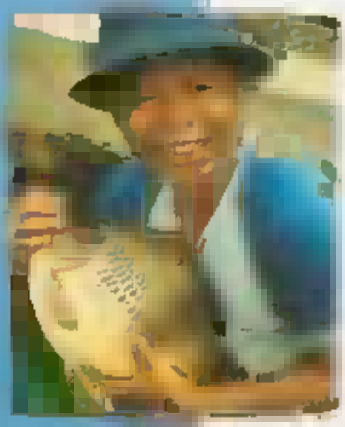
INSTITUTE OF AQUACULTURE

Final Report to the Department for International Development

DFID Final Report - Project R5998: Studies on the Susceptibility of Farmed *Rana tigerina/rugulosa* to frog Septicaemic Disease and its Control



UNIVERSITY OF STIRLING

Period of Report: March 1994 - March 1997

Dr. Valerie Inglis

Final Report to the Department for International Development

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Project R 5998: Studies on the susceptibility of farmed *Rana tigerina/rugulosa* to frog septicaemic disease and its control

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Executive Summary

Bacterial septicaemia is a major problem in frog farming particularly in South East Asia. Losses in hatcheries and grow-out farms are enormous and antibiotic prophylaxis and therapy, although widely practised, is largely ineffective. This project aimed to find out how this disease could be controlled.

Bacteriology and histology were conducted on normal and diseased frogs from farms in Thailand. A range of bacteria was isolated including an unspeciated aeromonad, which was highly virulent for frogs. On the evidence available so far this bacterium is an obligate pathogen, actively haemolytic and, based on rDNA analysis, unique. Other bacteria were less virulent or non-virulent in laboratory challenges but a wide range of viable bacteria could be recovered from macrophages. While the individual virulence of the carried strains is not known, they were able to grow out and kill frogs when the animals were stressed. Bacteria were observed in macrophages from most of the Thai frogs examined. Although conventional bacteriological sampling often was negative, viable bacteria could be recovered following macrophage separation and a bacterial resuscitation step. Histology of 'normal' tissue in this and in a related study showed a very high incidence of abnormalities consistent with on-going low-grade infection.

The non-specific immune system of the ranids was investigated in some detail and found to be functionally similar to fish and mammals but the values of the parameters measured varied widely. When all the results are considered together; the abnormal histology, the low and variable respiratory burst of macrophages, the failure of the frog innate defences to respond to chemical non-specific immunostimulation and the explosive outbreaks of bacterial disease in stocks when stressed; they point to on-going, sub-clinical bacterial challenge of the frog macrophages. This presents a major threat to health.

The effect of selected stressors on macrophage function was investigated but no relationship was demonstrated in the limited work carried out. However inadvertant stress, due to transportation and/or heat shock, did give rise to bacterial disease throughout the stock tanks affected. In the Stirling vivarium, environmental conditions and nutrition were shown to be critical in establishing a healthy population.

The conclusions drawn from these findings are that frog phagocytes are highly susceptible to bacterial colonisation. This either may remain undetected and the frogs continue to grow or, under stress, the system may break down. Nothing is known about the cost of this pressure in physiological terms nor how much better the frogs would perform if this challenge were substantially reduced. Further work is needed to confirm that farmed frogs can be relieved of this bacterial load and to define the environmental and nutritional conditions necessary for this. Then more specific work can follow to screen stocks for the presence of identified virulent pathogens, such as that found in this study, and to prepare and administer protective vaccines. The future of frog farming depends on producing stocks with a better standard of general health and in particular, a lower systemic bacterial load. Chemotherapy or vaccination cannot usefully be applied until this is achieved.

1.0 Background

Demand for frogs and the development of frog farming.

Frog populations have been in serious decline over the past twenty-five years. The reasons for this are not fully understood, but loss of habitat and widespread dissemination of chemicals are thought to be contributory factors, exacerbated by massive exploitation of natural populations for human consumption. Several countries in Asia and South America earn large amounts of foreign exchange by exporting live frogs and frozen frog legs and in recent years frog farming has grown to help meet this demand.

Frog farming was established in California early this century but growth has been slow. It was developing in Taiwan in the late 1950s and in Brazil in the 1980s. Growth in Thailand and Indonesia is more recent still and commercial success was not achieved there until the 1990s. In Bangladesh frog farming has not yet started and there ever dwindling wild populations have seriously reduced the numbers available to be caught by trappers.

Farms and farming practices differ greatly between and even within countries. In Brazil the government provided funding to help develop new systems and establish this new farming industry. The farmers there find it best to work in an integrated culture scheme with central breeding units producing young frogs and supplying them to farms for fattening. In Thailand while farms may vary considerably in size, from 1-2 ponds to larger spreads over 0.5 hectares, they usually are run as family units carrying out the whole operation.

