers colour chart. Percentages of positive

reactions and strong positive reactions (i.e. 3, 4 and 5) were calculated for each

enzyme of each *Aeromonas* species. The enzymatic composition of each species and

the relative enzymatic activities were also determined. Enzymatic composition was

considered to be the number of enzymes present in a given isolate. Enzymatic activity

was the value derived for each isolate based on the degree of activity of the enzymes

present in a given isolate.

6.2.7 HAEMAGGLUTINATION (HA) AND HA- INHIBITION (HAI) ASSAYS

This assay was carried out following the method described by Majeed and MacRae

(1994).

Blood cells were collected as described in section 6.2.2.2. Cells were washed twice

and resuspended in phosphate buffered saline (PBS), at 3% (v/v). Bacterial

suspensions were prepared by adding two loopfuls of an overnight *Aeromonas* culture

on TSA to 0.5 ml PBS, to provide a concentration of *c*. 10^{11} cfu/ml. Working solutions

of fucose, galactose or mannose (Sigma) 1% w/v in PBS were prepared on the day of

the test.

HA tests were performed at room temperature by mixing 20µl of the blood cell

suspension with the same volume of a bacterial suspension on a glass slide. A control

consisting of equal volumes of blood suspension and PBS was included on each slide.

Aeromonas isolates were considered to be HA-negative if HA had not occurred within

5 minutes. HA was recorded as ++ if the reaction was immediate and complete, or as + if the reaction was incomplete or not instantaneous but occurred within 5 minutes.

HAI tests were carried out at room temperature by mixing 20µl of blood suspension, 20µl of a solution of the test sugar 1% w/v in PBS plus 20µl of each bacterial suspension on a glass slide. A negative control consisting of two volumes of PBS and one volume of blood suspension was included on each slide. Inhibition was recorded if previously positive HA became negative or if strong HA (++) became weak (+) in the presence of the sugar solutions.

6.2.8 DATA ANALYSIS

The haemolysin and cytotoxin data were analysed in the statistics package SigmaStat®, (Jandel Scientific) using anova and Dunn's all pairwise mutiple comparison procedures. The API-ZYM data were analysed in the statistics package Statistica (Statsoft Inc.) using discriminant function analysis.

6.3 RESULTS

6.3.1 DEVELOPMENT OF THE HAEMOLYSIN ASSAY

A pilot study was carried out to determine whether red blood cells (RBC) from animals other than *Rana* spp. could be used as models to assess frog RBC lysis. (At this point in the study *Rana* spp. were proving difficult to import and maintain). The results are presented in Table 6.3.1.1.

Table 6.3.1.1 Haemolytic titre (reciprocal of maximum dilution producing haemolysis) of *Aeromonas* spp. ECP against RBC of different origins.

Isolate	Identification	horse	sheep	trout	toad	frog
1D4	Au	8	128	256	128	128
1D12	Au	4	256	256	128	128
1D33	Au	16	256	512	64	128
1D54	Au	8	128	256	64	128
2D1	Au	ND	16	512	64	128
2D8	Au	4	64	1024	32	128
2N12	*A. caviae*	16	0	128	32	8
2N36	*A. veronii* bv sobria	16	0	4	4	0
1N15	*A. caviae*	ND	0	64	512	0
2D20	*A. hydrophila*	ND	8	32	64	32
NCIMB 89	*A. hydrophila*	64	64	64	128	16

ND = not determined

The red blood cells from horse, sheep, trout, toad and frog were all sensitive to haemolysins produced by the Au isolates, although the degree of sensitivity varied greatly. The horse red blood cells were least sensitive, with titres ranging from 4 to16, while the trout red blood cells were most sensitive with titres ranging from 128 to 1024. Of all the species tested only the frog RBC yielded consistent results with the Au haemolysins, all of which produced a titre of 128. Haemolytic titres for the other motile aeromonads tested were varied. Frog and sheep RBC proved the least sensitive with titres ranging from 0 to 64, while toad were the most sensitive with titres ranging

from 4 to 512. The results indicated that none of the other RBC were suitable models
to indicate sensitivity of frog cells.

In the pilot study RBC were diluted 1 in 100 with sterile physiological saline as
recommended in the literature. The end point of the assay was taken as the maximum
dilution of ECP producing signs of haemolysis although this proved very difficult to
determine. A further small scale trial therefore was carried out to establish whether
more dilute suspensions of RBC would give clearer readings (more dilute suspensions
would also have the advantage of reducing the amount of frog blood necessary). The
results from this trial are presented in Table 6.3.1.2.

Table 6.3.1.2 Comparison of RBC dilution factors and source in determining
haemolytic actvity of *Aeromonas* ECPs.

Dilution factor	Sheep (2D20)	Trout (1D4)	Toad (1D4)	Frog (1D4)
1/100	ND	128	32	32
1/200	8	128	64	32
1/400	16 (good)	unreadable	128 (very faint)	128 (good)
1/800	16	ND	ND	ND
1/1600	unreadable	ND	ND	ND

ND = not determined, 2D20 = *A. hydrophila* ECP, 1D4 = Au ECP

The assays carried out with 1 in 400 dilutions of trout RBC and toad RBC were very
difficult to read. In contrast the 1 in 400 dilutions of sheep RBC and frog RBC
produced clearly visible haemolysis with an easily definable end point. The previous
trial had demonstrated that the sheep RBC lysis was a poor model for frog RBC lysis.
Further haemolysin assays were therefore carried out using a 1 in 400 dilution of frog
RBC in physiological saline. The reciprocal of the maximum dilution of *Aeromonas*

ECPs producing partial haemolysis was scored as the haemolytic titre (HT). The

results from these assays are presented in Table 6.3.2.

6.3.2 HAEMOLYTIC, CYTOTOXIC AND ELASTINOLYTIC PROPERTIES

The haemolytic, cytotoxic and elastinolytic activities of the individual *Aeromonas*

isolates are displayed in Table 6.3.2 and depicted graphically in Figures 6.3.2.1 to

6.3.2.6. Haemolysin production determined against frog RBC allowed division of the

isolates into three groups of high, moderate and low activity. The high activity group

were all Au isolates from internal locations in adult frogs. Those with moderate

activity were *A. hydrophila* isolated from external locations in adult frogs and

tadpoles and three internal isolates from tadpoles. The remaining group of low

haemolytic activity were from the same locations as the *A. hydrophila* isolates but

were different species; mainly *A. veronii* biovar sobria with a few isolates of *A.*

caviae, *A. veronii* biovar veronii and *A. schubertii*. Analysis of the data using all

pairwise multiple comparison procedures (Dunn's Method) revealed that the

differences in haemolytic titre between the three groups were highly significant (P =

<0.01).

Elastinolytic activity was demonstrated in 90% of the Au isolates examined, 60% of

the *A. hydrophila* isolates and in none of the other motile aeromonads. The range of

zone ratios was similar for Au and *A. hydrophila*; Au ratios ranged from 0 to 3.6 and

A. hydrophila ratios from 0 to 3.2. Zone ratios were very consistent, eight of the nine

Au isolates and 2 of the three positive *A. hydrophila* had zone ratios greater than 3.

Table 6.3.2 Haemolytic, cytotoxic and elastinolytic properties of *Aeromonas* spp.

	Haemolysin	Protease	Cytotoxin					
	Frog RBC	Elastin	Vero	RTG-2	SSN-1	HCT		
Species	Titre	Zone ratio	Titre	Titre	Titre	Titre	Isolate	Origin
Au	64	3.1	2	256	4	64	1D12	frog liver
Au	64	3.4	2	256	8	128	1D33	frog liver
Au	64						1D54	frog muscle
Au	64	3.5	2	256	4	64	2D1	frog liver
Au	64	3.5	0	256	16	128	2D8	frog heart
Au	64	3.1	0	256	32	256	2D16	frog liver
Au	64	3.4	0	256	16	64	2D25	frog liver
Au	64	3.2	2	128	32	32	3D29	frog liver
Au	64	3.6	0	128	32	64	4D3	frog spleen
Au	64	0	0	256	64	64	5N21	frog muscle
Au		2.5	0	128	64	512	T71	frog liver
A. h	0	0	128	8	32	64	1D75	frog intestine
A. h	8	3.2	16	4	4	32	2D20	frog intestine
A. h	32		128	16	32	32	3D5	tadpole liver
A. h	8		0	0	8	1	3D7	tadpole intestine
A. h	32						3D12D	tadpole kidney
A. h	32	2.1	32	2	2	64	3D14	tadpole skin
A. h	16		128	64	32	128	3D15	tadpole intestine
A. h	32		16	64	32	32	3D16	tadpole skin
A. h	0		0	2	8	0	3D18D	tadpole skin
A. h	32						3D19D	tadpole liver
A. h	32		32	16	16	64	3D37L	tadpole skin
A. h	16	3.1	128	32	8	32	5W1	water
A. h	32	0	8	16	8	32	9240	NCIMB
A. vbvs	0	0	8		4	32	2D12	frog intestine
A. vbvs	0	0	8	2	0	8	2N36	frog skin
A. vbvs	0						3D6	tadpole skin
A. vbvs	0						3D12L	tadpole kidney
A. vbvs	0						3N29	frog skin
A. vbvs	0						3N36	frog intestine
A. vbvs	0	0	32	4	8	64	3W1	water
A. vbvs	1						4D10	tadpole intestine
A. vbvs	0	0	4	2	0	16	4D11	frog skin
A. vbvs	0						4D17	frog skin
A. vbvs	0	0	2	4	4	8	4N15	frog intestine
A. vbvs		0	32	4	8	32	37	NCIMB
A. c	0	0	0	2	2	0	1N15L	frog skin
A. c	0						1N36	frog intestine
A. c	0	0	32	16	8	64	2N14	frog skin
A. c	0	0					1N15D	frog skin
A. c	8						2N12	frog intestine
A. c	0	0	0	4	1	1	838T	CECT
A. vbvv	0	0	0	1	1	0	2N1	frog intestine
A. vbvv	0	0	2	0	1	32	3D37S	tadpole skin
A. vbvv	0						3N24	frog intestine
A. vbvv		0	32	4	4	128	4257T	CECT
A. sch	0	0	8	1	1	32	5N11	frog skin
A. sch		0	64	2	16	32	4240T	CECT

Figure 6.3.2.1 Haemolytic activity of Aeromonas isolates against frog RBC
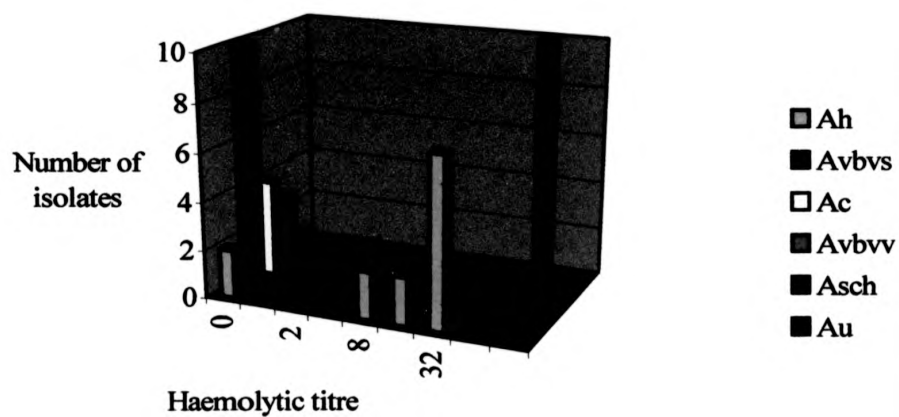
Figure 6.3.2.2 Elastinolytic activity of *Aeromonas* isolates
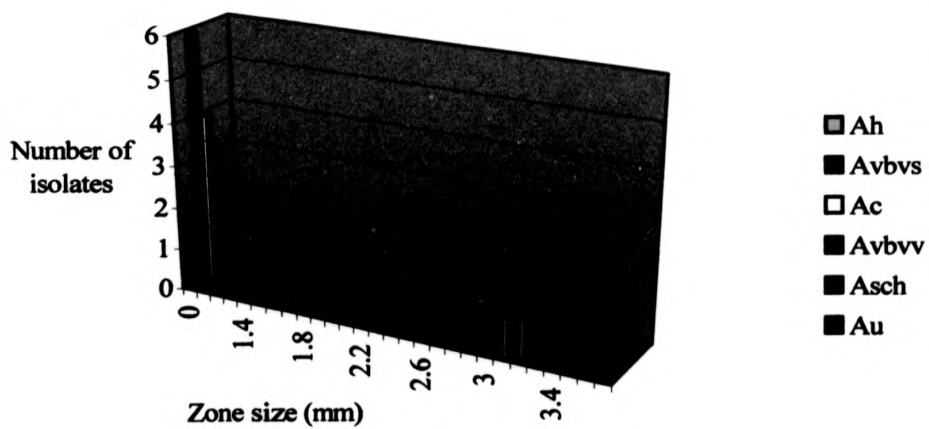
Figure 6.3.2.3 Cytotoxic activity against Vero cells
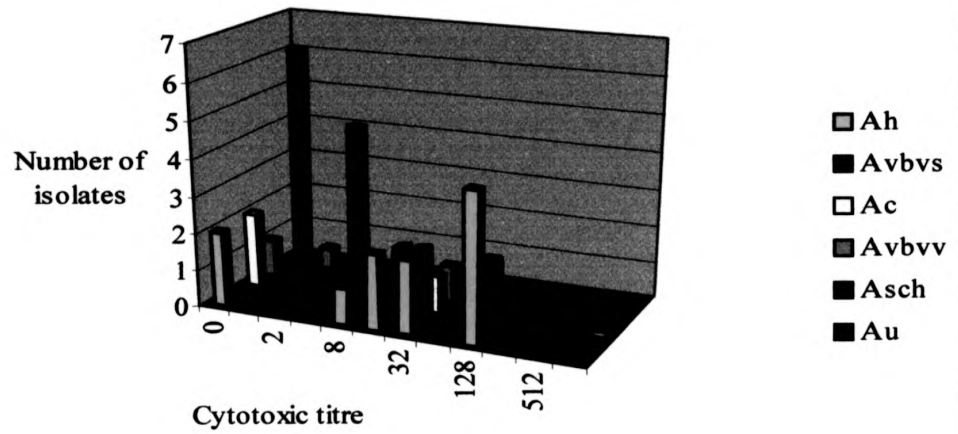
Figure 6.3.2.4 Cytotoxic activity against RTG-2 cells
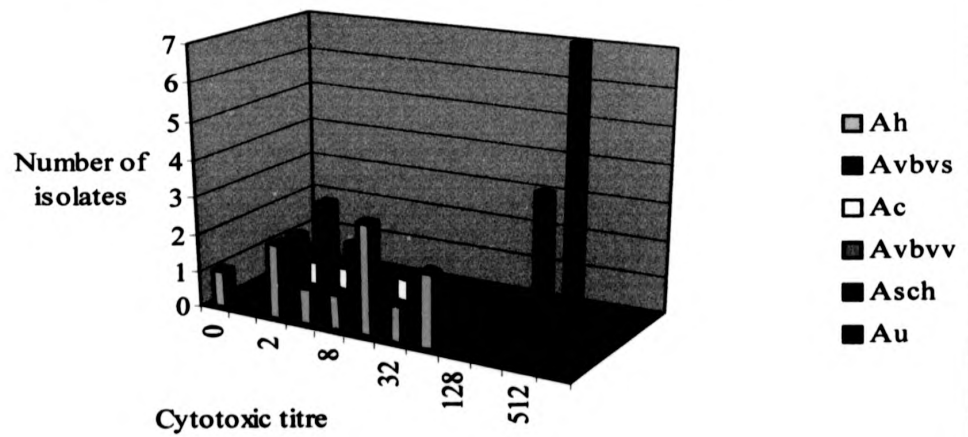
Figure 6.3.2.5 Cytotoxic activity against SSN-1 cells
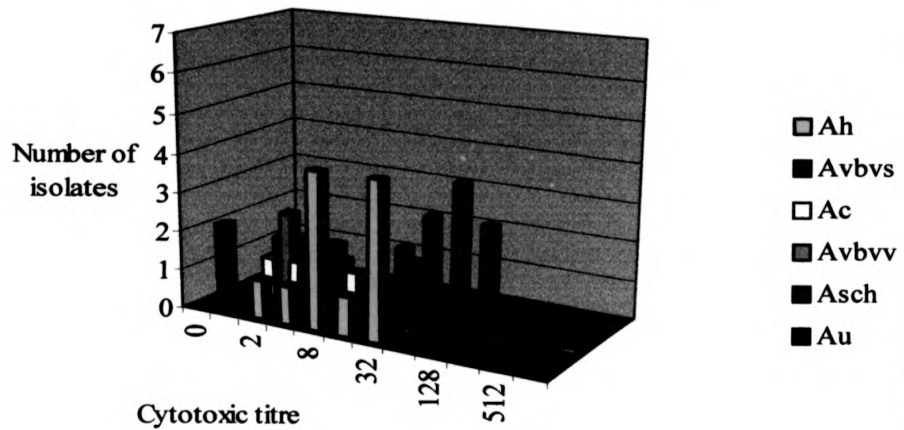
Figure 6.3.2.6 Cytotoxic activity against HCT cells
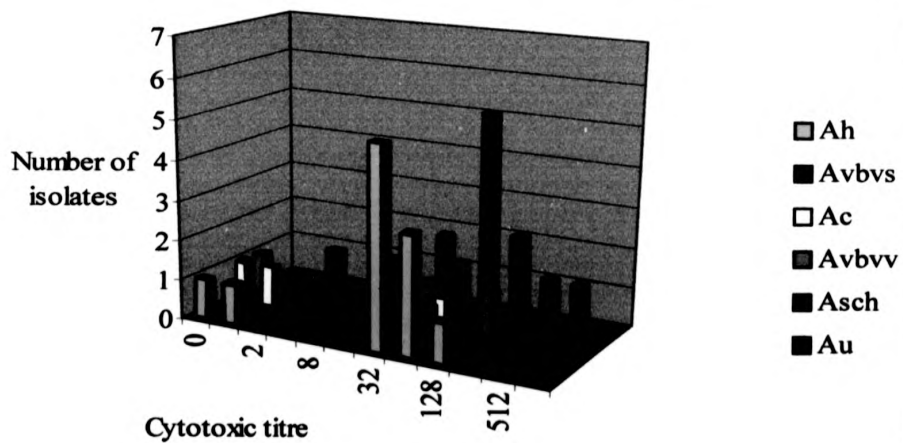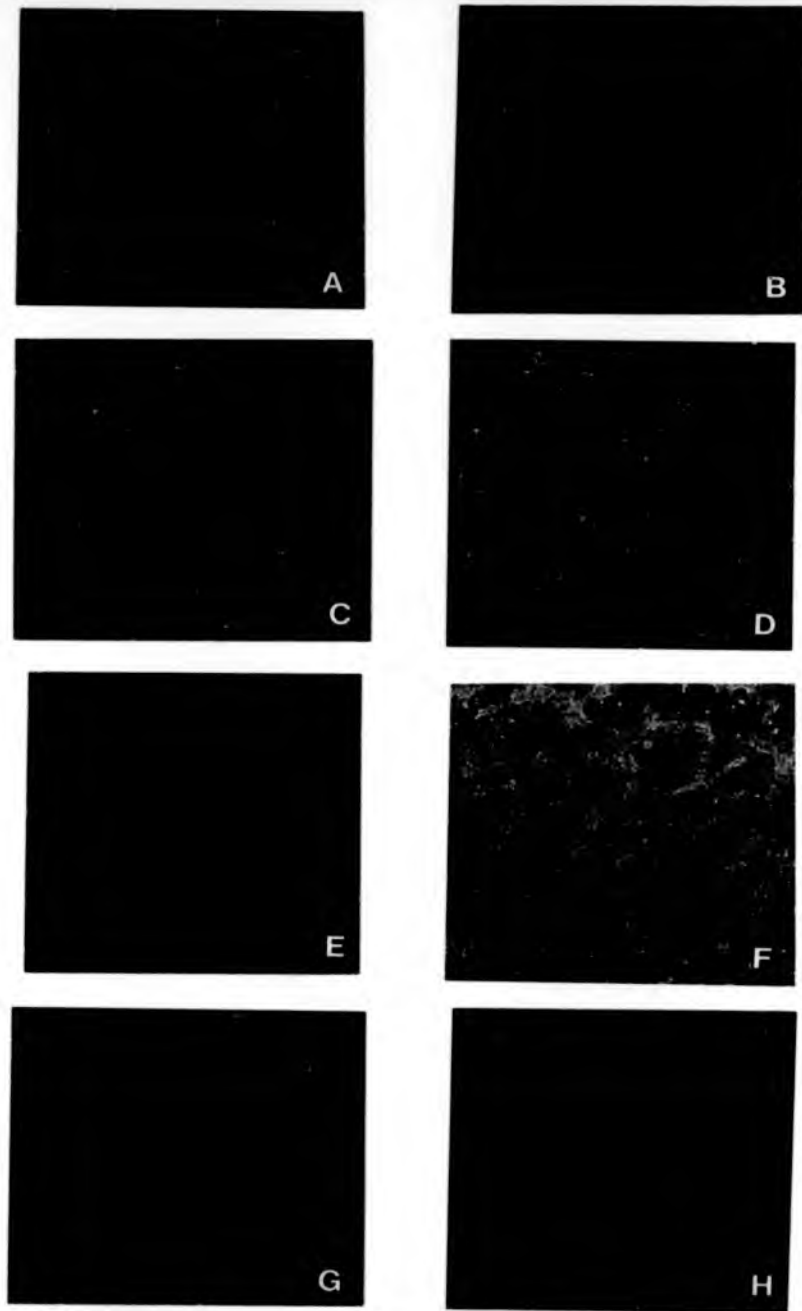
Figure 6.3.2.7 Cytotoxicity of *Aeromonas* ECP on cell lines. A: control Vero cells; B: Vero cytotoxin response; C: control RTG-2 cells; D: RTG-2 cytotoxin response; E: control SSN-1 cells; F: SSN-1 cytotoxin response; G: control HCT cells; H: HCT cytotoxin response. In general degenerative changes were manifested by clusters of rounding cells, cells shrinkage, dendritic elongation and detachment from the surface of the plate.