Frog farming is well suited to small-scale producers. The main output is frog meat, a high protein food for local consumption and for export; but there is also a range of by-products used in medicines and cosmetics and a specialised leather. Further it creates employment in ancillary services such as feed supply, support and marketing services. Although the potential is great it does not have large scale financial backing and recent fortunes have been subject to enormous fluctuations. While there have been very profitable harvests in the industry there have also been substantial losses. Frog culture is afflicted by disease, inadequate nutrition, abuse of chemicals, a variable market and an inadequate support structure.

Infectious disease, a principal problem in frog farming

The species farmed varies between countries but most commonly they are *Rana* spp. and in particular *R. catesbeiana* and the closely related Chinese and Indian bullfrogs *R.rugulosa* and *R. tigerina*.. These frogs can be grown to a marketable size of 160-180 g, in four months.

Most farms are arranged as concrete corrals using natural water sources. Often canals are shared between farms as water sources and drainage ditches are shared as repositories of waste. Water quality, water exchange practices and diet have all evolved on a basis of trial and error rather than on systematic study. Sometimes a hatch may yield a grow-out rate as high as 80%; often disease outbreaks result in 100% losses.

Although viruses, fungi and parasites have been isolated from frogs and protozoa can lead to 50-70% mortalities in tadpole nurseries, it is bacteria which have been associated with the greatest and most numerous epizootics and the cause of huge financial losses. The agents most often incriminated are the group of bacteria known collectively as the motile aeromonads. It is recognised that bacterial disease is husbandry related and is affected by nutrition and environmental conditions. However opinion of their differential impact on frog culture has been formed by empirical observation: here too there has been little systematic study. Field response has been to try to control disease with chemical disinfectants and antibacterial agents. There is little advice and even less control on use of these substances. They are often used prophylactically or as 'cure-alls'. The wide indiscriminate use of antibacterial agents has led to problems of bacterial resistance, untreatable disease and the risk of residues in flesh which renders the product highly undesirable for human consumption locally and unacceptable by the export market.

The pattern of expansion in frog farming is similar to that seen in the early years of the shrimp industry. Large profits have been made in satisfying the easily accessed local market but processing technologies and standards have not advanced sufficiently to satisfy a wider international market. Quickly supplies exceeded local demand, prices fell, stocking densities increased in an attempt to compensate and disease problems exploded. If frog culture is to become a sustainable industry, developments of plant and processing must take place to ensure

an international market and production strategies must be set in place to guarantee a steady supply of high quality and adequate volume. As a prime requisite the sweeping bacterial epizootics decimating the frog farms must be controlled and healthy frogs reared to marketable size.

2.0 Project purpose

Very little is known about the health of the frog. Most experience has come from work with laboratory animals often where survival is accepted as an indicator of health. There has been even less work done on the health of the tropical farmed varieties. In the domestication of any species for intensive culture a knowledge of the pathology is essential. To manage stocks effectively in relation to infectious disease it is important to have information on the:-

normal histology of the cultured species
pathological changes associated with disease
normal microflora of the frog and its environment
infectious disease agents of importance
innate defence mechanisms
effective therapeutic regimes.

This information did not exist for farmed frogs and this project was designed to contribute significantly to providing this knowledge base with particular reference to bacterial septicaemia, the disease of paramount importance in frog farming. It aims to elucidate factors predisposing to outbreaks and lead to development of strategies for control; then to start to disseminate this information.

The study was underpined by an extensive review of frog farming and frog diseases carried out in 1993-94 (Pearson and Inglis 1994)[1] which was extended to include in-depth reviews of bacterial diseases of frogs and the frog immune system which will be included in the PhD thesis to be submitted to the University of Stirling by M.Crumlish. Research was then conducted to meet the objectives stated in the project memorandum *viz.* to

[1] Pearson, M.D. and Inglis, V. 1994. A report on the current status of frog culture with emphasis on disease.

- identify the bacteria consistently associated with epizootic and enzootic septicaemic disease of frogs
- establish the incidence of disease-associated bacterial species in healthy frogs and their environment
- investigate selected pathogens and their virulence mechanisms
- elucidate the innate defences of frogs to bacterial infection
- assess the effect of stressors on the innate defences
- evaluate non-specific chemical immunostimulants in enhancing disease resistance.

During the second year of the study an unexpected finding was made in that frog spleen macrophages taken from animals which appeared healthy contained numerous bacteria. After this initial observation had been confirmed many times, its significance in the predisposition of animals to stress-related outbreaks of disease was investigated as a matter of high priority. It was felt by the project research group, that if this occurrence was common, it may be central in the failure of farmed frogs to withstand environmental stress. It would moreover have a major bearing on the possibility of prophylaxis by non-specific immunostimulation and may make the process of vaccination problematic. Therefore although this was not one of the objectives stated initially, the occurrence and significance of viable bacteria in frog macrophages became one of the main issues investigated in the second half of the study.

Opportunities provided by formal and informal exchanges with colleagues at the Aquatic Animal Health Research Institute (AAHRI) in Bangkok were taken to share resources and experience on other aspects of work on frog health so that complementary work at both centres contributed to a substantial body of findings. At the outset provision was made to disseminate the information generated by the project to the scientific and frog farming communities through publications, a workshop, a manual, lectures and laboratory sessions. This has been done.

3.0 Research Activities

All project research was co-ordinated to elucidate development of bacterial disease in tropical farmed frogs, susceptibility of frogs to these diseases and possible control strategies.

- The foundation of all activities lay in access to relevant frog populations. This was achieved in farms in Thailand, in the wet laboratories at AAHRI and in a frog vivarium which was established at the Institute of Aquaculture (IOA), University of Stirling.
- The bacteria to which tropical farmed frogs are exposed and which may pose a risk to health were assessed following a field survey conducted in Thailand at the outset of the project and from AAHRI laboratory records which were analysed in association with the AAHRI staff. A highly virulent frog pathogen was identified.
- Histology of normal and farmed frogs was started on tissue collected in conjunction with the bacteriology field survey and was developed later more fully in a PhD thesis being submitted to the University of Chulalongkorn by Kantimanee Phanwichien..
- The non-specific immune system of tropical farmed frogs, their first line defences, was investigated to provide basic information on their ability to respond to bacterial challenge.
- The efficacy of immunostimulants in enhancing the activity of components of the non-specific immune system of farmed ranids was investigated.
- The effects of physical stress on the ability of farmed ranids to withstand bacterial challenge was assessed.
- The frequency of occurrence of viable bacteria in frog macrophages and the significance of this finding was investigated in depth.
- The susceptibility of farmed frogs to bacterial challenge by identified pathogens and other bacteria of their general environment was elucidated.

The vulnerability of frogs to infectious disease and the reason for this was the major finding of this project with implications for successful frog farming, survival of the species and fundamental understanding of the non-specific immune system.

3.1 Research Staff

These studies have been conducted in close collaboration with AAHRI staff engaged on related ODA funded work in Thailand.
Key personnel were:-

Institute of Aquaculture

Dr V. Inglis, University Senior Research Lecturer, Project Co-ordinator - 3 years

Ms M. Crumlish, ODA funded PhD student - 2 years 6 months

Ms M. Pearson, ODA funded research fellow - 6 months

Mr D. Colquhoun, IOA M Sc student - 4 months

Aquatic Animal Research Institute

Dr T. Somsiri, Bacteriologist, Associate on related work

Dr K. Panwichien, Histologist. Associate on related work.

3.2 Location of work

Work was carried out at IOA, University of Stirling and in Thailand based at AAHRI, Kasetsart University, Bangkok. Dr Inglis visited Thailand twice during the project: in the first year, during an Resource Centre Scheme consultancy and after two and a half years to conduct a workshop on Frog Health for scientists from the Region. On both occasions there were sessions to plan and review progress of this project and Project R6206Cb, *Study of bacterial disease in Frog Hatcheries and Evaluation of Strategies to Reduce Hazards Associated with Antibiotic Usage.*

Ms Pearson spent 5 months in Thailand from May 1994 sampling frogs from farms throughout Thailand for bacteriology and histology and conducting bacterial challenge studies on frogs at the AAHRI wet laboratories. She returned to assist at the Frog Health workshop in October 1996 and to continue work on a pathogen which had by then become recognised as significant. At this stage she was no longer funded by this project.

Although Ms Pearson was awarded a Veterinary Research Fellowship by the Biotechnology and Biological Sciences Research Council (BBSRC) under the supervision of Dr Inglis, 6 months after the start of the project, she remained a close associate. Her contribution to many aspects of the work and particularly to the establishment and running of the frog vivarium at Stirling cannot be over estimated. It was decided at this stage that the most advantageous way forward was for Ms Pearson to pursue in-depth studies on the unspeciated frog pathogen which had been found in the initial survey in Thailand and allow Ms Crumlish, who had been

appointed to the project, to investigate the innate immune system of farmed fogs much more fully than otherwise would have been possible.

Further support on the bacteriological studies was provided by a fellowship awarded by the Japanese Society for the Promotion of Science to allow Ms Pearson to carry out genetic analysis of the pathogen in Japan and also by research on this issue by D. Colquhoun as part fulfilment of his work for an M Sc at IOA, Stirling.