Analysis of the data using all pairwise multiple comparison procedures (Dunn's Method) revealed that there was a significant difference between the zone sizes of the Au isolates and those of the other *Aeromonas* species (P<0.05) but not between Au and the *A. hydrophila* isolates nor between the *A. hydrophila* isolates and the other *Aeromonas* species.

The production of cytotoxins was determined in different mammalian and fish cell lines. The cytotoxic activity of all the aeromonads varied according to the cell line used, however this variation was most marked in the Au isolates. All the Au isolates displayed strong cytotoxic activity against rainbow trout cells (RTG-2). Titres were remarkably consistant with seven isolates yielding a titre of 256 and the remaining three isolates yielding a titre of 128, a difference of only one two-fold dilution. In comparison a selection of ten *A. hydrophila* from Thailand plus one type strain yielded titres ranging from 0 to 64 with little consistency in the results. The remaining group of motile aeromonads consisting of *A. veronii* biovar sobria, *A. veronii* biovar veronii, *A. caviae* and *A. schubertii* displayed much lower cytotoxicity titres ranging from 0 to 16. This division of the cytotoxic activity into three groups of high, moderate and low activity parallels that of the hemolytic titres of *Aeromonas* ECPs against frog blood.

In contrast results from the mammalian cell line (Vero) produced a different profile. All the Au isolates displayed very little cytotoxic activity against this cell line although the results were again consistent. Six isolates yielded a titre of 0 while the remaining 4 isolates yielded a titre of 2. Cytotoxin positivity was defined as a reciprocal titre of ≥ 4 (Wilcox *et al.*, 1994), therefore 100% of Au isolates did not

possess a cytotoxin active against Vero cells. In contrast several of the *A. hydrophila* isolates displayed strong cytotoxic activity, four of the eleven isolates displayed titres of 128, although titres were again variable ranging from 0 to 128. Titres for the remaining *Aeromonas* species were lower from 0 to 64 with 5 isolates yielding titres of less than 4.

The division of titres into high, moderate and low activity groups were less distinct with the snakehead (SSN-1) and catfish (HCT) cell lines. Au titres appeared higher than those for *A. hydrophila* and the other aeromonads but showed more variation, ranging from 4 to 64 against SSN-1 and 32 to 512 against HCT. The SSN-1 cell line titres ranged from 2 to 32 with *A. hydrophila* titres and from 0 to 16 with the other *Aeromonas* species (seven of which were less than 4). The HCT titres showed even less distinction with *A. hydrophila* and the other motile aeromonads yielding the same range from 0 to 128.

6.3.3 HAEMOLYSIN COLONY HYBRIDISATION

Results are shown in Table 6.3.3. Under high stringency the AHH1 probe (cloned from *A. hydrophila*) hybridised with all the Au strains tested; with three out of four *A. hydrophila* isolates from Thailand; and with the CECT type strains of *A. hydrophila* and *A. salmonicida*. In contrast the aerolysin homologue AHH4 (cloned from *A. hydrophila*) hybridised with only two *A. hydrophila* isolates from Thailand and with the CECT type strain of *A. salmonicida*. The ASA1 probe (cloned from *A. sobria*) hybridised with four out of five *A. veronii* bv sobria isolates from Thailand; with one out of four *A. hydrophila* from Thailand; with the single *A. schubertii* from Thailand; and with the CECT type strains of *A. sobria, A. veronii* bv sobria, *A. veronii* bv

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veronii and *A. schubertii.* The ASH1 gene (cloned from *A. salmonicida*) hybridised

with all ten of the Au isolates but did not react with any of the other Thai aeromonads

or type strains examined.

Table 6.3.3 Number of *Aeromonas* isolates reacting with haemolysin gene probes

Species	Source	n	AHH1	AHH4	ASA1	ASH1
Au	Internal frog	10	10	0	0	10
A. hydrophila	External frog	3	2	2 ´	1	0
	Environment	1	1	0	0	0
	Type strain	1	1	0	0	0
A. veronii bv sobria	External frog	4	0	0	4	0
	Environment	1	0	0	0	0
	Type strain	1	0	0	1	0
A. schubertii	External frog	1	0	0	1	0
	Type strain	1	0	0	1	0
A. veronii bv veronii	External frog	2	0	0	0	0
	Type strain	1	0	0	1	0
A. caviae	External frog	2	0	0	0	0
	Type strain	1	0	0	0	0
A. salmonicida	Type strain	1	1	1	0	0
A. sobria	Type strain	1	0	0	1	0
A. media	Type strain	1	0	0	0	0
A. eucrenophila	Type strain	1	0	0	0	0
A. jandaei	Type strain	1	0	0	0	0
A. trota	Type strain	1	0	0	0	0
A. allosaccharophila	Type strain	1	0	0	0	0
A. encheleia	Type strain	1	0	0	0	0

AHH1 gene cloned from *A. hydrophila*, low sequence homology (42%) with aerolysin
gene.
AHH4 gene cloned from *A. hydrophila*, high sequence homology (93%) with
aerolysin gene.
ASA1 gene cloned from *A. sobria*, significant sequence homology (66%) with
aerolysin gene.
ASH1 gene cloned from *A. salmonicida*, no significant sequence homologies with
bacterial haemolytic proteins in GenBank-EMBL DNA database.

6.3.4 API-ZYM

The enzymatic activities of the 35 isolates examined are summarized in Table 6.3.4.1

The results are presented as the percentage of isolates for each group giving a positive

reaction (+) and a strong positive reaction (++).

Table 6.3.4.1 *Aeromonas* enzymatic profiles as determined by API-ZYM system.

	Au = 10		Ah n=10		Avbvs n=6		Ac n=4		Avbvv n=3		A sch n=2	
	+	++	+	++	+	++	+	++	+	++	+	++
AP	100	30	100	70	100	83.3	100	0	100	66.7	100	50
E	100	20	100	0	100	0	100	0	100	0	100	0
ELi	100	100	100	80	100	100	100	100	100	0	100	100
LeA	100	100	100	50	100	66.7	100	25	100	0	100	100
APh	100	0	100	40	100	33.3	100	0	100	0	100	0
Li	100	40	90	0	100	0	100	0	100	0	100	50
βgal	100	60	90	20	100	33.3	100	0	100	0	50	50
Nac	100	100	90	80	100	100	100	100	100	100	100	100
VAr	90	0	20	0	16.6	0	25	0	33.3	0	50	0
CAr	100	0	50	0	100	0	50	0	66.7	0	100	0
Try	100	70	80	10	100	0	25	0	100	0	100	0
αglu	10	0	0	0	16.6	0	0	0	33.3	0	0	0
βglu	0	0	70	0	0	0	25	0	33.3	0	0	0

AP, alkaline phosphatase; E, esterase; ELi, esterase lipase; LeA, leucine arylamidase;
APh, acid phosphatase; Li, lipase; βgal, β galactosidase; Nac, *N*-acetyl -β
glucosaminidase; VAr, valine arylamidase; CAr, cystine arylamidase; Try, trypsin;
αglu, α glucosidase; βglu, β glucosidase.

In general the *Aeromonas* isolates produced similar enzymes. Valine arylamidase,

cystine arylamidase, trypsin, α glucosidase and β glucosidase gave variable reactions.

Ninety per cent of the Au isolates were capable of producing valine arylamidase

whereas only 20% (2 of 10) of *A. hydrophila* and 25% (4 of 16) of the total remaining

Aeromonas species were positive. Conversely none of the Au isolates produced β

glucosidase as opposed to 70% of the *A. hydrophila* isolates. The extent of enzymatic

activity also varied between the groups. Table 6.3.4.2 shows the differences in

enzymatic composition and activity among the species. The Au isolates yielded

slightly higher values in all categories.

Table 6.3.4.2 Enzymatic composition and activity of *Aeromonas* isolates

Isolate	n	Mean enzymatic composition[1]	Mean sum of enzymatic activity[2]	Mean enzymatic activity[3]
Au	10	11	25.9	2.4
Ah	10	9.9	21.4	2.2
Avbvs	6	10.2	23.8	2.3
Ac	4	9.3	19	2.0
Avbvv	3	10.7	22.5	1.8
Asch	2	10	19.7	2.3

1 Average number of enzymes present per isolate
2 Average total enzymatic activity per isolate. Enzymatic activity was scored as 0, 1, 2, 3, 4 or 5.
3. Average relative activity of the enzymes present per isolate

Multivariate statistical analyses were carried out on the enzyme activities of all the individual isolates in the Au group, *A. hydrophila* group and other *Aeromonas* spp. using Discriminant Function Analysis. This test identified eight significant variables (enzyme activites which could be used to distinguish between the three groups of bacteria) and compared their discriminatory powers. Two successive discriminant functions were computed which together contributed 100% to the overall discriminatory power Table 6.3.4.3. Both discriminant functions (roots) were highly significant ($p < 0.00000$) indicating that the three groups distinguished were statistically distinct.

Table 6.3.4.3 Discriminant function coefficients for API-ZYM enzymes

	Discriminant Function 1	Discriminant Function 2
Try	1.47555	0.60045
Aph	-1.80000	0.32650
βglu	-0.51587	1.99646
Nac	0.24041	-1.04678
βGal	0.78393	0.5081
LeA	0.55340	0.2121
CAr	-0.58717	0.24295
Li	0.20851	-0.40979
Cumulative proportion	0.77257	1.00000

Try, trypsin; βGlu, β glucosidase; APh, acid phosphatase; Li, lipase; Nac, *N*-acetyl -β glucosaminidase; βgal, β galactosidase; LeA, leucine arylamidase; CAr, cystine arylamidase;

The first discriminant function was weighted most heavily by trypsin and acid phophatase, the second function was marked mostly by β glucosidase. The first function accounts for over 77% of all discriminatory power and is therefore the most important interpretation of enzyme discrimination. Figure 6.3.4.1 presents a scatterplot of the two discriminant functions confirming this interpretation. The Au group are plotted much further to the right in this ordination diagram. Thus the first discriminant function distinguishes between Au and the other aeromonads, while the second function provides some discrimination between *A. hydrophila* and the other *Aeromonas* species. The most significant and clear discrimination possible distinguishes Au by the first function. This function is marked by a positive coefficient for trypsin and a negative coefficient for acid phosphatase, indicating that isolates with low trypsin and high acid phosphatase activity are unlikely to be Au.

Figure 6.3.4.1 Ordination Diagram of API-ZYM Discriminant Functions
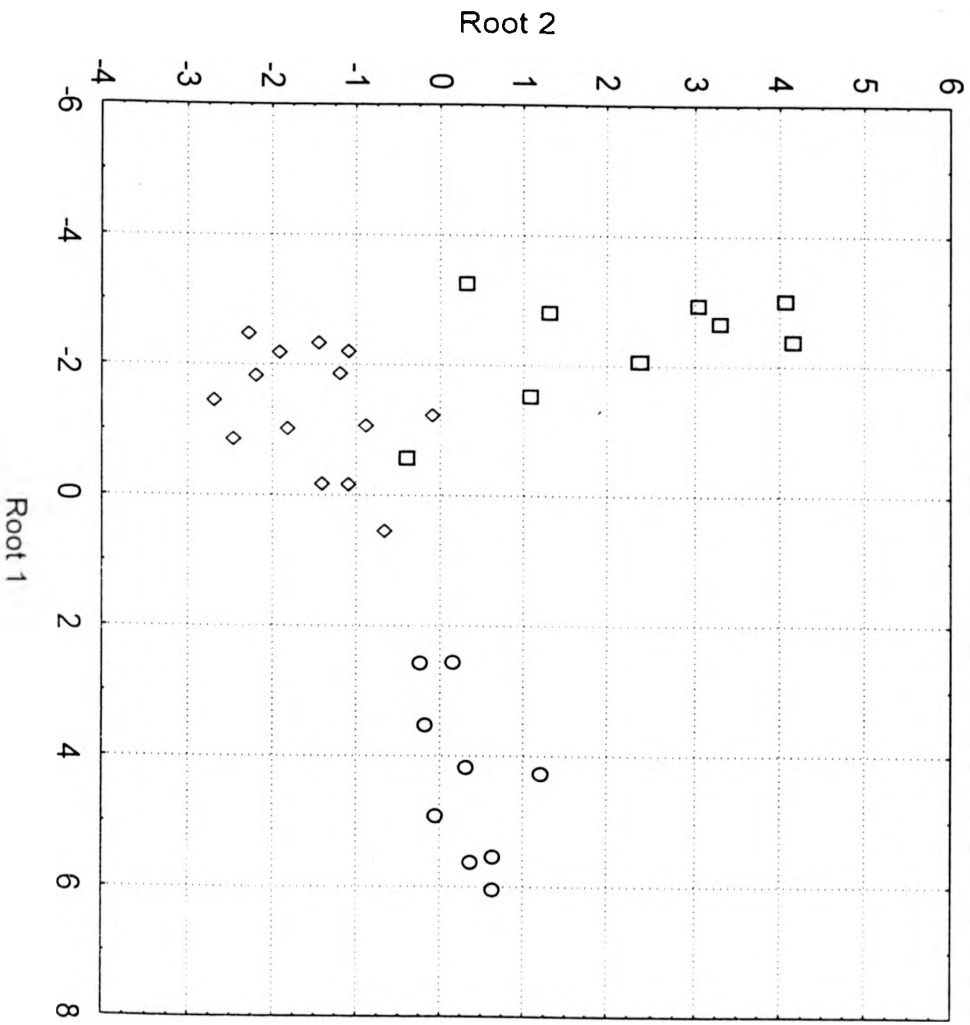
Legend:
○ Au
□ A. hydrophila
◇ Other Aeromonas spp.

6.3.5 Haemagglutination and haemagglutination inhibition

Haemagglutination activity of the *Aeromonas* isolates against frog, trout and horse

RBC are displayed in Table 6.3.5.1.

Table 6.3.5.1 Number of isolates displaying haemagglutination activities.

	n	Frog		Trout		Horse	
		+	++	+	++	+	++
Au	*10*	10	0	9	0	9	0
Ah	*5*	3	2	3	1	3	1
Avbvs	*6*	0	5	3	2	0	5
Ac	*4*	1	0	0	0	0	1
Avbvv	*3*	0	2	1	1	1	2
Asch	*2*	0	1	1	0	1	0

++ reaction immediate and complete
+ reaction incomplete or not instantaneous but occurred within 5 minutes.