Ms Crumlish visited Thailand twice during the project; for the month of June 1995 and for 4 months from July to November 1996. During these times she investigated the innate immune system of frogs as a defence against bacterial infection. She started work on the effects of stressors and non-specific immunopotentiators and at the end of the second visit played a major role in the conduct of the Frog Health workshop.

All other work by her was done at Stirling. She developed assays to measure the parameters of the innate immune system of frogs. This base-line data not yet available in the literature, was essential as the level of activation of the non-specific defences of animals is a good indicator of general health status. Assays were adapted from fish and refined on the frogs at Stirling. The intention was to repeat these measurements on the AAHRI frogs , animals in their natural climatic conditions and which had not been subjected to long transportation by air. If the range was similar to that found with the frogs at Stirling, this would provide a good indication that they had adapted well to the changed environment and could be regarded as normal.

All parameters of the non-specific immune system showed a much wider range than has been found for other species of animal and viable bacteria were found repeatedly in the spleen macrophages of animals considered free of disease. This project then was redirected in an attempt to elucidate the significance of these findings and to measure the state of activation of the innate defences in relation to the incidence of bacteria in the internal organs and the consequent susceptibility to stress-induced disease.
This aspect was pursued in association with colleagues at AAHRI in studies *in vivo* and by retrospective scrutiny of laboratory records there.

3.3 Experimental frog populations

3.3.1. Frog vivarium at Institute of Aquaculture, Stirling.

Tropical frogs of the species farmed are not immediately available in the UK, therefore a small number of tropical African clawed toads, *Xenopus laevis,* were obtained from the Biology Department, University of St Andrews. These were used to refine the bacteriological and histological techniques necessary for field work in Thailand. This experience was useful also in designing and setting up a tropical vivarium at Stirling.

All the necessary UK Home Office licences to import and work with tropical frogs were obtained and an arrangement made with AAHRI to supply frogs.

Eighty-seven post-larval *Rana rugulosa*[2] were transported from Thailand by Ms Pearson. A small number of mortalities occurred in the first two weeks after transfer and the first grading, but it was established that frogs could be brought in successfully, at least by personal courier. In the first year a large body of information was acquired on keeping these experimental frogs. Much of the work was empirical for although there are other people in the UK who keep tropical frogs, none do so in the numbers which this project required nor do they work with the species which are farmed.

In the second year the frog holding facility was substantially enlarged to incorporate a quarantine facility for new-arrivals and an system for improved grading.

With the improved vivarium and better understanding of husbandry, more frogs were brought in. Initially they were transported from AAHRI by courier as before. These were established successfully, grew and were used in immunological studies.

Two groups were then shipped as unaccompanied cargo.

The first shipment was successful but in a subsequent group many frogs were dead or dying on arrival. Only about half this population survived and settled. The reason for these losses initially was thought to be that the transfer was made at the end of the season for these animals; they were considered, as a consequence, not to be in good condition and moreover

[2] Stocks of frogs from Thailand cannot be defined genetically with certainty. The species *Rana rugulosa,* the Chinese bullfrog and *Rana tigerina,* the Indian bullfrog, are very close and interbreeding has occurred in the Thai farming industry. Throughout this report animals from Thailand will be referred to *Rana rugulosa.*

had had a difficult journey. Later as the understanding of susceptibility of frogs to stress-related bacterial disease increased, the reasons for this and other disease episodes could be interpreted more fully.

Thus a means of importing ranids to Stirling was established. It was only dependant on a ready supply. During the third year of this project AAHRI had pressing demands for their own frogs and consequently were unable to meets the needs of this project as well. A new source was found in a UK supplier who imported frogs caught in the US. Although the interrupted Thai supply initially was seen as a problem, it has since proved to be valuable to the project in allowing some important observations which had been made in the Thai farmed ranids to be followed up in other related species; viz. *Rana pipiens* trapped in the U.S. and *Rana temporaria* trapped in the U.K..

3.3.2 Sampling frogs in Thailand on farms and at AAHRI

Ms Pearson sampled frogs on farms during five field trips in Thailand at the outset of the project *vide infra.* Ms Crumlish carried out immunological studies on ranids reared and held at AAHRI during two periods in Thailand.

Establishing a frog holding facility in the U.K. and setting up a import system was much more difficult than had been anticipated, but it has been achieved. These frogs were used to establish all techniques of immunology, bacterial challenge and chemotherapy used at IOA and at AAHRI. Without this the findings which have been derived from this study could not have been made.

The opportunities for extended work in Thailand during this project were invaluable in giving access to frogs in sufficient numbers to allow reliable bacteriological and immunological work to be carried out.

4.0 Project Outputs

The expected outputs as stated in the project memorandum were:-

- scientific papers in peer review journals on
  histology of normal farmed frogs
  histopathology associated with frog septicaemic disease
  influence of stressors on the innate defences of frogs
  immunomodulation
- a frog health manual
- a workshop for frog health scientists in South East Asia
- general reports in aquaculture newsletters.

Four scientific papers have been produced, two posters presented at international conferences, four articles published in Aquaculture News, the manual produced and the workshop conducted. Of the scientific papers, one is published, one has been accepted for publication, one has been accepted for oral presentation with a view to publication in the conference proceedings and the fourth has been submitted. Histopathology has been included in one paper, but because of the nature of the findings of this project, the main content of the papers is on bacteriology, the innate immune system and bacterial challenge of this system. This is elaborated further below and details of publications are given on pages 41-42. The Frog Health Manual is included as Appendix 3 and details of the workshop including an evaluation are included in the RCS Consultancy report submitted by Dr Inglis to the ODA in November 1996.

The direction of the project changed to some extent as it progressed both as a result of scientific findings and also because of additional resources becoming available. The significance of the on-going bacterial challenge of the frog non-specific immune system was considered such that this issue was pursued as a priority. This decision was justified further by obtaining a BBSRC award to concentrate on bacteriological studies: this effort in turn was enhanced by obtaining an award from the Japanese Society for the Promotion of Science and by work done by an MSc research student from the University of Stirling. The output on histology was complemented by work done concurrently by a University of Chulalonkorn PhD student. Details on these developments also are provided in this section.

4.1 Bacteria associated with septicaemia of farmed frogs in Thailand

4.1.1 Field Work in Thailand

Five field trips were made to farms in different geographical locations in Thailand; to Kam Pam Petch, Pak Thong Chai, Petchburi, Pathun Thani and Chanburi. On each farm bacteriological and histological samples were taken from 10 diseased and 10 clinically normal frogs.
Samples for bacteriological examination were taken from inner thigh skin, lesions, thigh muscle, heart blood, kidney, liver, spleen and intestinal contents.

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 reaction and by testing the ability of isolates to produce oxidase and to metabolise glucose fermentatively or oxidatively. Further biochemical tests were performed using the API 20 E *enterobacteriaceae* system (BioMerieux, France).

Results

First stage identification tests on samples from the field trips revealed the presence of many putative Aeromonads in the diseased frogs and a range of isolates from the clinically normal frogs. Further classification with the API 20 E identification kit showed that the sugar fermentation patterns of many of the suspected Aeromonads from the internal organs of the diseased frogs did not fit that of either *A. hydrophila* or *A. sobria*. This "unknown" Aeromonad had been isolated from the organs of four septicaemic frogs on the first farm visited, four septicaemic frogs on the second, one septicaemic frog on the third, one septicaemic frog on the fourth and one septicaemic frog on the fifth farm visited. It has been isolated from one skin sample only and from no intestinal samples. In contrast although *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.

A. sobria was isolated from the skin and intestine and *A. hydrophila* was isolated from the skin of septicaemic tadpoles. Three isolates of *A. sobria* were obtained from the internal organs of two septicaemic tadpoles. These isolates may have been the result of sampling artefacts as the small size of the tadpoles made aseptic technique difficult.

A yellow pigmented bacterium (YPB) was isolated consistently from the skin, and occasionally from the intestine of diseased and clinically normal frogs. This bacterium was frequently isolated in pure culture 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 and subsequently become septicaemic. The organism was similar to a *Flavobacterium* sp. but test results did not match all the characteristics of this genus.

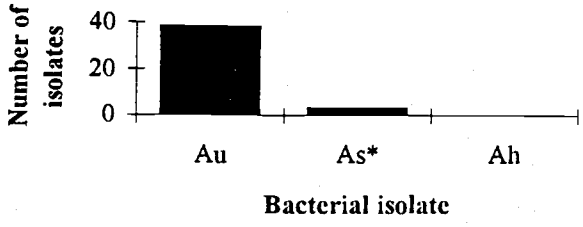
These findings are summarised in Table 1 and Figures 1-3.

Table 1. Number of bacterial isolates from internal and external organs

	Unidentified Aeromonad	*Aeromonas sobria*	*Aeromonas hydrophila*
Liver	9	1*	
Heart	7		
Spleen	9		
Kidney	8	1*	
Muscle	3	1*	
Pericardial fluid	1		
Peritoneal fluid	1		
Total internal isolates	**38**	**3**	**0**
Intestine		10	3
Skin	1	12	3
Ulcer	1		
Total external isolates	**2**	**22**	**6**

* isolated from tadpoles

Figure 1. Total number of isolates from internal organs



*(Tadpoles only.)