Haemagglutination actvity (HA) against all three cell types was a common property of

the thirty aeromonads screened, with the exception of the *A. caviae* isolates, only one

of which displayed activity against frog red cells, and one of which displayed activity

against horse cells. Strong (++) HA was common amongst both biovars of *A. veronii*.

Only 25% of HA positive *A. hydrophila* isolates exhibited strong activity against

horse and trout blood while 60% exhibited strong activity against frog cells. None of

the Au isolates displayed strong HA against any of the blood cells examined.

Table 6.3.5.2 shows the pattern of haemagglutination seen in the presence of fucose,

galactose and mannose. The sugar inhibition studies showed that, in general HA was

inhibited by the presence of mannose. 87.5% of the isolates HA positive for frog RBC

and 79% of the isolates HA positive for trout RBC displayed the F-G-M+ pattern.

100% of the HA positive Au strains demonstrated this pattern of HA inhibition

against both frog and trout RBC.

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Table 6.3.5.2 Percentage of isolates displaying haemagglutination inhibition in the

presence of various sugars.

	n	n	F-G-M+		F-G+M+		F+G-M-		F+G-M+		F+G+M+		F-G-M-	
	Fg	T	Fg	T	Fg	T	Fg	T	Fg	T	Fg	T	Fg	T
Au	10	9	10	9	0	0	0	0	0	0	0	0	0	0
Ah	5	4	4	3	1	1	0	0	0	0	0	0	0	0
Avbvs	5	5	3	1	1	3	0	0	0	0	1	0	0	1
Ac	1	0	0	NA	0	NA	0	NA	1	NA	0	NA	0	NA
Avbvv	2	2	1	1	1	0	0	1	0	0	0	0	0	0
Asch	1	1	1	1	0	0	0	0	0	0	0	0	0	0

F, fucose; G, galactose; M, mannose; + inhibition óf haemagglutination; -, no

inhibition; NA, non agglutinator

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6.4 Discussion

The pathogenicity mechanisms of *Aeromonas* spp. involve a complex interaction of bacterial and host components (Janda 1991). There are a multitude of putative virulence factors, which may have a role in the aetiology of disease; however their precise combination has yet to be determined (Gosling 1996). The findings presented in this section address some of those factors and illustrate the complex, polygenic nature of *Aeromonas* pathogenicity.

The aeromonads used in the present study were divided, on the basis of their reactions in the various assays, into three major groups; Au, *A. hydrophila* and other *Aeromonas* spp. A number of differences were noted among the three groups.

Haemolysin activities against frog RBC were significantly different between the collection of aeromonads. Groups of high haemolytic activity (Au), moderate haemolytic activity (*A. hydrophila*) and low haemolytic activity (other *Aeromonas* spp.) were noted. The fact that the isolates with high haemolytic activity were all found internally in septicaemic frogs indicates that haemolysin production may be correlated to origin and potential virulence of *Aeromonas* isolates. Janda *et al.* (1994) in a study of 20 isolates of *Aeromonas* from human blood found that, among other factors, elevated levels of protease and haemolysin activity to sheep RBC correlated with higher virulence.

The pilot study using RBC from a range of origins indicated that haemolytic activity of the *Aeromonas* isolates varied according to the source of RBC used in the assay, with trout being the most sensitive to Au haemolysins and horse the least. A

subsidiary study was therefore carried out in conjunction with the present work and published in an MSc thesis by Miranda (1997). Miranda investigated the haemolytic activity of a selection of aeromonad isolates from the present study against rabbit, sheep, catfish, trout and frog RBC. He discovered that the *Aeromonas* isolates showed differing haemolytic activities against the blood samples tested. Of the two mammalian blood samples, only rabbit red blood cells showed sensitivity to haemolysins produced by the aquatic *Aeromonas* isolates. Both the catfish and frog RBC appeared sensitive to the haemolysins while the trout cells were extremely sensitive.

These findings supplement and support those of a much earlier study by Bernheimer *et al.*, (1975) who investigated the sensitivity to aerolysin of erythrocytes from eleven different animal species (all mammalian). Their results showed that RBC from different mammals differed greatly in sensitivity to lysis by purified aerolysin. Those from the rat were 300 times more sensitive than those from the sheep, whereas RBC from other species exhibited intermediate degrees of sensitivity or resistance.

This has implications for laboratories investigating the haemolytic activity of aeromonads, particularly non-mammalian and environmental isolates. The standard screening method at present is to examine zones of diffusion on blood agar. Agar plates require much larger volumes of blood than microtitre plates, therefore commercially prepared mammalian bloods are generally used, commonly horse in the UK and sheep in the USA. These substrates may however be inappropriate to the isolates under investigation.

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For example Paniagua *et al.,* (1990) examined virulence factors of motile *Aeromonas* spp. isolated from a river and evaluated their ability to produce haemolysins on agar plates containing sheep erythrocytes. Karunakaran and Devi (1994) investigated the haemolytic capabilities of *A. caviae* on sheep RBC. However Bernheimer *et al.* (1975) and Miranda (1997) both demonstrated that sheep RBC are relatively insensitive to *Aeromonas* haemolysins.

These papers illustrate the importance of selecting reagents appropriate to the strain and origin of isolates under investigation. Paniagua *et al.,* (1990) found no correlation between caeseinase, haemolytic and cytolytic activities and virulence for fish in the ninety-seven motile *Aeromonas* strains examined. However the findings may have been different had the authors used RBC from a source other than sheep.

In the present study the sensitivity of different RBC to haemolysins was reflected in the sensitivities of some cell lines to *Aeromonas* cytotoxins. In particular the RTG-2 were extremely sensitive to Au cytotoxin but less so to the toxins produced by other species. This is of interest because the RTG-2 cell line originates from rainbow trout and the blood cells showing the most sensitivity to the Au haemolysins were the rainbow trout RBC. In contrast the mammalian cell line (Vero) showed very little sensitivity to the cytotoxins produced by the Au isolates but were more sensitive to those toxins produced by the *A. hydrophila* group.

In the past Vero cells have been recommended as the cell line most appropriate for screening *Aeromonas* cytotoxins (Kuijper *et al.* 1989, Noterdaeme *et al.* 1991). Kuijper *et al.* (1989) compared the cytotxic activity of 100 *Aeromonas* strains on Vero

(monkey), HeLa (human) and Y_1 mouse adrenal cells. They found that microscopic
changes were more pronounced and present earlier in Vero cells than in HeLa or
mouse adrenal cells. These workers were studying human faecal isolates therefore the
primate cell line may have been appropriate. However a later study by Noterdaeme *et
al.,* (1991) characterised twenty-five *A. hydrophila* from freshwater fish and
freshwater samples. They distinguished three clusters of bacteria and compared the
production of cytotoxin on Vero, HeLa, HEp-2 (human) and BB (fish). The Vero and
BB cell lines gave opposing results, the Vero cell line was least sensitive to the
isolates which caused 100% lysis of the BB cells and vice versa. The authors did not
discuss the BB cell line results and concluded that Vero cells were more suitable for
detection of *Aeromonas* cytotoxins because degeneration and vacuolation was more
pronounced and faster than in HeLa cells. While this assay may be appropriate for
studies on human isolates, caution should be exercised when interpreting results for
isolates of aquatic origin. Many studies have used Vero cells alone to screen for
cytotoxins (Majeed and MacRae, 1994, Vadivelu *et al.* 1991, 1995). The studies
presented here however have shown that red blood cells and cell cultures from
homeotherms may not be appropriate to investigate the cytotoxins associated with
disease in poikilotherms.

The ECP of the Au group were particularly active against both rainbow trout RBC and
cell cultures. It may be that the haemolysin and the cytotoxin are the same protein.
However Vadivelu *et al.,* (1991) in a study of human clinical isolates found that some
isolates produced either cytotoxin or haemolysin alone and concluded therefore that
the haemolysin and the cytotoxin may be two different proteins. In their study only
Vero cells and rabbit erythrocytes were used to determine toxic activity and this may

have influenced the results. It will not be possible to determine whether the cytotoxin and haemolysin are the same factor until each toxin has been purified and assayed against a range of RBC and cell cultures.

The results presented in this study demonstrated that the haemolytic abilities of the Au isolates against frog RBC were significantly higher than those of the *A. hydrophila* isolates and other *Aeromonas* species. The colony hybridisation studies revealed that Au isolates possessed haemolysin genes which differed from those of the other *Aeromonas* isolates. In particular, the gene ASH1 was present in Au and in none of the other Thai aeromonads or type strains tested. Previous work with haemolysin gene probes had indicated that almost all motile aeromonads possess either the sobria gene ASA1 (or one of its homologues) or the hydrophila gene AHH4 (Hirono *et al.* 1992, Hirono and Aoki 1993). Au therefore is unusual among the motile aeromonads in possessing neither of these genes although both were present in other motile aeromonads from Thailand.

It is interesting that all 10 of the Au isolates possessed the ASH1 gene as this had previously only been found in a single *A. salmonicida* isolate and two pathogenic *A hydrophila* isolates, one from a septicaemic eel in Japan and one from Thailand (Hirono pers. comm.). It was originally thought that this gene did not originate in aeromonads but was acquired from another genus during bacterial passage in the laboratory. The fact that all 10 of the Au isolates possessed this gene indicates that it does in fact belong to the genus *Aeromonas* and may explain the higher haemolytic activity against frog and trout RBC of the Au isolates in comparison to the other motile aeromonads from Thailand. It is possible that the products of differing

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haemolysin genes have a range of activities against cells from various sources. The aerolysin gene product may be active against cells of mammalian origin while the ASH1 product is active against cells from aquatic animals. Further work is necessary to investigate the significance of the presence of the ASH1 gene and whether it may be associated with bacterial virulence.

Charkraborty *et al.*, 1987 presented genetic evidence that the aerolysin structural gene *aerA* is found in all members of the genus. Howard *et al.*, 1996 suggest that this gene is not found in other bacteria and that it may be considered a distinguishing feature of the genus *Aeromonas*. However DNA hybridisation studies by Hirono *et al.*, (1992) indicated that only 4 of 14 (28.6%) clinically isolated strains of motile *Aeromonas* spp. contained nucleotide sequences homologous to the *A. hydrophila* aerolysin gene, therefore raising doubts about the use of this gene to distinguish the genus. The present study confirms this finding, only 3 of the 37 isolates tested contained the AHH4 homologue of aerolysin and only a further 10 isolates contained sequences homologous to the closely related *A. sobria* aerolysin ASA1. The Au isolates although highly haemolytic contained genes AHH1 and ASH1. The AHH1 gene has low homology with aerolysin while ASH1 does not have homologous sequences with any bacterial haemolytic proteins present in the DNA data banks. The use of aerolysin as a marker for genus *Aeromonas* is therefore questionable.

An enzyme which is known to be extremely haemolytic for fish (but not mammalian) erythrocytes and is of prime importance in the pathogenesis and virulence of *Aeromonas salmonicida* isolates is glycerophospholipid:cholesterol acyltransferase (GCAT). This phospholipase is the most lethal component of the *A. salmonicida*

exoenzymes (lethal dose 45ng/g fish). *In vitro* this toxin has high leucocytolytic and

cytolytic (RTG-2 cells) activity. Its precise mode of pathogenesis is uncertain and

appears complex (Ellis, 1991).

The ASH1 haemolysin gene differs from the GCAT gene in its molecular mass and

nucleotide sequences (Hirono and Aoki, 1993), however the exotoxins may have

similar modes of action. The GCAT toxin of *A. salmonicida* possesses extremely high

haemolytic activity for fish but not rabbit erythrocytes, while Au ECPs demonstrated

high haemolytic activity for rainbow trout but not sheep RBC with moderate activity

against rabbit erythrocytes.

The selective haemolytic activity of GCAT is probably related to the phospholipid

content of erythrocyte membranes. The optimal substrate for the GCAT has been

reported to be phosphotidylcholine (PC) substituted with unsaturated fatty acids (Ellis

1991). Fish tissues are much richer in polyunsaturated fatty acids than are those of

mammals. The proportion of PC in the erythrocyte membranes of Atlantic salmon is

58.6% of total phospholipids compared with 29.5% in human erythrocytes. Therefore

over half of the fish cell membrane phospholipids are highly susceptible to the action

of GCAT and possibly the ASH1 product. Exposure to the enzyme could cause the

membrane to lose its integrity resulting in cell lysis. The mammalian erythrocyte

membranes may remain intact because they contain only a minority of suitable

substrates (Ellis 1991).

Although many proteases have been implicated in the pathogenesis of bacterial

disease, there is currently no rapid *in vitro* system to assess levels of enzymatic

activity in relation to virulence of isolates. The present study assessed whether

particular enzymes assayed in the API-ZYM system could be correlated with clinical

and non-clinical isolates and hence be potential virulence factors. Multivariate

statistical analysis revealed that the API-ZYM system differentiated between the three

groups of *Aeromonas* isolates defined by the haemolysin and cytotoxin studies and

that a specific group of enzymes were responsible for those differences.

The enzyme which received the heaviest weighting in the discriminant function

analysis was trypsin. Most of the *Aeromonas* isolates possessed this enzyme (except

A. caviae), however 70% of the Au isolates produced strong positive reactions

whereas only one *A. hydrophila* isolate and none of the other aeromonads yielded high

levels of this enzyme. Waltman *et al.* (1982) in a study of *Aeromonas* isolates from

various sources, found trypsin was slightly more prevalent in clinical isolates from

humans than in isolates from the environment or fish. The present studies also found

trypsin levels are slightly higher in clinical isolates from frogs and could possibly be

correlated with potential virulence in these animals.

The enzyme with the second heaviest weighting, acid phosphatase, was present in all

isolates, however only *A. hydrophila* and *A. sobria* isolates produced strong positive

reactions with this enzyme. Interestingly alkaline phosphatase was also present in all

isolates, but only 30% of Au gave strong reactions while 70% and 83.3% of *A.*

hydrophila and *A. veronii* biovar sobria respectively gave strong reactions. These

enzymes have similar functions (hydrolysis of esters of phosphoric acid) but different

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pH optima, possession of both indicates an extensive phosphatase activity. Both enzymes are widely distributed among living organisms, including many bacteria.

The enzyme given the third heaviest weighting was β glucosidase. Neither Au nor *A. sobria* produced this enzyme while 70% of the *A. hydrophila* isolates produced the enzyme at low levels. This finding agrees with that of Waltman *et al.*, (1982) who found that *A hydrophila* were positive for the enzyme while *A. sobria* isolates were negative. The enzyme hydrolyses the (β1→4) linkages of glucosides such as cellulose or plant starch. Thus possession of the enzyme would not be of particular benefit to a bacterium adapted to infect animal tissues.

In general the API-ZYM enzymes which conferred the distinction between the *Aeromonas* groups (with the exception of trypsin) could not be said to promote bacterial invasion or spread. Many of the enzymes which distinguished Au from *A. hydrophila* were shared with other *Aeromonas* species. Thus although the API-ZYM tests confirmed the phenotypic distinctiveness of the Au isolates, the enzymatic activities examined are not the only required determinants for the development of septicaemic disease in frogs, other factors must be involved in pathogenesis and invasiveness of isolates.

Previous studies have indicated that high elastase activity can be correlated with virulence of bacterial strains (Hsu *et al.*, 1981, Santos *et al.*, 1988, Esteve *et al.*, 1995b). In the present study all but one of the Au isolates demonstrated elastinolytic

activity, as did three of the five *A. hydrophila* isolates examined. None of the other

motile aeromonads displayed any elastinolytic activity. There was a significant

difference between the elastinolytic capabilities of the Au group and the other

Aeromonas species but not between Au and *A. hydrophila*. It is probable that

possession of elastase enhances bacterial pathogenesis through degradation of critical

host proteins but that elastinolytic abilities alone do not confer virulence on an isolate.

Differences in cell surface properties may be related to host specificity and

invasiveness of bacteria. In the present study haemagglutination activity against blood

cells from frog, fish and mammalian sources was a common property amongst the

aeromonads with the exception of *A. caviae*. However strong agglutination reactions

were more common amongst *A. veronii* biovars than the other isolates, none of the Au

isolates displayed strong agglutination reactions for any of the blood cells tested. This

corresponds with the findings of previous studies. Majeed and MacRae (1994)

demonstrated that strong agglutination was more common among *A. sobria* isolates

and Nishikawa and Kishi (1988) found that haemagglutination was more common in

A. sobria than in *A. hydrophila* or *A. caviae*. Pin *et al.* (1994) demonstrated that

agglutination activity was preferentially associated with *A. hydrophila* and *A. sobria*

while very few *A. caviae* had agglutinating activity. It is unlikely therefore that

haemagglutination alone can be linked to virulence.

Atkinson and Trust (1980) demonstrated by haemagglutination inhibition of 11

selected strains of *A. hydrophila* with 17 different sugars that a spectrum of different

attachment mechanisms are present in aeromonads. Specificity of attachment

mechanisms may be associated with virulence, as the recognition of different surface

receptors determines selection of target cells and efficacy of adherence.

Pin *et al.* (1994) found that haemagglutination was inhibited in general by the

presence of mannose, 86% of their human clinical isolates and only 56% of the strains

from food showed mannose sensitive agglutinins. Majeed and MacRae (1994) found

that approximately 50% of 114 *Aeromonas* isolates from lamb meat products were

capable of haemagglutinating human erythrocytes. They showed a strong correlation

(100%) between cytotoxin production by *Aeromonas* strains and haemagglutination

inhibition in groups F-G-M+ and F-G+M+. In the present study most of the

Aeromonas isolates displayed haemagglutination which could be inhibited by

mannose (M+), only two *A. veronii* biovars were capable of haemagglutinating trout

RBC in the presence of mannose (M-). The Au isolates were again very consistent in

their reactions, all displayed the same pattern of F-G-M+ for both trout and frog

blood. The other aeromonads exhibited a larger range of inhibition patterns however

F-G-M+ and F-G+M+ were predominant groups for both trout and frog blood. It was

not possible therefore to correlate haemagglutination and sugar inhibition patterns

with potential virulence of isolates

In conclusion the finding that Au was highly haemolytic for frog and trout blood and

cytotoxic for rainbow trout cells indicates that these isolates are more likely to be

virulent for these species. The possession of the ASH1 gene may be related to these

properties as it was only present in the Au isolates and had previously only been found

in another three aeromonads, all of which were associated with disease. The ASH1

gene was first cloned from a strain of *A. salmonicida* isolated by S.F. Snieszko from a

diseased brook trout, indicating that the gene product may be toxic for trout

(American Type Culture Collection, 1992). If the gene does encode for a toxin active

against both trout and frog cells then further studies are indicated to isolate the pure

gene product by genetic manipulation and identify of its role in the disease process.

Chapter 7. Pathogenicity Models

7.1 INTRODUCTION

Pathogenicity models aim to reproduce bacterial disease *in vivo* by challenging

selected host species with bacterial isolates. These models enable the disease process

to be monitored and thereby fulfil many important functions. They are essential for the

evaluation of virulence of bacterial strains. In addition they can be used to test the

efficacy of prophylactic and chemotherapeutic treatments and to investigate the

relationship between environmental stressors and bacteria in the aetiology of disease

(Janda 1991).

7.1.1 MODE OF CHALLENGE

7.1.1.1 Injection challenge

Under laboratory conditions many bacterial diseases, particularly those prevalent in

farmed aquatic species, are difficult to reproduce and injection of the pathogen is the

easiest way to establish an infection. However studies using this method have major

flaws as the bacteria are introduced in a manner that does not adequately reflect

natural routes of infection (Wise *et al.* 1993). The normal processes of bacterial

invasion and multiplication are circumvented and the host response may be unnatural

as a result. Large numbers of bacteria introduced simultaneously could overwhelm the

host, and induce a toxic response rather than cause a bacterial septicaemia. Despite

these major limitations, this method is thought useful to demonstrate the pathogenicity

of micro-organisms.

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7.1.1.2 Bath challenges

As an alternative to injection challenges, aquatic animals can be challenged by immersion in suspensions of potential pathogens. These bath challenges have the advantage of being more representative of natural infections. Unlike challenge by injection (which imposes the additional stress of handling each individual fish) they more closely simulate the natural routes of infection because none of the natural barriers in the fish is by-passed. However researchers using a bath challenge with catfish (*Ictalurus punctatus*) and *Edwardsiella ictaluri* found that the method was associated with a high degree of variability (Wise *et al.* 1993). The authors postulate that challenge responses vary between aquaria due to differences in fish stocks and their stress responses and conclude that such systems are effective at detecting only large differences in treatment. Such limitations would, however, apply to any experimental model, irrespective of the mode of bacterial transmission. Comparisons between bath challenges and controlled natural infections in fish show similar infectivity kinetics indicating that disease development is relatively consistent after the infection has been established (Wise *et al.*, 1993; Meade *et al.* 1994). The main disadvantage of the immersion challenge is that it requires a larger bacterial inoculum to accomplish the challenge than any of the other procedures. This becomes an important consideration if the challenge pathogen is difficult to grow in bulk.

7.1.1.3 Co-habitation challenges

The co-habitation challenge is a modification of the bath challenge and represents a yet more natural route of infection. This type of challenge is accomplished by holding groups of deliberately infected aquatic animals in water with non-infected animals to ensure a natural source of challenge for the non-infected "cross-over" animals. These

models best mimic a natural water borne exposure and disease outbreak. They result in a subtle challenge that is not likely to overwhelm minor differences in resistance that might occur among different groups of fish (Murray *et al.*, 1992). However challenge methods based on cohabitation take longer to complete than other methods and have the disadvantage that the challenge dose received by the fish is unknown.

Investigations into the complex aetiology of aquatic bacterial diseases should use test challenge methods that closely mimic natural exposure to the pathogen. Bath and co-habitation challenges best fulfil this requirement. Water-borne exposure enables the host to demonstrate its total repertoire of immune mechanisms located both externally and internally (Nordmo and Ramstad, 1997). On the other hand, these methods are more difficult to control and standardise than injection methods. The pathogenicity of some strains of bacteria may also be insufficient to induce a satisfactory outbreak of disease in the target species by these methods (Nordmo, 1997). It is frequently very difficult to obtain sufficiently high levels of morbidity in experimental water-borne challenges, to evaluate different stressors or treatments and researchers are often compelled to use injection challenge (Ellis, 1988).

7.1.2 PATHOGENICITY MODELS USING MOTILE AEROMONADS

7.1.2.1 Variation in host susceptibility and strain virulence

Most investigations determining the relative pathogenicity of motile *Aeromonas* strains have used either mouse or fish models, although limited studies have also been performed with chicks and turkey poults (Janda 1991). In general these studies have concentrated on the use of injection challenges. Values obtained from such

investigations indicate that between 10- and 1000- fold fewer bacteria are required to cause mortality in susceptible fish than in susceptible mice (Janda 1991). Brenden and Huizinga (1986) compared the virulence of three strains of *A. hydrophila* by intramuscular injection of normal and x-irradiated mice and goldfish. The 50% lethal dose in normal animals ranged from 6.3×10^7 to 2.8×10^8 in mice and from 5.9×10^5 to 6.2×10^6 cfu in goldfish. The authors suggested therefore, that a tropism for fish cells may exist in aeromonads.

Many different fish species have been used in *Aeromonas* challenge models, including: channel catfish (De Figueiredo and Plumb, 1977; Hsu *et al.*, 1981), rainbow trout (Mittal *et al.*, 1980; Peters *et al.*, 1988), walking catfish (Supriyadi, 1986, Angka *et al.*, 1995), giant gourami (Supraydi, 1986), various carp species (Suprayadi, 1986; Ramaiah and Manohar, 1988), milk fish (Lio-Po and Duremdez-Fernandez, 1986), European eels (Esteve *et al.*, 1993, Esteve *et al.*, 1995b), tilapia and blue gourami (Leung *et al.*, 1995).

Some studies have demonstrated a range of susceptibility to aeromonads among fish species. Supriyadi (1986) tested the susceptibility of four fish species to infection by *A. hydrophila* at three levels 10^3, 10^5 and 10^7 bacterial cells per fish. He found that the walking catfish were most susceptible to infection by *A. hydrophila* at 10^5 cells per fish. Giant gourami were more resistant than the walking catfish but less resistant than two strains of common carp tested, which did not differ in susceptibility. Esteve *et al.*, (1993) demonstrated that rainbow trout were less susceptible to infection by *A. hydrophila* and *A. jandaei* isolates than European eels, perhaps because of the lower

water temperatures at which they were held during challenge. In contrast Leung *et al.* (1995) found that the LD_{50} values (the number of bacteria required to kill 50% of challenge animals) of *A. hydrophila* in tilapia and blue gourami were similar. The LD_{50} values of the virulent isolates were in the range 10^4-10^6, the LD_{50} values of the avirulent strains ranged from $10^{7.5}$ to $>10^8$.

Many studies have demonstrated significant variations in strain to strain virulence potential of *Aeromonas* isolates. De Figueiredo and Plumb (1977) investigated the virulence of different isolates of *A. hydrophila* obtained from diseased fish and water, for channel catfish. The water isolates were significantly ($P< 0.05$) less virulent than the isolates from diseased fish although all the isolates were similar in a range of biochemical tests.

Mittal *et al.* (1980) studied the virulence of *A. hydrophila* isolated from moribund and healthy fish, and *A. sobria* isolated from healthy fish, for their virulence to rainbow trout. The LD_{50} values for the two *A. hydrophila* strains isolated from moribund fish were $10^{4.5}$ and $10^{5.5,}$ while those for the isolates from healthy animals were all $>10^7$cells.

Hsu *et al.* (1981) studied the virulence of 20 *A. hydrophila* isolates for channel catfish. The authors had found two discrete groups of *A. hydrophila* when they studied elastase activity. They discovered that cells from elastase positive strains produced lesions and mortalities when injected into channel catfish. High elastase activity isolates caused up to 80% mortality, within a one week period of inoculation into

groups of channel catfish. On the other hand, only one of the low activity isolates caused mortality in the inoculated channel catfish.

Angka *et al.* (1995) assessed the virulence of eighteen *A. hydrophila* isolates isolated from walking catfish with ulcerative disease. The isolates were grouped as virulent, weakly virulent and avirulent strains according to the LD_{50} of $10^{4.5-5.5}$, $10^{5.5-6.5}$ and > 10^7 cfu/ml respectively. For virulent strains, 30% of the fish injected with 10^4 cfu/ml died between 18 and 120 hours while no fish died when injected with 10^4 cfu/ml of the weakly virulent and avirulent strains. The results showed that different isolates of *A. hydrophila* obtained from ulcerated walking catfish showed a great variation in pathogenicity and virulence.

Esteve *et al.*, (1995b) undertook virulence and lethality assays for 24 strains of motile aeromonads (*A. hydrophila, A. jandaei, A. sobria, A. caviae* and *A. allosaccharophila*) in European eels. For each strain, six juvenile European eels were injected intraperitoneally with 0.1ml of a suspension of bacteria containing 10^{10} - 10^4 cells/ml. The authors found that all *A. hydrophila* strains and most *A. jandaei* isolates (71%) were virulent for eels. Mortalities began at 18h post-challenge and "red fin disease" was produced in elvers that died during the experiment. Pure cultures of inoculated *A. hydrophila* and *A. jandaei* strains were recovered from kidney and liver of all dead and moribund eels. Isolates of *A. sobria, A. caviae* and *A. allosaccharophila* were avirulent for European eels since no mortalities were recorded in challenged elvers at doses > $10^{8.4}$ cfu per fish.

7.1.2.2 Comparisons of *Aeromonas* challenge methods

The studies described above all used injection challenges, either intraperitoneal or intramuscular, to induce disease. However in nature bacterial pathogens may gain entry by more than one route. Therefore other studies have investigated the effects of different routes of bacterial challenge.

Lio-Po and Duremdez-Fernandez (1986) tested the virulence of two *A. hydrophila* strains isolated from milk fish against transport stressed *Chanos chanos* fingerlings. Various combinations of bacterial concentration and different routes of inoculation (intraperitoneal injection, bath treatment of scaled fish and bath treatment of undamaged fish) were tested. Results showed that bacterial entry was more effective in fish with scales removed than in uninjured fish or through intraperitoneal injection. The minimum lethal dose of *A. hydrophila* to scaled fish appeared less than 10^5 cells/ml water. When *A. hydrophila* was injected intraperitoneally into undamaged fish a dose of 10^7 cells per 2g fish was needed to cause significant mortalities. In general the virulence of the isolates was directly proportional to dose.

Esteve *et al.*, (1993) conducted studies to determine the pathogenicity for European eels of *A. hydrophila, A. sobria, A. caviae* and *A. jandaei*. Virulence of isolates obtained from diseased eels, clinically healthy eels and water were evaluated by two challenge methods: intraperitoneal injection and bath exposure. The i.p. experiments were used to assess the potential virulence of isolates. Only the most virulent strains in i.p. experiments were selected for bath challenges. Of the isolates tested by i.p. all strains of *A. hydrophila* and those of *A. jandaei* isolated mainly from disease outbreaks, were pathogenic for eels (LD$_{50}$ dose i.p. $10^{5.4}$ to $10^{7.5}$ cfu per fish). The

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disease was acute in eels infected by i.p. challenge. Strains of these species caused

lower mortality rates and progressed more slowly, giving rise to an ulcerative disease,

when bath challenges of 10^7 to 10^8 cfu/ml were used

Ramaiah and Manohar (1988) tested the ability of *A. hydrophila* to produce infection

and cause mortality in Indian major carp by immersion exposure with or without

scarification and through intramuscular injection. No mortality or any sign of disease

was observed during 10 days in any undamaged bath challenged fish. Mortalities of

60% to 100% were observed in the scarified groups exposed to bath challenge. When

A. hydrophila suspensions were injected at 1.4×10^7 cells per fish, 100% mortalities

occurred in all groups by day 5. In this study clinically normal fish held in pathogen

rich waters did not succumb to infection, the authors emphasised therefore that apart

from strain virulence and host susceptibility, access to the internal milieu of the host

may be essential to establish infection.

On the basis of the cumulative results of these studies, the general conclusions can be

drawn that significant variations in strain to strain virulence potential exist within

aeromonad phenospecies and genospecies, and fish species vary in their susceptibility

to *Aeromonas* species. It is difficult however to make direct comparisons between the

various studies as protocols varied greatly. In studies on diseases of fish, temperature

is a particularly important variable. Bacterial growth is very much affected by

temperature, as are most aspects of the physiology of fish (Horne and Ellis,1988).

Many studies have shown that the immune response of fish (cell mediated and

humoral) is slower at lower temperatures (Ellis, 1988). Therefore incubation periods

and outcomes of bacterial challenges are intimately linked to the temperature of the environment.

One of the main limitations in evaluating the pathogenicity of *Aeromonas* isolates is the lack of consistent protocols for challenging host species with bacterial isolates. The use of fish models to establish virulence potential of *Aeromonas* species is appropriate for aquatic isolates, however pathogens may induce disease by different mechanisms in different hosts (Brenden and Huizinga, 1986). The very different physiology of the amphibian and the fish means that caution should be exercised when extrapolating data from fish challenges to frogs.

7.1.3 PATHOGENICITY MODELS USING FROGS

Unfortunately few studies have been reported which use frogs as pathogenicity models for aeromonads. Glorioso *et al.,* (1974a) set up a challenge model using *Rana catesbeiana* adults and larvae. A very small number of animals were used to test the pathogenicity of bacterial isolates belonging to a number of genera, including *Aeromonas, Pseudomonas, Proteus, Flavobacteria,* and Enterobacteriaceae, which had been taken from a range of septicaemic bullfrogs caught in the wild. One tadpole and one adult were given a bath challenge at 10^6 bacteria / ml. A second pair of frogs were force fed 10^{10} bacteria and a third pair were injected with 10^6 bacteria in 0.2ml isotonic saline solution. Death of any frog within two weeks was considered to be a presumptive test for pathogenicity of the individual bacterial isolates. Pathogenicity was then confirmed with up to 25 additional frogs exposed to the pathogen by the same routes used in the presumptive test. Death of 70% of these frogs within 2 weeks was considered to be a confirmed test for pathogenicity. These authors found that

force feeding bacteria to the frogs had no apparent effect on health. Infection induced

by injection of *Flavobacterium* sp. was not uniformly fatal. These frogs developed

open skin lesions, and a few recovered. With every other genus tested, infection

induced by injection of the pathogen was fatal within 10 to 18 days and mixed

cultures including the inoculated pathogen could be isolated from the heart blood of

the dead frogs. In each frog the inoculated pathogen was the predominant isolate and

the clinical signs of septicaemia were observed. Immersion of the test frogs in

individual suspensions of *A. hydrophila, P. aeruginosa, C. fruendii* and

Flavobacterium sp. resulted in the death of 50% of the frogs. However death occurred

within 20 hours and the challenge pathogens could not be re-isolated.

Somsiri *et al.* (1997) challenged *Rana tigerina* with *A. hydrophila* and *A. sobria* and

found that both induced the symptoms of red-leg in frogs. Injection and immersion

challenges were used in frogs of different ages. Age of tadpoles or frogs and water

temperature were considered to be the important factors determining severity of the

syndrome after immersion challenge. The authors found that tadpole sensitivity to *A.*

hydrophila and *A. sobria* was affected by reduced temperature, however neither high

levels of ammonia nor pH fluctuation enhanced susceptibility to disease. Adult frogs

had to be stressed by both low temperature and physical injury before infection would

occur after immersion challenge.

These reports reflect those of the fish pathogenicity models, in that injection

challenges establish disease more readily and produce higher mortalities than bath

challenges, which yield more variable results.

7.1.4 AIMS

In the 19th century Robert Koch formulated three postulates to determine the cause of infectious disease. These postulates state that an organism is causal if:

1. it is present in all cases of the disease.

2. it does not occur in another disease as a fortuitous and non-pathogenic parasite

3. it is isolated in pure culture from an animal, is repeatedly passaged and induces the same disease in other individuals.

This is a relatively rigid framework for demonstrating that an organism is a potential pathogen. The influence of environmental factors is ignored, although these are extremely important in the aquaculture situation. However the postulates are a standard by which disease association can be assessed (Thrushfield 1995).

The aim of this part of the study was therefore to reinoculate Au in *Rana* spp., establish whether the symptoms of frog septicaemic disease could be reproduced and whether Au could be re-isolated in pure cultures thereby fulfilling the third of Koch's postulates.

7.2 MATERIALS AND METHODS.

7.2.1 STOCK FROGS

Adult (35g-40g) and post-larval (2g-7g) *Rana rugulosa* were imported from the

Aquatic Animal Research Institute (AAHRI) in Bangkok and maintained in the frog

house at Stirling (Figure 7.2.1). Post-larval frogs were reared in nursery tanks of

propylene (0.5m x 0.7m x 0.35m) with heated pads underneath to maintain a constant

water temperature of 28°C. Initially Eheim™ pumps with biological filters were used

in an attempt to maintain water quality, but these proved unable to deal with the

amount of organic waste generated by the frogs. The pumps were therefore discarded

and approximately 70% water changes were carried out daily, using dechlorinated tap

water from header tanks preheated to 28°C. Water was maintained at a level sufficient

to allow the animals to swim freely, this therefore increased as the animals grew.

Large polystyrene floats with hides were placed in the tanks to provide feeding and

sitting areas. Adult animals were maintained in two large fibre glass tanks (1.2m x

1.0m x 0.7m) in water at an approximate depth of 15cm. Fifty percent of the water

was changed daily using the supply from the header tanks. The ambient temperature

was maintained as close to 28°C as possible. This did sometimes fluctuate (especially

in the Winter when on one occasion the temperature outside dropped to -20°C)

therefore 150-300 watt heaters were placed within the tanks to maintain the water

temperature at a constant 28°C. All animal were fed once daily *ad libitum* on pelleted

feed.