Figure 2. Total number of isolates from skin

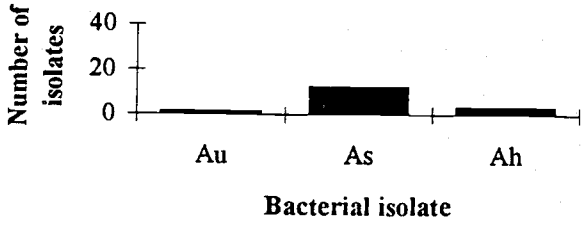
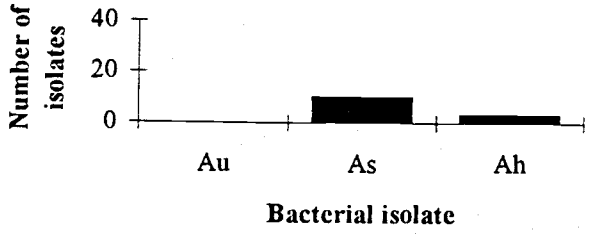


Figure 3. Total number of isolates from intestine



Au = unidentified Aeromonad

As = *Aeromonas sobria*

Ah = *Aeromonas hydrophila*

4.1.2 Bacterial Challenge

Yellow pigmented bacteria

A small challenge experiment using the YPB was set at AAHRI. Three frogs were injected intramuscularly with an isolate from the liver of a septicaemic frog. The challenged frogs showed no signs of ill health. Experimental frogs were kept under optimal conditions at very low stocking densities. It is probable that the YPB is pathogenic only to stressed frogs in poor environmental conditions.

Unspeciated aeromonad

A small scale study to assess the pathogenicity of this bacterium was arranged in collaboration with AAHRI staff. Three groups of three frogs were placed in separate aquaria under optimal conditions. Three suspensions of the challenge organism were prepared with the optical densities of 1, 0.5 and 0.1 at 610 nm. Each group of frogs received intraperitoneal injections of 0.2 ml of one of the suspensions. Within 24 hours the frogs which had been treated with the highest dose were all dead. Two frogs dead in the group receiving the 0.5 suspension, the remaining frog was moribund with clinical signs of bacterial septicaemia i.e. petechial haemorrhages in the thigh muscles and internal organs. One frog died in the group which received the lowest dose, the two remaining were moribund and displayed clinical signs of septicaemia. Bacteriological sampling yielded almost pure cultures from the liver, spleen and kidney of all the frogs in the high and middle dose groups and two frogs in the low dose group. Identification with the API 20 E system confirmed these as the unspeciated pathogen. Some other bacteria were recovered and these were mainly enterobacteriaceae. The dead frogs when sampled showed considerable *post mortem* changes and under these conditions it is to be expected that there would be outgrowth of intestinal bacteria. The dominance of the challenge isolate in the bacteria recovered was significant.

4.1.3 Virulence and DNA analysis of the unspeciated pathogen

In vivo virulence studies

Two methods of infecting frogs have been explored. Firstly by intraperitoneal injection (i.p.) and secondly by bath exposure. Both were successful in achieving a dose related incidence of infection. Both have advantages: i.p. challenge is very effective in establishing infection and is highly reproducible. Bath challenge reflects natural exposure very much more closely and is better suited to evaluation of treatments and in particular methods to enhance the innate defences or vaccination. The latter method has been used in the present studies where possible.

Groups of frogs were challenged by bath exposure to *A. hydrophila*, *A. sobria* or the unspeciated virulent pathogen (AU). A control group remained unchallenged. The concentration of *A.hydrophila* and *A. sobria* was 100-fold greater than that of AU. AU killed 50% of the frogs while all the frogs in the other groups survived.

Aeromonad speciation

Motile aeromonads have been classified by different systems into as many as 13 groups. The most highly discriminating method, albeit the slowest, is by determining the nucleotide sequences in the bacterial DNA. The arbitrarily primed polymerase chain reaction or randomly amplified polymorphic DNA (RAPD) analysis, has shown promise in providing a more limited but still useful degree of DNA fingerprinting. Using RAPD analysis selected fragments of the DNA are amplified allowing differences between like isolates to be detected. It has great potential as a precursor to more detailed nucleotide sequencing.

Some work on RAPD analysis has been done in collaboration with Aoki's group in Tokyo. This was developed further in an MSc project (D. Colquhoun). Working with M. Pearson he was able to show amplification of identical DNA fragments from several AU isolates. All of these had similar phenotypic expression but had come from different sources in Thailand. The RAPD profiles were the same as each other but distinct from those of the other aeromonads tested.

These findings have been published by Inglis, Colquhoun, Pearson, Miyata and Aoki, 1996 (see Appendix 1).

More recent work by M. Pearson with additional support from the Japanese Society for the Promotion of Science has been carried out to sequence the nucleotides in the highly conserved 16s ribosomal DNA of the unspeciated pathogen and a collection of other *Aeromonas* spp.. It has been found to differ from all type species tested so far. One easy marker which is useful in screening is the inability of this pathogen to ferment sucrose; this allows differentiation from *A. hydrophila* which was found, otherwise, to be phylogenetically closely related.
The unspeciated pathogen produces significantly more haemolysin than either *A. hydrophila* and *A. sobria*, two common fish pathogens, and this is very active against frog, toad and trout red blood cells. Genetic analysis has identified the haemolysin gene as different from the two commonly found in motile aeromonads. It is the same as has been found in *A. salmonicida* but in only one other motile aeromonad isolate and one which had been held in a laboratory collection for many years and until now was thought to have arisen from inadvertent gene transfer within the laboratory. Further studies are being carried out with different funding and are as yet incomplete. However it is clear that the pathogen of interest can be screened for easily by a biochemical test (sucrose negative), confirmed by 16s rDNA sequencing and that a DNA probe for rapid identification could be developed directed at the haemolysin gene.

The haemolysin gene already has been cloned into *E. coli* and its role in virulence currently is under investigation. Pathogenicity for fish also is being explored. A small survey is underway to determine how widespread this organism is in frog farms in Thailand but these findings will not be available within the duration of this project (ends 31 March 1997). Details of biochemical characterisation and DNA analysis of the organism are given in Appendix 2.

4.1.4 Discussion and conclusions

Frogs are very vulnerable to bacterial infection because of their highly permeable skin and nature of their amphibian habitat. In farming conditions in tropical and sub-tropical countries, shallow ponds of static water provide ideal conditions to support a heavy bacterial load. Motile aeromonads have been widely incriminated in the literature as the causative agents of

septicaemic disease in frogs and it was found in this survey of Thai farms that they do occur widely on frogs and in their environment. However the taxonomy of this group is very complex and it is recognised that the umbrella term *Aeromonas hydrophila*-complex is often used to describe only approximately the bacteria concerned. The findings of this survey indicate considerable variation in virulence to frogs between isolates and in particular has revealed one phenotype from sites far apart in Thailand, which was consistently associated with disease and which is distinct from *A. hydrophila* and *A. sobria* and. Moreover this pathogen was not found in the environment or commensally on frogs, but only in the internal organs of diseased animals. It can be identified easily in screening and confirmation tests. It is unique and therefore a good candidate for development of both a vaccine and a rapid diagnostic probe. Before further work is expended in either of these directions it is essential that a substantial survey be conducted among the frog farms of Thailand and elsewhere to determine the prevalence of this agent is and also to elucidate whether it is a pathogen of frogs only or also of fish farmed in the same environment.

The pathogenic status of the yellow pigmented bacterium which had been recovered frequently from the internal organs as well as from the skin and intestine also was considered. Disease and intracellular spread of a range of bacteria occurred after frogs were stressed: however in an experimental challenge the YPB did not cause disease under conditions where the unspeciated aeromonad rapidly caused deaths. This was the first observation in this project of the high susceptibility of frogs to fatal infections by bacteria of low virulence when the animals were stressed.

4.1.5 Histology of *Rana rugulosa*

Tissue samples were taken from heart, spleen, kidney, lungs, liver, skin, and small intestine. Samples were fixed in 10% neutral buffered formalin, processed by standard methods and sectioned at 5μm. Sections were stained with haemotoxylin and eosin (H&E).

Samples from clinically normal frogs.

Examination of clinically healthy animals revealed signs of gross pathology internally in many individuals. Many had bronzing and yellowing of the liver implying a lot of fatty deposition and a nutritionally imbalanced diet, others showed signs of parasitic infestation indicated by the presence of fibrotic tracts and white raised plaques on the surface of the liver and other abdominal viscera.

On histological examination the frog tissues were found to have features found both in fish and mammalian tissues.

1. Liver

The histological structure of the amphibian liver is similar to that of the teleost fish:

1.1 Hepatocytes are not organised into well defined lobules.
1.2 Cytoplasmic appearance can be variable depending on nutritional status of individuals.
1.3 Haemopoeitic tissue complete with melanomacrophage centres, is found in varying amounts around the blood vessels.

2. Kidney

The histological structure of the amphibian kidney appears to more closely resemble that of the mammal than the teleost:

2.1 In adult frogs the paired kidneys are compact, lobulated organs which unlike the teleost kidney are relatively mobile within the abdominal cavity.
2.2 Like the mammalian kidney, the frog kidney can apparently be divided into an outer cortex and an inner medulla. In mammals the medulla contains part of the renal tubules (loops of Henle) and the cortex contains the glomerulus and proximal and distal tubules. In frogs the medulla contains the glomerulus and the distal tubules while the cortex apparently contains the proximal tubules.