FIGURE 7.2.1

FROG HOUSE LAYOUT
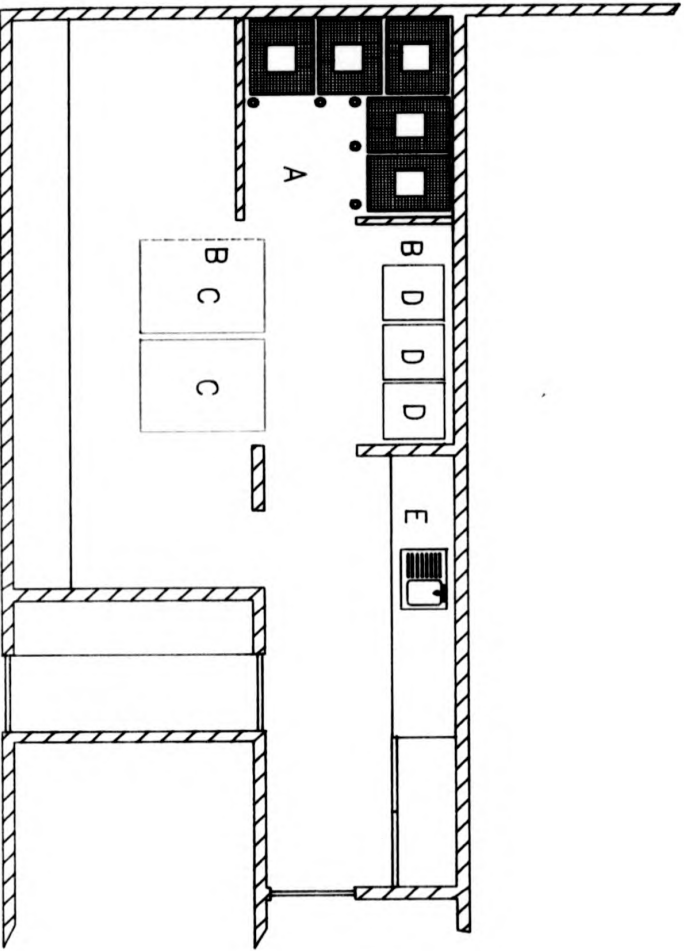
A   Nursery

B   Header tank

C   Growout tanks

D   Glucan exposure tanks

E   Bench space and sink

•   Eheim recirculating pump

7.2.2 EXPERIMENTAL FROGS

Experimental animals were transferred to 24l plastic tanks in a challenge room remote from the frog rearing facilities. Water supply was dechlorinated tap water in a closed static system for each tank. Daily water changes of 100% were performed, animals were fed daily *ad libitum* on pelleted feed. Effluent water from the tanks was treated with an iodophor disinfectant.

7.2.3 BACTERIAL ISOLATES

An isolate of Au, 2D8, was selected for the pathogenicity studies. This strain had been isolated in pure cultures from the liver, heart-blood, kidney, spleen and muscle of a large frog exhibiting signs of septicaemia on farm 2 in the North of Thailand. Isolates 2N14 and 2N36 were taken from the skin of normal frogs on the same farm. 2N14 was initially identified as *A. hydrophila* however later tests indicated that the isolate was in fact *A. caviae*. 2N36 was identified and confirmed as *A. sobria*. Culture conditions were as described in section 4.2.2.

7.2.4 PREPARATION OF CHALLENGE INOCULA

Standard curves for estimation of the number of viable bacteria in a suspension were prepared as described in Appendix 5. Overnight cultures of the bacterial isolate in TSB were centrifuged at 2200g for 10 minutes. The pelleted cells were resuspended in sterile physiological saline and the optical density of the suspension adjusted to yield the desired bacterial concentration which was then verified by viable counts.

7.2.5 INJECTION CHALLENGES

To ensure that the internal isolate of Au had not loss any virulence on laboratory subculture, the strain was passaged through two frogs. The first frog (74g) received 0.1ml of a suspension containing 1.3×10^9 cells by intraperitoneal (i.p.) injection. Au was re-isolated and injected into a second frog (68g) i.p. in 0.1ml of a suspension containing 3.6×10^2 cells. This was followed by a small range finding challenge of four frogs (average weight 75.3g) injected i.p. with 0.1ml of suspensions containing respectively; 1.5×10^2, 1.5×10^3, 1.5×10^4, 1.5×10^5.

Two further large scale i.p. challenges were undertaken at AAHRI in conjunction with M. Crumlish as part of an efficacy trial on immunostimulants. In the first trial five replicates of five frogs (average weight 40g) were injected i.p. with 0.1ml of an Au suspension containing 1×10^5 cells. Five replicates of five frogs were injected with saline to act as control animals. In the second challenge four groups of five frogs were injected i.p. with 0.1ml of an Au suspension containing 1×10^2 cells. Two groups of five frogs were injected i.p. with saline to act as controls. Frogs were maintained in glass tanks with 4l of water in the AAHRI wet lab, daily (100%) water changes were undertaken and the animals were fed a standard commercial pellet.

7.2.6 AU BATH CHALLENGES

A bacterial suspension in sterile physiological saline was added to three litres of water in two tanks in quantities sufficient to give 1.6×10^8 and 1.6×10^6 cells ml^{-1} respectively. A third tank containing 3 litres of water only was set up as a control tank. Three groups of five frogs (average weight 66.5g) were transferred to the tanks. The frogs were exposed to the bath challenge overnight (4pm - 10am). 100% water

changes were then carried out and repeated daily in all the tanks. Room temperature was maintained at a constant 25°C and water temperature for each tank was constant at 23°C. The animals were therefore at a slightly suboptimal temperature, this was not however a deliberate attempt to stress the animals it was merely the highest room temperature which could be achieved in that aquarium at the time. Mortalities were recorded daily and samples were taken from ulcers, livers, heart-blood and spleens.

7.2.7 COMPARATIVE BATH CHALLENGES

The objective of this part of the study was to determine the comparative virulence of three aeromonad isolates; Au (2D8), *A. sobria* (2N36), and 2N14 which at the time of the trial was identified as *A. hydrophila* but at a later date, after more extensive phenotyping, was re-identified as *A. caviae*. Bacterial suspensions in sterile physiological saline were added to 3 tanks each containing 3l of water in quantities sufficient to give Au 1.2×10^7 cells/ml, *A. caviae* 6.5×10^7 and *A. sobria* $>1 \times 10^{10}$. A fourth tank containing 3 litres of water only was set up as a control tank. Four frogs (average weight 76.6g) were placed in each tank and exposed to the bath challenge overnight. Frogs were maintained as described above, except that room temperature for the first 16 days was maintained at 28±1°C with water temperature of 25±1°C, water temperature was then dropped to 19°C±0.5°C for one week. At the end of this period the frogs were further stressed by an injection of corticosteroids at a dose rate of 30mg/kg body weight. The animals were maintained for a further eight days at the reduced temperature after which the experiment was terminated and the animals destroyed and sampled.

7.3 RESULTS

7.3.1 STOCK ANIMALS

Eighty seven *Rana rugulosa* were initially transported from Thailand as hand luggage. They were the first laboratory population of this species to be established in this country and some initial health problems were encountered. Several mortalities occurred in the first weeks after transfer which were attributed to the stress of the journey. However low grade mortalities continued throughout the month following transfer, in particular rectal prolapses were a frequent occurrence. It was felt that the initial rearing system allowed too much temperature variation, therefore the nursery system described in section 7.2.1 was constructed. The animals improved immediately on transfer to this system and the majority were reared successfully.

Unfortunately many of the growing frogs developed erosions on prominent areas such as the eyes and tip of the snout. It was thought that these were initially caused by trauma and then either an infection or a subclinical physiological problem delayed healing. Topical applications of human ulcer treatments such as Bongel™, salt baths and oxytetracycline baths had little effect. However when the frogs where switched from the commercial frog feed to a floating carp pellet the erosions resolved suggesting an underlying nutritional element.

Acquisition of new stock developed into a serious problem as the project progressed. Airlines became increasingly less co-operative about the transport of live animals as hand luggage and subsequent animals had to be imported as unaccompanied cargo. This was not ideal as the animals became chilled on the journey, although the cargo hold was supposed to be heated. The first cargo shipment was successful but a second

group suffered from a severe bacterial septicaemia resulting in the death of about half the population. The onset was very acute and Au was isolated in pure culture from the moribund and dead animals. (An isolate from this outbreak was later used to replace the lost strain 1D54 in the virulence studies.) A bath treatment of enrofloxacin (50mg/l for one hour) was administered which stopped the mortalities immediately. The remaining animals regained their health and were reared successfully.

The supply of frogs was seasonal and the following year when animals were again imported a further outbreak of septicaemic disease occurred, the cause of which was not identified, and which proved intractable to treatment by enrofloxacin. This entire population had to be culled out.

7.3.2 AU INJECTION CHALLENGES

On the first bacterial passage a large adult frog was injected intraperitoneally with 1.3×10^8 cells in 0.1ml physiological saline. The frog died within 24 hours and heavy pure cultures of the pathogen were isolated from the liver, heart, and spleen. It was felt that the animal had probably died from toxic shock before the pathogen had had a chance to multiply and regain virulence characteristics lost during the subculturing and storage process. Therefore a second passage was prepared using a much lower dose of the bacteria.

The second frog was injected intraperitoneally with 0.1ml of a suspension containing 3×10^2 cells. The frog died on the fourth day after the injection and exhibited gross clinical signs of septicaemia i.e. flushed thighs, an engorged, friable liver and ascites. The pathogen was isolated in heavy pure cultures from the heart, liver and spleen.

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It was apparent from the bacterial passage that a very few cells could kill healthy frogs when injected intraperitoneally. A small range finding test was therefore carried out to give an indication of the minimum number of cells necessary for a successful i.p. challenge. Four frogs (average weight 75.3g) were injected with 0.1ml of suspensions ranging from $1.52 \times 10^{5\text{-}2}$ cells ml^{-1}.

Table 7.3.2.1 Mortality of *R. rugulosa* following i.p. challenge with Au at a range of concentrations.

Bacterial dose (cfu/ml)	1.52×10^5	1.52×10^4	1.52×10^3	1.52×10^2
Time to death	24 hours	36 hours	2 days	5 days

All frogs showed signs of bacterial septicaemia and heavy pure cultures of the pathogen were isolated from the heart, liver and spleen.

The pathogen appeared to be virulent when injected intraperitoneally even in very low numbers. It is difficult to consistently inject very low numbers of bacteria, particularly isolates like Au which autoagglutinate. Therefore in view of these results, and given that numbers of experimental animals were severely limited, the remaining experiments undertaken in Stirling were bath challenges. These were thought to produce a more natural model of the disease process and were therefore a better use of limited resources. However two more injection challenges were undertaken at AAHRI in conjunction with M. Crumlish, as part of her studies on the innate immune system of frogs. The results from these confirmed those of the range finding trial at Stirling.

Table 7.3.2.2 Mortality of *R. rugulosa* following i.p. challenge with Au at 1x 10^5 cells

Tank no.	Treatment	24hrs	2days	3days	4days	5days	% affected	% mortality
1	Au	5	-	-	-	-	100	100
2	Au	5	-	-	-	-	100	100
3	Au	4	1	-	-	-	100	100
4	Au	5	-	-	-	-	100	100
5	Au	5	-	-	-	-	100	100
6	saline	0	0	0	0	0	0	0
7	saline	0	0	0	0	0	0	0
8	saline	0	0	0	0	0	0	0
9	saline	0	0	0	0	0	0	0
10	saline	0	0	0	0	0	0	0

Au was isolated in heavy pure cultures from the livers of the challenge mortalities.

The saline injected controls were sacrificed on day 5 and sampled, there was no

significant growth from their internal organs.

Table 7.3.2.3 Mortality of *R. rugulosa* following i.p. challenge with Au at 1x 10^2 cells

Tank no.	Treatment	24hrs	2days	3days	4days	5days	6days	7days	% affected	% mortality
1	Au	1	2	1	1	-	-	-	100	100
2	Au	0	4	1	-	-	-	-	100	100
3	Au	0	4	0	1	-	-	-	100	100
4	Au	0	4	0	0	0	1*	-	100	80
6	saline	0	0	0	0	0	0	0	0	0
7	saline	0	0	0	4	0	0	0	0	80

*This animal was moribund and sacrificed for bacteriological sampling

Au was isolated in pure heavy growth from the livers of all the challenge tank

mortalities. No significant growth was obtained from the control animal mortalities,

these mortalities were not explained. The surviving control animals were sacrificed

and sampled on day 7. No significant growth was isolated from any of these animals.

7.3.3 AU BATH CHALLENGES

Mortalities began within 24 hours during the overnight challenge in both high and low dose tanks. Results are shown in table 7.3.3. During the overnight challenge one frog from the 1.6×10^8 bath escaped. He was identified by his pigmentation and replaced in the tank the following morning. Both the high and low dose challenge tanks showed signs that frogs had regurgitated food during the night, there was a lot of organic debris on the feed pallet and in the water, which smelt very bad.

Table 7.3.3 Frog mortalities during Au bath challenge

Bacterial dose cfu ml^{-1}	18 hrs	2 days	3 days	4 days	5 days	6 days	7 days	8 days	% affected	% mortality
1.6×10^8	1	2	1	1*	-	-	-	-	100	100
1.6×10^6	1	1	-	-	-	1	-	1	100	80
control	-	-	-	-	-	-	-	-	0	0

*escapee didn't get full challenge

All dead frogs showed clinical signs of bacterial septicaemia and the challenge bacterium was re-isolated in pure cultures from the liver, heart-blood and spleen of all mortalities. The surviving frog from the 10^6 tank developed severe ulceration around the mouth. This was very different to the erosions noticed in stock animals during the growout period. The erosions were always associated with prominent areas such as the eyes and the tip of the snout, they progressed very slowly and did not appear to involve more than the superficial epithelial layers. The ulcers were located around the mouth, had a very bloody, active appearance, and involved underlying tissues. On day 14 this frog was anorexic and lethargic with severe ulceration, it was therefore destroyed. No significant growth was isolated from the internal organs, however Au was isolated in heavy dominant culture from the ulcer.

Control frogs initially appeared healthy and were eating well. However one control

frog developed ulcers around the mouth very similar in appearance to those observed

in the surviving 10^6 frog. These ulcers progressed in a similar manner and by day 18

post challenge the frog was lethargic and anorexic with severe ulceration (Figure

7.3.3.1). The animal was destroyed. No significant growth was isolated from the

heart-blood, liver or spleen however Au was isolated in heavy dominant growth from

the ulcer. The remaining control frogs were maintained in the challenge aquaria and

used to provide blood samples in the following months. They were sacrificed three

months after the start of the trial and bacterial samples were taken from the liver, heart

blood, kidney and spleen. There was no significant growth on any of the plates.

7.3.4 COMPARATIVE BATH CHALLENGES

Table 7.3.4 Frog mortalities during comparative pathogenicity trial

Isolate	Species	24 hrs	2days	3days	4days	5days	6days	7days	17days	% affected	% mortality
2D8	Au	1	0	1	0	0	U+	U++	U+++	75	50
2N14	A. c	0	0	0	0	0	0	0	0	0	0
2N36	A. vbvs	0	0	0	0	0	0	0	0	0	0
Control	control	0	0	0	0	0	0	0	0	0	0

U+, ++, +++ = one frog with ulcers mild, moderate, severe

Two frogs in the Au challenge tank developed signs of septicaemia and died rapidly,

one further frog developed ulcers around the mouth very similar in appearance to

those observed in the previous experiment. These ulcers progressed in a similar

manner and the animal also developed ulcers on its front and hind feet. By day 17 post

challenge, the jaw (Figure 7.3.3.2) and all the feet were exhibiting severe ulceration.

The animal was destroyed. No significant growth was isolated from the heart-blood,

liver or spleen, however Au was isolated in heavy dominant growth from all the

Figure 7.3.3.1 Ulceration associated with chronic Au infection

Figure 7.3.3.2 Ulceration associated with chronic Au infection

ulcerated areas. The remaining frogs (1 in the Au tank and 4 each in the other challenge tanks and controls) showed no adverse effects, apart from inappetance, despite temperature stress and corticosteriod injection. These animals were destroyed four weeks after the start of the trial and sampled for bacteriology. There was no significant growth from any of their internal organs.

7.4 DISCUSSION

The cumulative results from the injection challenges demonstrated that Au was highly pathogenic for farmed *R. rugulosa*. At doses of $\geq 1 \times 10^5$ cfu 100% mortality was achieved within 48 hours. High mortality rates (>50%) were achieved even at very low doses; 1×10^2 cfu yielded 70% mortality within 48 hours and 95% mortality within 4 days.

Other investigators have demonstrated that very low numbers of *Aeromonas* spp. can kill aquatic animals. Daly *et al.,* (1996) in a study on *in vitro* survival and virulence of *A. salmonicida* strains found the LD_{50} in 4 days of one strain to be less than 10 cfu by intraperitoneal injection; virulence was also observed with this strain following bath and co-habitation challenges. Another five isolates examined in the same study, gave LD_{50}s higher than 1×10^7 cfu by intraperitoneal injection and were avirulent by bath challenges, even if the challenges were carried out at 10°C, a stressful temperature for brook trout. The authors state that *A. salmonicida* with an $LD_{50}/4$day of <10 cells, is the most virulent bacterial pathogen of fish (Daly *et al.,* 1995). The injection challenges carried out at AAHRI indicate that the $LD_{50}/4$days for Au would be less than 10^2 cfu as 70% mortalities had occurred within 48 hours at this dose rate, indicating, by the above criterion, that it is a virulent pathogen for frogs.

However, as previously discussed, although injection challenges may give an indication of the virulence of bacterial isolates, they are not an accurate reflection of the natural processes of infection. The Au isolates also presented a further problem for low dose injection challenges in that they strongly autoagglutinated. The injection

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studies indicated that if comparative virulence studies and LD_{50}s were to be attempted by this method then very low doses of Au would be needed.

The decision was therefore made to undertake further challenges using a bath model, with the added advantage of establishing a more natural model of the disease process. The initial bath challenges indicated that Au was virulent when the animals were subjected to slightly suboptimal temperature without the necessity of damaging the skin. A bath challenge of 1×10^8 cfu/ml produced 80% mortalities within 3 days, and as the remaining animal had not encountered the full challenge, the results could have been even higher. The 1×10^6 cfu/ml challenge produced 60% mortalites within 6 days and 80% within 8 days. The health of the remaining animal was compromised due to severe ulceration and it had to be sacrificed.

It is interesting that one of the control animals also developed a severe, progressive, ulcerative condition from which Au was isolated. At present the reservoir of Au is uncertain. Isolates taken from skin and intestine of frogs in Thailand revealed numerous *A. sobria* isolates and *A. hydrophila* isolates but no Au. However the outbreak of septicaemia in the recently imported stock animals, implies at least some carrier animals exist. It is possible that the control frog was also carrying Au and that the stress of the challenge room precipitated the disease. Alternatively the control animal could have received a contaminating dose from one of the challenge tanks. The animals were maintained within plastic tanks on the same bench in the challenge room and although different cleaning implements were used for each tank, it is possible that water from a challenge tank had contaminated the controls, possibly from splashes caused by the frogs jumping around the tanks. However if contamination had occurred

it must have been at a much lower dose than the 10^8 and 10^6 cfu/ml present in the challenge tanks, which would suggest a very virulent pathogen.

The results from the initial bath challenges were encouraging however the numbers of frogs available were too few to allow $LD_{50}s$ to be calculated. A further experiment was therefore undertaken to compare the virulence of Au with that of other motile aeromonads from the same farm. Similar results were again observed, the challenge with Au produced 50% mortalities within 3 days.

One further animal was sacrificed at 17 days post challenge due to severe ulceration. The remaining animal did not succumb to either the acute septicaemic or ulcerative form of the Au infection. The animals challenged with *A. caviae* and *A. sobria* did not show any adverse effects despite being subjected to rigorous temperature stress (19°C) and corticosteroid injections.

The chronic ulcerative condition demonstrated by three frogs infected with Au is an interesting development and reflects the results of Esteve *et al.*, (1993). The workers found that strains of *A. hydrophila* and *A. jandaei* caused acute septicaemic disease in European eels when injected intraperitoneally, but produced an ulcerative disease with lower mortality rates which progressed more slowly when bath challenges of 10^7 to 10^8 cfu/ml were used. These authors concluded that the ability to reproduce disease in eels under laboratory conditions using 2 challenge methods: i.p. injection and bath exposure supported the role of *A. hydrophila* and *A. jandaei* as primary pathogens of European eels. In the Au bath challenges more frogs were affected by acute septicaemic disease than by the chronic ulcerative condition.

The genetic analyses described in Chapter 5 indicate that Au is more closely related to *A. hydrophila* HG1 than to any other *Aeromonas* genospecies. Somsiri *et al.* (1997) found that adult frogs challenged with *A. hydrophila* needed both stress by low temperature and physical injury before infection could occur. Previous studies on *A. hydrophila* bath challenges of fish have indicated that it is often necessary to damage the skin of fish before bacterial septicaemias can be established (Ramaiah and Manohar, 1988; Lio-Po and Duremdez-Fernandez, 1986). Access to the host is therefore important in the pathogenesis of *A. hydrophila* septicaemias. Although Au is closely related to *A. hydrophila*, it is unusual in that septicaemic infections could be established in clinically healthy animals by bath challenge without prior skin damage.

It is possible that the Stirling animals were more stressed than the frogs challenged with *A. hydrophila* in Thailand and therefore were more susceptible to bacterial invasion without skin damage. The interaction of stress and the immune system has been extensively explored in mammals and the increasing importance of aquaculture has generated many recent studies on fish; however the effects of stress on ranids is a subject which has barely been touched.

Stressors have profound and diverse effects on the defence mechanisms in fish. The inhibitory effects on disease resistance (immune suppression) have been demonstrated in many studies and are well summarised by Wendelaar Bonga (1997). More specifically some studies have investigated the effects of stress on the response of fish to bacterial challenge. Peters *et al.*, (1988) exposed juvenile rainbow trout simultaneously to a stressor and to infection by *A. hydrophila* through injection and bath challenges. Tissue and blood samples from stressed trout that had been

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experimentally infected were more frequently contaminated with bacteria than those of unstressed fish. The pathogens in both challenge methods were able to overcome the complex defence mechanisms of the stressed fish more easily than those of the unstressed ones.

Wise *et al.*, (1993) examined the effects of stress on the susceptibility of channel catfish fingerlings to *Edwardsiella ictaluri* during immersion challenges. Stress and increased time of static bacterial exposure significantly decreased the survival of channel catfish after immersion challenge. This study established a relationship between a behavioural stressor (confinement) and increased susceptibility of channel catfish to *E. ictaluri* infection.

Somsiri *et al.*, (1997) found that low temperature was one of the most significant stressors in frogs. The Stirling frogs were maintained at a water temperature of 23°C during the initial challenge period and 25°C for the first few weeks of the comparative challenge; the optimum water temperature for these animals is 28-30°C. In addition the Stirling stock suffered fluctuating temperatures in the frog house during the growout period. It is probable that the problems associated with establishing a new laboratory population of animals resulted in a subliminally stressed group of animals. However Somsiri *et al.*, (1997) found that low temperatures (20°C) had to be associated with skin damage before bacterial infections could be established in adult frogs. Therefore temperature stress alone cannot explain the fact that Au rapidly caused an acute systemic infection in undamaged frogs.

The challenge studies indicate that Au is a more virulent pathogen for frogs than the *A. hydrophila* isolate used by Somsiri *et al.,* (1997) in Thailand. These results support the findings presented in Chapter 6 on virulence factors, which showed that ECPs from Au were more haemolytic and cytotoxic to frog cells than those from *A. hydrophila.*

Although *A. hydrophila* challenges often require prior skin damage in fish before septicaemias can occur *A. salmonicida* challenges do not. Au has been shown to have several parallels with the *A. salmonicida* group. The collection of Au isolates have been demonstrated to be phenotypically and genotypically homogeneous, and like *A. salmonicida* are associated with a septicaemic disease syndrome. In addition the Au isolates all carry a gene which was originally cloned from a virulent isolate of *A. salmonicida* (ASH1) and may confer cytotoxic activity against frog and fish cells. It is probable that Au, like *A. salmonicida,* represents a homogeneous group of specific pathogens within the genus *Aeromonas.*

The aetiology of Au septicaemia is complex and a number of factors both bacterial and host associated can influence the ultimate development of the disease, however the present studies have demonstrated the virulence of Au for *R. rugulosa.* Koch's postulates were fulfilled to a limited extent, in that the bacterium was administered to clinically normal animals by injection and bath challenge. The animals then developed acute septicaemic disease and Au was reisolated from the diseased animals. More work needs to be done to produce a reliable bath challenge with which to establish LD_{50}s for Au against frogs. Once a reproducible challenge model is established

investigations into prophylactic and chemotherapeutic treatments for frog septicaemic

disease may be undertaken.

Chapter 8. Discussion

8.1 GENERAL DISCUSSION

Frog farms, although small scale, made a significant contribution to rural economic

development in Thailand during the 1990s. However the rapid intensification of frog

culture, coupled with a lack of information on the animal and its pathogens, resulted

in epidemics of bacterial disease which decimated production in certain districts. If

frog culture is to be a sustainable industry, effective techniques for disease control

must be developed. One of the first steps in this process is to establish the taxonomy

and biology of pathogens associated with frog disease.

A primary objective of this study was therefore to undertake field research in Thailand

to isolate and identify bacteria consistently associated with frog septicaemic disease.

The survey described in Chapter 2 revealed that, although bacterial cultures from

septicaemic frogs yielded a number of different genera, only the motile aeromonads

were isolated in pure cultures from more than one septicaemic animal on more than

one farm.

Aeromonads are ubiquitous in the aquatic environment and have long been recognised

as a cause of disease in fish and frogs (Carnahan and Altwegg, 1996). Indeed the

earliest acknowledged reference to a motile aeromonad was Sanarelli's report in 1891

of *Bacillus hydrophilus fuscus* isolated from the blood and lymph of a septicaemic

frog. The association of aeromonads with farmed frog septicaemia was not therefore

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surprising. However to consistently isolate a phenotypically unique motile aeromonad

from the internal organs of frogs in diverse geographic areas was unexpected.

Information on the characterisation of aeromonads isolated from animals is limited,

with the exception of the non-motile *A. salmonicida* group (Gosling, 1996).

Aeromonas salmonicida, is the causative agent of furunculosis, a fatal epizootic

disease of salmonids, and one of the most important fish pathogens (Roberts, 1993).

Identification of *A. salmonicida* isolates has been relatively easy in comparison to the

confusion that has existed over the taxonomy of the motile isolates. The non-motile

aeromonads can be identified by phenotypic analysis and have not therefore been

difficult to characterise and associate with disease outbreaks. More recently genetic

studies have demonstrated that the phenotypic homogeneity of *A. salmonicida* is

reflected in a genomic homogeneity for isolates from diverse geographic origins

(Miyata *et al.*, 1995, Inglis *et al.*, 1996).

Although motile aeromonads are also well recognised as a cause of fish disease, they

represent a far more diverse group. The absence of adequate rapid phenotyping

methods for characterising clinical species, led to the use of common nomenclature

such as *A. hydrophila, A. hydrophila*-group or *A. hydrophila* complex. When DNA

studies revealed the existence of a large number of distinct species, many

investigations were undertaken to develop schema which phenotypically differentiated

recognised genospecies (Altwegg *et al.*, 1990, Abbott *et al.*, 1992, Kampfer and

Altwegg 1992, Wilcox *et al.*, 1992, Carnahan and Joseph, 1993, Magee *et al.*, 1993,

Millership and Want 1993, Mulla and Millership 1993). These studies into the

systematics of *Aeromonas* made it possible to identify isolates in a more accurate

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fashion; however the successful characterisation of the genus has mainly involved human isolates. For example, of the 138 isolates studied by Altwegg *et al.*, (1990) 112 were human clinical specimens, 13 were environmental, 9 were type strains and the origin of four strains was not known. Similarly of the 133 strains described by Abbott *et al.*, (1992) 102 were human, 15 were environmental and 16 were isolated from unnamed "animals". Motile aeromonads are pathogenic to a large number of animal species, yet few systematic studies to characterise isolates in naturally occurring infections have been conducted. The characterisation in this study of 42 motile aeromonads from clinically normal and diseased frogs therefore extends the taxonomic database of the genus. Many workers anticipate that animal isolates will provide new *Aeromonas* taxa (Gosling, 1996, Austin *et al.*, 1996). The isolation, phenotyping and genotyping of the Au isolates from frogs lends credence to this prediction.

The Au isolates are unusual among the motile aeromonads in that they can be phenotypically differentiated from other isolates at an early stage in the characterisation process. The biochemical reactions of the group are distinctive in that they utilise a very small number of sugars and notably do not metabolise sucrose. The inability to utilise sucrose is being increasingly reported among potentially pathogenic Gram negative bacteria. Neither *A. jandaei* nor *A. schubertii* make use of sucrose. These bacteria appear to be of clinical significance in human medicine; *A. jandaei* has been isolated from human blood, wound infections and diarroheal stools whereas *A. schubertii* strains have been isolated from human abcesses, wounds, pleural fluid and blood (Hickman-Brenner *et al.*, 1988; Carnahan *et al.*, 1991b). A similar trend has been observed in bacteria affecting marine crustacea, the most pathogenic species of

which are non-sucrose utilisers. An emerging bacteria of considerable interest to crayfish farming is *Vibrio mimicus*. In a five year period *V. mimicus* has been isolated from 14 out of 15 disease outbreaks in crayfish, whilst *Vibrio cholerae* was isolated from 3 of the 15. The two bacteria differ only in their ability to utilise sucrose (*V. mimicus* being a non-utiliser) (Owens and Edgerton, 1997).

The *Aeromonas* frog isolates continue this theme; in that commensal, sucrose utilising motile aeromonads were present in the gut and on the skin of diseased and clinically normal adult animals but were not isolated from the internal organs of any septicaemic adults. Whereas the non-sucrose utilising Au was isolated from internal organs of septicaemic animals, but was only found in one skin sample from a severely septicaemic animal and was not isolated from any gut samples or from any samples of clinically normal frogs. Au therefore does not appear to be part of the commensal bacterial flora of the frog. Pathogenicity studies presented in Chapter 7 confirmed that the internal, non sucrose utilising Au isolates were frog pathogens.

The Au isolates resemble *A. salmonicida*, in that they are readily distinguished phenotypically, show a homology of the genome and are associated with a haemorrhagic, septicaemic disease syndrome. It is important for frog farmers and health advisers to be aware that a potentially virulent pathogen can be differentiated from other motile aeromonads omnipresent on the farm, at a very early stage using a standard commercial micromethod.

The findings in this study are however of interest to broader medical and scientific communities. The role of *Aeromonas* isolates in the pathogenesis of human disease

has generated much interest in the past two decades. This interest has led to the association of these bacteria with a number of distinct infectious processes (Joseph, 1996). An increasing body of scientific information has been generated implicating *Aeromonas* species as gastrointestinal pathogens of humans (Janda and Duffey, 1988). In addition reports of *Aeromonas* sepsis, wound and ocular infections, peritonitis and meningitis, as well as a number of miscellaneous infections have appeared in the literature. Although rare, these infections, are often fatal or have serious debilitating outcomes, such as amputation (Janda and Abbott, 1996).

Despite the increase in information doubts remain about the role aeromonads play in human infections. There are numerous sources of *Aeromonas* spp. which suggests that there should be more illness in humans than is observed. No animal models have been successful in mimicking the gastro-enteritis produced in humans by *Aeromonas* isolates. Some studies have failed to find a significant difference in the isolation rate of *Aeromonas* species between symptomatic and asymptomatic humans, others have been unable to firmly establish the clinical involvement of faecal isolates in the gastrointestinal process (Janda and Duffey 1988). Morgan *et al.*, (1985) failed to induce diarrhoea in human volunteers despite using *A. hydrophila* isolates possessing accepted virulence factors. The authors concluded that additional virulence properties of *A. hydrophila* strains need to be sought and characterised.

It has been suggested that there are particular biotypes of *Aeromonas* which cause illness (Janda and Duffey 1988, Joseph, 1996). The findings of the present study support the hypothesis that particular biotypes of *Aeromonas* are associated with

clinical syndromes. Many different aeromonads were isolated from the frog intestine and skin, however only the Au biotype was found internally in septicaemic animals.

Evidence was presented in Chapters 4, 5 and 6 that the Au isolates were phenotypically and genotypically distinct from other motile aeromonads but closely related to *A. hydrophila*. It is possible that Au diverged from *A. hydrophila* when amphibians made the transition from water to land, and has since diverged enough to be classified as a separate species, adapted to infect the terrestrial stage of the frog life cycle. If Au reflects the transition between aquatic animal pathogens and mammalian pathogens, the characterisation and study of virulence factors from such strains could provide information on pathogenic mechanisms relevant to both poikilotherms and homeotherms.

The Au isolates cause a toxaemic septicaemia, rather than the gastro-enteritis most frequently associated with *Aeromonas* infections in humans. It is interesting however that Biolog's Microlog™ 1 database identified all the Au isolates as *A. schubertii*. This indicates that Au is following many of the metabolic pathways of these human clinical isolates. *Aeromonas schubertii* exhibits an unusual disease spectrum in that it has only been recovered from extraintestinal sites in humans such as blood, wounds and cellulitis. Janda (1991) suggests that the species may have a propensity for causing serious extraintestinal disease in humans since no faecal isolates have so far been identified. The distribution of Au in frogs reflects the extraintestinal distribution of *A. schubertii* in humans. Studies on the action of Au virulence factors could therefore help to elucidate the pathogenesis of *Aeromonas* septicaemias in humans.

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The virulence mechanisms of aeromonads are, as yet, only partially understood,
however the role of haemolysins, proteases and other extracellular proteins in
pathogenesis is well recognised (Gosling 1996). Several workers have demonstrated
that *Aeromonas* ECPs are capable of producing pathological changes in fish tissues
similar to those seen in natural infections, thus confirming the importance of the ECPs
enzymatic activities in the pathogenesis of disease (Allan and Stevenson, 1981, Santos
et al., 1988, Esteve *et al.*, 1995b). Many of the histopathological changes associated
with Au septicaemias, and described in Chapter 3, were reproduced by Miranda
(1997) in the organs of *Rana pipiens* after injection with Au ECPs. Miranda injected
frogs intramuscularly with ECPs from Au and *A. hydrophila*. He showed that Au
ECPs caused severe pathological change with necrosis in most of the internal organs,
particularly in the heart, spleen, liver, kidney and thigh muscle. The changes produced
by injection with *A. hydrophila* ECPs were much milder. The *A. hydrophila* isolate,
which originated from frog skin, had no elastase activity and was much less
haemolytic to frog RBC than the Au isolate, indicating that these extracellular
enzymes are extremely important in the pathogenesis of Au septicaemia in frogs.
The studies on the virulence factors presented in Chapter 6 further illustrate the
difference in enzymic capabilites between Au and the other frog isolates. The enzymes
assayed by the API-ZYM system are not implicated in the pathogenesis of disease.
They are, however, a phenotypic expression of the genetic differences described in
Chapter 5 and demonstrate that the Au isolates form a distinct group.

Haemolytic and cytotoxic activities are related to disease producing potential of the
isolates. The ECPs produced by the Au isolates were less active against mammalian
cells than aquatic animal cells. Conversely the majority (but not all) of the *A.*

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hydrophila isolates were more active against mammalian cells. The suggestion

therefore that particular *Aeromonas* biotypes affect particular species is well supported

by this data. This knowledge is valuable not only to aquaculturists but also to the

medical community as it supports the hypothesis that although many biotypes are

present in the environment only some are active against humans.

The varied enzymic capabilites demonstrated in the present study among isolates of

different origins have a further and more general significance. The cytotoxins from the

internal Au isolates were much less active against cells of mammalian origin than

against cells from aquatic animals. There is sporadic evidence that similar strains of

human and environmental origin may not always react alike in media or cause the

same reactions at particular incubation temperatures (Joseph and Carnahan, 1994).

This has wide ranging implications for scientists characterising aquatic and

environmental bacteria and even raises questions about nutrient media preparation.

The use of media substrates which are inappropriate to the origin of bacteria under

investigation may produce misleading results.

In recent years workers have recognised that that the expression of most virulence

factors is regulated by environmental conditions. (Williams 1988; Garduno *et al.*,

1993; Mateos and Paniagua, 1995). Pathogenic aeromonads have to survive within

three very different domains, only one of which relates to disease in aquatic animals:

a) laboratory culture conditions; b) environmental conditions where nutrients may be

very restricted and c) within the hostile surroundings encountered *in vivo* in host

tissues. In laboratory culture, bacteria multiply rapidly in nutritionally sufficient

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media. Whilst to establish *in vivo* infections, pathogenic bacteria must overcome host defense mechanisms and compete for nutrients with host tissues.

Williams (1988) states that bacteria have a remarkable ability to alter their metabolism in response to changing environmental conditions. The prime structure through which this adaptation occurs is the bacterial cell envelope. The envelope is crucial to the establishment of infection, it is involved in promoting adhesion to and colonisation of host tissues, in uptake of essential nutrients and in conferring resistance to host defences. Bacteria growing *in vivo* will manufacture envelopes characteristic of that environment, which will differ markedly in physiology, biochemistry and immunogenicity from those cells grown in standard laboratory media.

The phenotype expressed by a bacterium is therefore a direct product of its environment. In addition, the expression of essential virulence factors is known to respond to various environmental signals. The increase of bacterial virulence following animal passage, as well as attenuation of strains after prolonged cultivation *in vitro,* occurs frequently among microorganisms.

Ishiguro *et al.* (1981) demonstrated that growing *A. salmonicida* at a higher than optimal temperature (26-27°C) resulted in the selection of spontaneous attenuated derivatives in the initial bacterial population. Subasinghe *et al.,* (1993) studied the effect of serial passage on the extracellular products and virulence of *A. hydrophila*. Serial passage through live fish was found to increase the production of toxins in a virulent strain. Passage through agar media resulted in a decreased toxin production for both virulent and avirulent strains. Mateos and Paniagua (1995) observed that,

after long term *in vitro* cultivation, a reduction in virulence for trout of *A. hydrophila* strains frequently occurred. The authors conversely found that repeated intramuscular injection enhanced the virulence of the isolate for rainbow trout. A progressive increase of resistance to the bactericidal activity of trout serum after each animal passage was also observed.

Researchers are therefore aware that *in vitro* growth is often not conducive to the expression of essential virulence determinants found during the normal course of infection. However few workers have considered the effect that substrate origin in growth media may have on bacterial phenotype and expression of virulence factors. Aquatic isolates are still routinely cultured on media containing substrates of mammalian origin e.g. sheep blood agar, brain heart infusion agar. Indeed the manufacturers of the Biolog system recommend that all gram negative isolates (with a very few exceptions) be cultured on sheep blood agar in order to standardise results. While this may be appropriate for mammalian isolates, it could alter the phenotypic expression of aquatic and environmental isolates and produce misleading results. Our studies clearly indicate that bacteria within the genus *Aeromonas* secrete a number of toxins which have differing activity against cells of differing origin. Repeated culture of these bacteria on media containing mammalian substrates could alter the expression of these virulence factors.

The findings in the present study emphasise the need for substrates in enzyme investigations and in growth media to reflect the origin of the isolates being cultured and examined. To date aquatic isolates have been treated very much as mammalian

isolates. Test methods such as API and Biolog are adapted from mammalian medicine. The data bases which define phenotypic criteria are based on mammalian and particularly human isolates. Aquatic and aquatic animal isolates should not be treated as mammalian isolates but should be acknowledged as a distinct group with their own attributes and requirements.

The striking contrast in haemolytic and cytotoxic activity of Au, *A. hydrophila* and other aeromonads is probably explained by possession of the ASH1 gene. Hirono and Aoki (1992) cloned this gene from *A. salmonicida* ATCC 14174, but had been unable to demonstrate its presence in any of the 104 other *A. salmonicida* isolates tested. A search of the DNA data banks failed to reveal any homologous sequences with the ASH1 gene (Hirono and Aoki, 1993). The authors speculated that the ASH1 haemolysin did not originate from *A. salmonicida* but might have been acquired from another genus or family of bacteria.

Aeromonas species possess a number of different haemolysin genes which have been the subjects of many investigations. Aerolysin-like haemolysins which have been sequenced and characterised include: aerolysin from *A. hydrophila* (Howard and Buckley, 1986), and AHH3, AHH4, AHH5 and ASA1 from *A. hydrophila* and *A. trota* (Hirono *et al.*, 1992). In addition there are two other haemolysin genes AHH1 and AHH2 cloned from *A. hydrophila* which are thought to have diverged from a common ancestor different to that of aerolysin (Aoki and Hirono, 1991, Hirono and Aoki 1991). The GCAT gene which is implicated in the pathogenesis of furunculosis,

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and is produced by *Aeromonas* and *Vibrio* spp. has been cloned, sequenced and reasonably well characterised biochemically (Buckely *et al.*, 1982).

These haemolysin genes are common to virulent and avirulent aeromonads making it difficult to determine which haemolysins are involved in the pathogenesis of septicaemias. It is therefore of particular interest that all 10 of the Au isolates possessed the ASH1 gene, while it was not present in any of the type strains or other motile aeromonads from Thailand. Unlike other haemolysin genes, ASH1 is not widely distributed among aeromonads and has only been identified in virulent isolates. It is possible therefore that this gene product is involved in the pathogenesis of frog septicaemias. Further studies are necessary to determine the distribution of the ASH1 gene in aeromonads isolated from farmed frog septicaemias. The sensitivity of fish cells, in particular rainbow trout cells, to Au lysins, indicates a wider possible distribution for the gene. Therefore surveys for the presence of the ASH1 gene should be extended to include *Aeromonas* isolates from septicaemic farmed fish in the sub-tropics and temperate zones. If the gene is widely found in strains associated with disease outbreaks, its presence could be used as a virulence determinant to distinguish isolates which are potentially pathogenic to aquatic animals from the ubiquitous aeromonads routinely found in the aquatic environment.

Knowledge of pathology is intrinsic to any domestication and intensification of culture (Edwards and Huisman, 1991). Many of the disease problems encountered in the frog farming industry result from a lack of basic understanding of the intricate harmony between the frog, pathogen and environment. This study has begun to

redress a small part of that imbalance by isolating and describing one of the pathogens

associated with frog septicaemic disease. More generally the characterisation of a

group of phenotypically and genotypically homogeneous motile aeromonads, extends

the scientific knowledge of a very complex genus. Study of this unique collection,

known to be associated with a septicaemic disease syndrome, should further the

detection of new *Aeromonas* toxins, and the understanding of their mechanisms of

pathogencity and their effect on humans and animals.

8.2 SUMMARY OF MAIN CONCLUSIONS

8.2.1 *AEROMONAS* SPP. IN FROG SEPTICAEMIA

8.2.1.1 A phenotypically distinct non-sucrose utilising motile aeromonad was consistently isolated from septicaemic farmed frogs in Thailand. The isolates were designated Au (*Aeromonas* unspeciated).

8.2.1.2 Histological examination of organs from frogs affected by Au septicaemia revealed a similar histological picture to Gram negative bacterial septicaemias observed in fish. Principal features include hyperaemia of the capillaries, and widespread necrosis, affecting particularly the lungs, heart and haemopoietic tissue.

8.2.1.3 Bacterial challenge of clinically healthy animals with Au reproduced the symptoms of septicaemic disease and resulted in mortalities, thereby fulfilling Koch's postulates and confirming that Au is one cause of septicaemic disease in frogs.

8.2.2. SPECIATION

8.2.2.1 The Au isolates were unusually homogeneous in their biochemical reactions for a geographically diverse group of motile aeromonads. All the Au isolates were unable to utilise sucrose, a reaction selected as a primary identification test for aeromonad genospeciation by Abbott *et al.* (1992). Molecular analysis indicated that Au is most closely related to HG1 however 100% of HG1 isolates utilise sucrose.

8.2.2.2 RAPD DNA analysis produced very homogenous patterns for the Au isolates whilst producing scattered profiles for type strains and other Thai aeromonads. The

RAPD profiles showed that the Au isolates were all closely related to *A. hydrophila* (HG1) but were more closely related to each other.

8.2.2.3 The Au 16s rDNA sequences showed a level of homology with the *Aeromonas* type strains that from ranged 98.1% to 99.8% indicating that these isolates are part of that evolutionary line. In the hypervariable region V3 of the 16srDNA sequence all the Au isolates were identical and differed from all previously published *Aeromonas* sequences. This 16s rDNA data indicates that all 10 Au isolates are the same species and that this species differs from previously recognised species.

8.2.3 VIRULENCE FACTORS

8.2.3.1 Au was highly haemolytic for frog and trout blood and cytotoxic for rainbow trout cells. Au displayed lower activity against cells of mammalian origin, indicating that these isolates are more likely to be virulent for aquatic animals.

8.2.3.1 The possession of the ASH1 gene may be related to these properties as it was only present in the Au isolates and had previously only been found in another three aeromonads, all of which were associated with disease in aquatic animals.

8.2.3.3 The source of cells used in haemolysin and cytotoxin assays is extremely important in relation to the origin of the bacteria under test. Aquatic bacterial enzymes should not be assayed against cells of mammalian origin.

8.3 FUTURE RESEARCH NEEDS

The aetiology of frog septicaemic disease is complex and involves more than the presence of a virulent bacterial isolate. Stresses to the animal and changes to the environment, imposed by farming practices, all enhance pathogen capability. The study of these subjects in farmed frogs has barely begun but is essential in the future, to ensure a holistic approach to disease diagnosis.

The present study has concentrated on the involvement of *Aeromonas* species in frog septicaemic disease. This does not mean that no other genera cause epidemics of bacterial disease in farmed frogs. Disease outbreaks in the industry should continue to be monitored: a) to confirm the presence of Au and whether it is a prime cause of septicaemia in farmed frogs; b) to investigate what other bacteria pathogenic to frogs exist and to establish the frequency with which such isolates occur.

Au was not isolated from frog skin or intestine, it is therefore very important to identify the reservoir of the bacteria and if possible eliminate them from frog farms. Surveys should be undertaken for the presence of the bacteria in water and sediments from the frog ponds. It would also be of interest to determine whether Au was present in wild populations of frogs and in water sources entering farms.

Other farmed aquatic species in S.E.Asia should be surveyed for the presence of Au, to establish whether it is a frog pathogen only. The virulence studies indicated that Au ECPs were cytotoxic to fish cells of different origins, therefore fish species could be affected by Au. A small scale survey will be undertaken in Bangladesh. A student conducting an epidemiological study of Epizootic Ulcerative Disease in many

different farmed fish species will be screening healthy and diseased fish for the presence of Au.

Further work is needed to develop a reproducible bath or co-habitation challenge in frogs. Larger scale frog challenges could then be carried out to establish LD_{50}s for Au and to compare Au pathogenicity with other motile aeromonads. Investigations into both chemotherapeutic and prophylactic methods of control of frog septicaemic disease could then be undertaken.

Extensive phenotypic and genotypic characterisation is necessary before the Au isolates can conclusively be described as a new species of motile *Aeromonas*. DNA-DNA hybridization is currently accepted as the best procedure for establishing new species. The genetic relatedness of Au within the genus *Aeromonas*, and in particular to HG1, must therefore be determined by this method. In addition the Ad Hoc Commitee on Reconciliation of Approaches to Bacterial Systematics (Wayne *et al.*, 1987), recommended that a distinct genospecies should not be named until specific phenotypic differentiation can be made from all other genospecies. Therefore much more extensive phenotyping needs to be undertaken to ensure that Au differs from definition strains for all known HGs and that the phenotypic criteria which define the group are shared by all Au isolates. Dr Geert Huys at Gent University in Belgium is currently undertaking fatty acid analysis of 10 Au isolates and further genetic analyses will be undertaken both at IASU and at Gent University.

Aeromonas unspeciated, and other *Aeromonas* isolates known to be associated with outbreaks of septicaemia in tropical and temperate farmed aquatic species, should be

screened for the presence of the ASH1 gene. The product of this gene is one possible

pathogenic determinant of motile aeromonad septicaemia of farmed frogs. The

development of a gene probe would make it possible to screen bacterial flora present

on the frog farm and predict the presence of potential pathogens. Thus farmers could

be forewarned if bacteria are likely to be virulent, allowing disease prevention

strategies to be employed, rather than costly chemical treatments and avoiding, or

considerably reducing, mortalities due to septicaemic disease.

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Appendix 1

FIRST STAGE IDENTIFICATION TECHNIQUES AND BACTERIAL MEDIA

GRAM'S STAIN (modification of Huker's method, Frerichs and Millar 1993)

Take a single bacterial colony from overnight growth on the appropriate agar at the appropriate temperature and emulsify in a drop of sterile distilled water on a glass slide. Spread the suspension was spread into a thin film on the slide, allow to air dry and then heat fix. Stain the slide with crystal violet solution (Crystal violet 2g, 95% ethanol 20ml, ammonium oxalate 0.5g, distilled water 70ml), fix with iodine solution (Iodine 1g, potassium iodide 2g, distilled water 300ml), destain with acetone and counterstain with safranin solution (Safranin 0.25g, 95% ethanol 10ml, distilled water 90ml). Examine the slide under oil immersion at 1000x.

MOTILITY "hanging drop" method (Frerichs and Millar, 1993).

Suspend a single colony from an overnight culture on the appropriate agar at the appropriate temperature in a drop of sterile distilled water placed on a cover slip. Place a small amount of soft paraffin wax at each corner of the cover slip and gently position a glass slide in contact with the wax but avoiding the drop. Quickly but carefully invert the whole preparation so that the drop hangs free under the coverslip above the glass slide. Examine the drop microscopically at 400x for evidence of bacterial motility. Isolates from agar plates which do not demonstrate motility should be subcultured into the appropriate broth and examined again after 24 hours, to ensure no false negatives are recorded.

CYTOCHROME OXIDASE

Cytochrome oxidase activity can be assessed using commercial strips impregnated with the redox dye tetramethyl-p-phenylenediamine (Pathotec^{TM}, Organon Teknika Corp, Durham). Smear a single colony from an overnight culture onto the reagent strip using a sterile platinum wire. The development of a dark purple colour within 1 minute indicates oxidation of the reagent and is recorded as a positive result. No change of colour is considered negative.

GLUCOSE OXIDATION-FERMENTATION (O/F) TEST

This test demonstrates the ability of the organism to metabolise glucose aerobically (by oxidation) or anaerobically (by fermentation). Supplement O-F basal medium (Difco) with 1% w/v glucose. Dispense ten ml aliquots of the medium into glass test tubes and sterilise. Inoculate duplicate tubes are inoculated a colony from an overnight culture by a single stab with a straight wire. Add sterile liquid paraffin to a depth of about one cm to one tube, leave the other uncovered. Incubate both tubes overnight at the appropriate temperature. Results are interpreted as follows:

	Open tube	Covered tube
Oxidative (O)	Yellow	Green
Fermentative (F)	Yellow	Yellow
No reaction (-)	Blue/green	Green

b

AESCULIN AGAR
Aesculin 0.5g
Ferric Citrate 0.25g
Peptone water 500ml
Agar bacteriological (Agar No.1) 1% w/v

ELASTIN MODIFIED AGAR PLATES (Hsu *et al.*, 1981)
Elastin	1g
Tryptone	2g
Yeast extract	2g
Cysteine hydrochloride	0.3g
Sodium chloride	1g
Agar	15g
Tris HCl pH 8.0	0.02M
Calcium chloride	0.04M

Disperse elastin in 20ml of 0.1N NaOH, heated at 100°C for 1 hour with frequent

shaking, adjust pH to 8.0 with 0.1N NaOH, add distilled water to make 400ml and

place solution in a blender at high speed for 3 minutes. Autoclave at 120°C for 15

minutes. Remaining ingredients were dissolved in 600ml of tris buffer and autoclaved

separately at 120°C for 15 minutes. Before pouring the two autoclaved solutions were

mixed.

c

Appendix 2

REAGENTS AND SOLUTIONS USED IN GENOTYPIC ANALYSIS

TE BUFFER

10mM Tris.Cl (pH 8.0) (10ml of 1M stock)
1mM EDTA-NaOH (pH 8.0) (4ml of 250mM stock)
deionized H_2O to 1 litre
Sterilise by autoclaving and store at room temperature.

TBE BUFFER

Stock solution:
10x: 107.79g Tris base
        55.03g boric acid
        7.44g EDTA.Na_2
        deionized H_2O to 1 litre

TBE is used at a working strength of 1x for polyacrylamide gel electrophoresis and

0.5x for agarose gel electrophoresis.

10% SDS (SODIUM DODECYL SULPHATE)

Dissolve 100g of electrophoresis-grade SDS in 900ml of H_2O. Heat to 68°C to assist

dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust

the volume to 1 litre with H_2O. Dispense into aliquots.

CTAB/NaCl SOLUTION

(10% CTAB in 0.7M NaCl)

Dissolve 4.1g of NaCl in 80 ml H_2O and slowly add 10g CTAB (hexadecyltrimethyl

ammonium bromide) while heating and stirring. If necessary, heat to 65°C to dissolve.

Adjust final volume to 100ml.

SOC MEDIUM

To 950ml of deionised H_2O, add:

tryptone	20g
yeast extract	5g
NaCl	0.5g

Shake until the solutes have dissolved. Add 10ml of a 250mM solution of KCl. (This solution is made by dissolving 1.86g of KCl in 100ml of deionized H_2O.) Adjust the pH to 7.0 with 5N Na OH ($\bullet$ 0.2ml). Adjust the volume of the solution to 1 litre with deionized H_2O. Sterilise by autoclaving, allow to cool to $\bullet$ 60°C and then add 20ml of a sterile 1 M solution of glucose and 5ml of a sterile solution of 2M $MgCl_2$. Aliquot into 1.5ml Eppendorf and store at -20°C.

(The glucose solution is made by dissolving 18g of glucose in 90ml of deionized H_2O. After the sugar has dissolved adjust the volume of the solution to 100ml with deionized H_2O and sterilise by filtration through a 0.22-μ filter. The $MgCl_2$ solution is made by dissolving 19g of $MgCl_2$ in 90ml of deionized H_2O. Adjust the volume of the solution to 100ml with deionized H_2O and autoclave).

L AGAR ANTIBIOTIC PLATES

Tryptone	10g
Yeast extract	5g
NaCl	10g
Agar	15g
H_2O to 1 litre	

Sterilise by autoclaving. Allow to cool and add: 1.5ml of a 50mg/ml X-gal solution, 100μl of 100mM solution of IPTG, ampicillin to a final concentration of 50μg/ml and tetracycline to 15μg/ml.

e

(X-gal = 5-bromo-4-chloro-3-indolyl-B-galactoside, 50mg/ml in dimethylformamide.

IPTG = isopropyl B-D-thiogalactopyranoside, 23.8mg/ml of H_2O gives a 100mM

solution).

2 x YT MEDIUM

To 900ml of deionized H_2O, add:

Tryptone	16g
Yeast extract	10g
NaCl	5g

Shake until the solutes have dissolved. Adjust the pH to 7.0 with %N NaOH. Adjust

the volume of the solution to 1 litre with deionized H_2O. Sterilise by autoclaving.

SOLUTION I (PLASMID DNA EXTRACTION)

50 mM glucose
25 mM Tris.Cl (pH 8.0)
10mM EDTA (pH 8.0)

Solution I can be prepared in batches of approximately 100ml, sterilised by

autoclaving and stored at 4°C.

SOLUTION III (PLASMID DNA EXTRACTION)

5M potassium acetate	60ml
glacial acetic acid	11.5ml
H_2O	28.5ml

The resulting solution is 3M with respect to potassium and 5M with respect to acetate.

DENATURING SOLUTION (COLONY HYBRIDISATION)

5M NaCl	6ml
NaOH	0.4
H_2O	to 20ml

NEUTRALISING SOLUTION (COLONY HYBRIDISATION)

5M NaCl	6ml
Tris.HCl pH 7.4	10ml
EDTA	0.0074g
H_2O	to 20ml

20x SSC (COLONY HYBRIDISATION)

NaCl	175.3g
Na_3citrate	88.2g
H_2O	to 1l

Adjust pH with 10N NaOH

COLONY HYBRIDISATION BUFFER

Formamide	10ml
20xSSC	5ml
20%SDS	200µl
0.5M EDTA pH8.0	40µl
100x Dernhardt's reagent	200µl
H_2O	4ml

DERNHARDT'S REAGENT

2% (w/v) bovine serum albumin (Fraction V; Sigma)
2% (w/v) Ficoll (Type 400, Pharmacia)
2% (w/v) polyvinylpyrrolidone

g

Appendix 3

16s rDNA SEQUENCES OF 10 Au ISOLATES

DNA sequence alignment

Row labels (top group): 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, AC, AE, AH, AJ, AM, AS, ASO, AT, AV

Row labels (bottom group): 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, AC, AE, AH, AJ, AM, AS, ASO, AT, AV

Seq	Start	Sequence	End
1	172	TAACTCCGTGCCAGCAGCCGCGGTAATTACGGA...GGGGTTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGATTAAG	261
2	173	TAACTCCGTGCCAGCAGCCGCGGTAATTACGGA...GGGGTTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGATTAAG	262
3	172	TAACTCCGTGCCAGCAGCCGCGGTAATTACGGA...GGGGTTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGATTAAG	261
4	172	TAACTCCGTGCCAGCAGCCGCGGTAATTACGGA...GGGGTTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGATTAAG	261
5	172	TAACTCCGTGCCAGCAGCCGCGGTAATTACGGA...GGGGTTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGATTAAG	261
6	171	TAACTCCGTGCCAGCAGCCGCGGTAATTACGGA...GGGGTTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGATTAAG	260
7	173	TAACTCCGTGCCAGCAGCCGCGGTAATTACGGA...GGGGTTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGATTAAG	262
8	173	TAACTCCGTGCCAGCAGCCGCGGTAATTACGGA...GGGGTTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGATTAAG	262
9	172	TAACTCCGTGCCAGCAGCCGCGGTAATTACGGA...GGGGTTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGATTAAG	261
10	173	TAACTCCGTGCCAGCAGCCGCGGTAATTACGGA...GGGGTTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGATTAAG	262
AC	173	TAACTCCGTGCCAGCAGCCGCGGTAATTACGGA...GGGGTTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGATTAAG	262
AE	173	TAACTCCGTGCCAGCAGCCGCGGTAATTACGGA...GGGGTTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGATTAAG	262
AH	173	TAACTCCGTGCCAGCAGCCGCGGTAATTACGGA...GGGGTTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGATTAAG	262
AJ	173	TAACTCCGTGCCAGCAGCCGCGGTAATTACGGA...GGGGTTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGATTAAG	262
AM	173	TAACTCCGTGCCAGCAGCCGCGGTAATTACGGA...GGGGTTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGATTAAG	262
AS	173	TAACTCCGTGCCAGCAGCCGCGGTAATTACGGA...GGGGTTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGATTAAG	262
ASO	173	TAACTCCGTGCCAGCAGCCGCGGTAATTACGGA...GGGGTTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGATTAAG	262
AT	173	TAACTCCGTGCCAGCAGCCGCGGTAATTACGGA...GGGGTTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGATTAAG	262
AV	173	TAACTCCGTGCCAGCAGCCGCGGTAATTACGGA...GGGGTTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGATTAAG	262

Seq	Start	Sequence	End
1	262	TTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTTAGAGGGGGGTAGAATTCCAGGTGTA	351
2	263	TTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTTAGAGGGGGGTAGAATTCCAGGTGTA	352
3	262	TTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGT-CAGCTAGAGTCTTGTTAGAGGGGGGTAGAATTCCAGGTGTA	350
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5	262	TTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTTAGAGGGGGGTAGAATTCCAGGTGTA	351
6	261	TTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTTAGAGGGGGGTAGAATTCCAGGTGTA	351
7	263	TTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTTAGA-GGGGGGTAGAATTCCAGGTGTA	349
8	263	TTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTTAGAGGGGGGTAGAATTCCAGGTGTA	352
9	262	TTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTTAGAGGGGGGTAGAATTCCAGGTGTA	352
10	263	TTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTTAGAGGGGGGTAGAATTCCAGGTGTA	352
AC	263	TTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTTAGAGGGGGGTAGAATTCCAGGTGTA	352
AE	263	TTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTTAGAGGGGGGTAGAATTCCAGGTGTA	352
AH	263	TTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTTAGAGGGGGGTAGAATTCCAGGTGTA	352
AJ	263	TTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTTAGAGGGGGGTAGAATTCCAGGTGTA	352
AM	263	TTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTTAGAGGGGGGTAGAATTCCAGGTGTA	352
AS	263	TTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTTAGAGGGGGGTAGAATTCCAGGTGTA	352
ASO	263	TTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCGGCTAGAGTCTTGTTAGAGGGGGGTAGAATTCCAGGTGTA	352
AT	263	TTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCGCTAGAGTCTTGTTAGAGGGGGGTAGAATTCCAGGTGTA	352
AV	263	TTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCAGCTAGAGTCTTGTTAGAGGGGGGTAGAATTCCAGGTGTA	352

DNA sequence alignment (two blocks, page rotated 90°).

Block 1

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1	352	GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGA	441
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5	352	GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGA	441
6	352	GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGA	441
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8	353	GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGA	442
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10	353	GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGA	442
AC	353	GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGA	442
AE	353	GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGA	442
AH	353	GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGA	442
AJ	353	GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGA	442
AM	353	GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGA	442
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ASO	353	GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGA	442
AT	353	GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGA	442
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Block 2

Label	Start	Sequence	End
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8	443	GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACG	532
9	442	GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACG	531
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AC	443	GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACG	532
AE	443	GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACG	532
AH	443	GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACG	532
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AM	443	GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACG	532
AS	443	GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACG	532
ASO	443	GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACG	532
AT	443	GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACG	532
AV	443	GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACG	532

Block 1 — sequence alignment (columns 712/713 … 799–802)

```
1   712: GTGCTGCATGGCTGTCGTCGTCGTCAGCTCGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTA  801
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3   711: GTGCTGCATGGCTGTCGTCGTCGTCAGCTCGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTA  800
4   712: GTGCTGCATGGCTGTCGTCGTCGTCAGCTCGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTA  801
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6   712: GTGCTGCATGGCTGTCGTCGTCGTCAGCTCGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTA  801
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9   712: GTGCTGCATGGCTGTCGTCGTCGTCAGCTCGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTA  801
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AC  713: GTGCTGCATGGCTGTCGTCGTCGTCAGCTCGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTA  802
AE  713: GTTCTGCATGGCTGTCGTCGTCGTCAGCTCGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTA  802
AH  713: GTGCTGCATGGCTGTCGTCGTCGTCAGCTCGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTA  802
AJ  713: GTGCTGCATGGCTGTCGTCGTCGTCAGCTCGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTA  802
AH  713: GTGCTGCATGGCTGTCGTCGTCGTCAGCTCGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTA  802
AS  713: GTGCTGCATGGCTGTCGTCGTCGTCAGCTCGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTA  802
ASO 713: GTGCTGCATGGCTGTCGTCGTCGTCAGCTCGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTA  802
AT  713: GTGCTGCATGGCTGTCGTCGTCGTCAGCTCGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTA  802
AV  713: GTGCTGCATGGCTGTCGTCGTCGTCAGCTCGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTA  802
```

Block 2 — sequence alignment (columns 800–803 … 888–892)

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1   802: ATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGG--CCTTACGGCCAGGGCTAC  890
2   803: ATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGG--CCTTACGGCCAGGGCTAC  891
3   801: ATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGG--CCTTACGGCCAGGGCTAC  889
4   802: ATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCC-CCTTACGGCCAGGGCTAC  891
5   802: ATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGG--CCTTACGGCCAGGGCTAC  890
6   800: ATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGG--CCTTACGGCCAGGGCTAC  890
7   802: ATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGG--CCTTACGGCCAGGGCTAC  888
8   802: ATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGG--CCTTACGGCCAGGGCTAC  890
9   803: ATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGG--CCTTACGGCCAGGGCTAC  891
10  802: ATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGG--CCTTACGGCCAGGGCTAC  890
AC  803: ATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGC--CCTTACGGCCAGGGCTAC  891
AE  803: ATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGC--CCTTACGGCCAGGGCTAC  892
AH  803: ATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGC--CCTTACGGCCAGGGCTAC  892
AJ  803: ATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGC--CCTTACGGCCAGGGCTAC  892
AM  803: ATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGC--CCTTACGGCCAGGGCTAC  892
AS  803: ATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGC--CCTTACGGCCAGGGCTAC  892
ASO 803: ATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGC--CCTTACGGCCAGGGCTAC  892
AT  803: ATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGC--CCTTACGGCCAGGGCTAC  892
AV  803: ATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGC--CCTTACGGCCAGGGCTAC  892
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 1    891: ACACGTGCTACAATGGGCGGGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGGTCGTAGTCCG...ATCGGAGTCTC  980
 2    892: ACACGTGCTACAATGGGCGGGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGGTCGTAGTCCG...ATCGGAGTCTC  981
 3    890: ACACGTGCTACAATGGGCGGGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGGTCGTAGTCCG...ATCGGAGTCTC  979
 4    892: ACACGTGCTACAATGGGCGGGTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGGTCGTAGTCCA...AATCGGAGTCTC  981
 5    891: ACACGTGCTACAATGGGCGGGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGGTCGTAGTCCG...ATCGGAGTCTC  961
 6    891: ACACGTGCTACAATGGGCGGGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGGTCGTAGTCCG...ATCGGAGTCTC  980
 7    889: ACACGTGCTACAATGGGCGGGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGGTCGTAGTCCG...ATCGGAGTCTC  978
 8    892: ACACGTGCTACAATGGGCGGGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGGTCGTAGTCCG...ATCGGAGTCTC  980
 9    891: ACACGTGCTACAATGGGCGGGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGGTCGTAGTCCG...ATCGGAGTCTC  980
10    892: ACACGTGCTACAATGGGCGGGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGGTCGTAGTCCG...ATCGGAGTCTC  981
AC    892: ACACGTGCTACAATGGGCGGGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGGTCGTAGTCCG...ATCGGAGTCTC  981
AE    893: ACACGTGCTACAATGGGCGGGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGGTCGTAGTCCG...ATCGGAGTCTC  982
AH    893: ACACGTGCTACAATGGGCGGGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGGTCGTAGTCCG...ATCGGAGTCTC  982
AJ    893: ACACGTGCTACAATGGGCGGGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGGTCGTAGTCCG...ATCGGAGTCTC  982
AM    893: ACACGTGCTACAATGGGCGGGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGGTCGTAGTCCG...ATCGGAGTCTC  982
AS    893: ACACGTGCTACAATGGGCGGGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGGTCGTAGTCCG...ATCGGAGTCTC  982
ASO   893: ACACGTGCTACAATGGGCGGGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGGTCGTAGTCCG...ATCGGAGTCTC  982
AT    893: ACACGTGCTACAATGGGCGGGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGGTCGTAGTCCG...ATCGGAGTCTC  982
AV    893: ACACGTGCTACAATGGGCGGGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGGTCGTAGTCCG...GATCGGAGTCTC  982


 1    981: CAACTCGACTCGTGAAGTCGGAATCGCTAGTAATCGAAATCAGAATGTTCGGTGAATACGTTCCCGGGCCTTGT  1057
 2    982: CAACTCGACTCGTGAAGTCGGAATCGCTAGTAATCGAAATCAGAATGTTCGGTGAATACGTTCCCGGGCCTTGT  1058
 3    980: CAACTCGACTCGTGAAGTCGGAATCGCTAGTAATCGAAATCAGAATGTTCGGTGAATACGTTCCCGGGCCTTGT  1056
 4    982: CAACTCGACTCGTGAAGTCGGAATCGCTAGTAATCGAAATCAGAATGTTCGGTGAATACGTTCCCGGGCCTTGT  1058
 5    981: CAACTCGACTCGTGAAGTCGGAATCGCTAGTAATCGAAATCAGAATGTTCGGTGAATACGTTCCCGGGCCTTGT  1057
 6    979: CAACTCGACTCGTGAAGTCGGAATCGCTAGTAATCGAAATCAGAATGTTCGGTGAATACGTTCCCGGGCCTTGT  1055
 7    981: CAACTCGACTCGTGAAGTCGGAATCGCTAGTAATCGAAATCAGAATGTTCGGTGAATACGTTCCCGGGCCTTGT  1057
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 9    981: CAACTCGACTCGTGAAGTCGGAATCGCTAGTAATCGAAATCAGAATGTTCGGTGAATACGTTCCCGGGCCTTGT  1058
10    982: CAACTCGACTCGTGAAGTCGGAATCGCTAGTAATCGAAATCAGAATGTTCGGTGAATACGTTCCCGGGCCTTGT  1058
AC    982: CAACTCGACTCGTGAAGTCGGAATCGCTAGTAATCGAAATCAGAATGTTCGGTGAATACGTTCCCGGGCCTTGT  1059
AE    983: CAACTCGACTCGTGAAGTCGGAATCGCTAGTAATCGAAATCAGAATGTTCGGTGAATACGTTCCCGGGCCTTGT  1059
AH    983: CAACTCGACTCGTGAAGTCGGAATCGCTAGTAATCGAAATCAGAATGTTCGGTGAATACGTTCCCGGGCCTTGT  1059
AJ    983: CAACTCGACTCGTGAAGTCGGAATCGCTAGTAATCGAAATCAGAATGTTCGGTGAATACGTTCCCGGGCCTTGT  1059
AM    983: CAACTCGACTCGTGAAGTCGGAATCGCTAGTAATCGAAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGT  1059
AS    983: CAACTCGACTCGTGAAGTCGGAATCGCTAGTAATCGCGGTGAATACGTTCCCGGGCCTTGT  1059
ASO   983: CAACTCGACTCGGTGAAGTCGGAATCGCTAGTAATCGCGGTGAATACGTTCCCGGGCCTTGT  1059
AT    983: CAACTCGACTCGNTGAAGTCGGAATCGCTAGTAATCGAAATCAGAATGTTTCGGTGAATACGTTCCCGGGCCTTGT  1059
AV    983: CAACTCGACTCGTGAAGTCGGAATCGCTAGTAATCGAAATCAGAATGTTCGGTGAATACGTTCCCGGGCCTTGT  1059
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Appendix 4

CALIBRATION CURVE

Inoculate 10mls of Tryptone Soya Broth (TSB) with a colony of the selected bacterial isolate and incubate overnight at 30°C. Measure the optical density of the bacterial suspension at 610nm and dilute the suspension with TSB to give optical densities of 1, 0.8, 0.6, 0.4, 0.2 and 0.1. At each optical density take a sample of the bacterial suspension and carry out viable counts (see below) to establish the number of bacteria in the suspension. Plot number of bacteria against optical density to give a calibration curve for the bacterial isolate. The regression equation obtained from the calibration curve may thereafter be used to estimate number of bacteria in broth cultures.

VIABILITY BY DROP COUNT OF A BACTERIAL SUSPENSION

Remove 0.5 ml of suspension to be counted and add to 4.5ml of saline. Make serial dilutions of suspension from 10^{-1} to 10^{-7} in saline. Split 7 TSA plates into 6 segments, label each plate with one dilution factor. Add 6, 20µl drops of the 10^{-7} dilution to the appropriately labelled plate and repeat the sequence with each following dilution. Leave on a level surface until drops are dry and then invert plates and incubate at the temperature appropriate for the species. Check plates the following day and count colonies on plates with between 10-50 colonies per drop. To calculate the number of colony forming units per ml (cfu/ml):

Average number of colonies per drop x 50 x dilution.

PREPARATION OF BACTERIAL CHALLENGE INOCULUM

Put up an overnight culture of bacterial isolate in TSB (if undertaking large scale bath challenges ensure you have sufficient volume). Measure the optical density at 610nm and, using the regression equation from the calibration curve, estimate number of bacteria in the broth suspension. Dilute suspension sufficiently to obtain $1x\ 10^8$ bacteria and then prepare appropriate volume of challenge suspension at required concentration. In addition make serial 10 fold dilutions of $1x10^8$ suspension in saline, from 10^{-1} to 10^{-7}, and place in refrigerator. Challenge fish with bacteria, then plate out dilutions as soon as possible for a viable count. Leave plates to dry and then incubate. Count colonies the next day to check that the challenge dose was at the correct concentration.

Calibration Curve Au 2D8

$y = 4.993x - 0.4439$