3. Spleen

The frog spleen has features of both the mammalian and the teleost spleen.

3.1 As in mammals, the frog spleen has a dense fibro-elastic outer capsule giving rise to supporting connective tissue trabeculae which conduct larger blood vessels throughout the pulp.

3.2 In the mammalian and amphibian spleen the lymphocyte-rich white pulp is clearly distinguishable from the erythrocyte-rich red pulp.

3.3 In both the teleost and amphibian spleen melanomacrophage centres occur, and are usually located close to vessels.

4. Skeletal muscle

The histological structure of skeletal muscle in frogs, mammals and fish does not differ and consists of long, multinucleated cells, called muscle fibres arranged in parallel and bound together by connective tissue into bundles which together form the whole muscle. In these connective tissue sheaths numerous blood capillaries and nerve fibres occur. Regular cross-striations are observed in the longitudinal skeletal muscle fibres due to the arrangement of contractile proteins within the muscle fibres.

5. Heart

The frog heart differs in structure from both fish (frogs have paired atria, fish have only one) and mammals (frogs have a single ventricle, mammals have two).

5.1 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.

5.2 The ventricle wall of the frog is composed entirely of compact cardiac muscle but on its inner surface has numerous trabeculae which probably help to keep separate streams of blood returning from the pulmonary circuit and the body.

5.3 In mammals, teleosts and amphibia the walls of all parts of the heart consist of three basic layers: an internal membrane, the endocardium; an intermediate layer, the myocardium; and an external membrane, the epicardium. The histological structure of which is similar in all three.

6. Lungs

6.1 The adult frog possesses simple paired saccular lungs opening directly into the pharynx.

6.2 Unlike mammals, the frog has no trachea or bronchial tree and hence no cartilage is found in the respiratory tract.

6.3 The vascularised epithelial lining of the lungs is thrown into folds (septae). The septae separate infundibula which are lined with thin squamous respiratory epithelium and thus form blind ended alveoli.

7. Small intestine

7.1 The principal site of digestion in the adult frog is the small intestine.

7.2 The surface area of the small intestine is increased by the formation of villi in the mucosa.

7.3 The intestinal villi are covered by a simple columnar epithelium which is composed mainly of absorptive enterocytes and occasional mucus producing goblet cells

7.4 A connective tissue layer, the lamina propria, extends into the core of each villus. and contains a rich network of blood vessels.

8. Skin

8. 1 The amphibian skin, as in mammals and fish, consists of the epidermis and the dermis.

8.2 The epidermis consists of a stratified squamous epithelium divided into three layers:

8.2.1 The stratum corneum, on the outside, composed of a single layer of flattened and keratinised cells.

8.2.2 The stratum intermedium, lying directly beneath the stratum corneum and made up of various layers of polyhedral cells.

8.2.3 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 laminar.

8.3 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.

8.4 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.

Incidental findings from clinically normal animals.

Although animals selected for the normal samples showed no external signs of disease, on histological examination, many of the tissues revealed evidence of pathological change. Many of the livers showed evidence of mild to severe inflammatory infiltrates. Some livers showed evidence of parasitic migration with fibrosis around the ducts. Pathology was less obvious in spleen sections although small granulomatous reactions were observed in some animals. Many kidneys showed mild to severe pathology. Inflammatory foci were frequently observed often around glomeruli. The inflammatory cells were predominantly macrophages although one or two eosinophilic granular cells were consistently present. Inflammatory infiltrates of varying severity were also frequently observed following lobular divisions of kidneys, tracking down the connective tissue pathways which transport blood vessels from the periphery of the organ. Many kidney tubules contained deposits which caused distortion and enlargement of the tubular lumens.

This pathological evidence suggests a low grade inflammation in many of the frog organs. The most common cause of such reactions in cultured fish is bacterial infection, however no bacteria were isolated from the organs of clinically healthy frogs using standard bacterial isolation techniques. This supports findings by M. Crumlish that bacteria may be present in healthy animals but an enrichment step is necessary to isolate them. Also consistent with the findings of M Crumlish, most of the inflammatory cells observed were macrophages suggesting that these cells are constantly activated and fighting infection.

Samples from diseased animals

Tissue samples from diseased animals showed a wide range of pathological changes. Many were similar to those seen in the clinically normal animals with varying degrees of inflammatory cell infiltration, of most organs. However the changes observed in animals suffering from septicaemia caused by the unspeciated aeromonad all showed similar and quite distinct pathological changes. The changes were severe and acute. The livers in particular showed severe hyperaemia, with pooling of blood in the sinusoids and also haemorrhages between the hepatic cords. Many necrotic erythrocytes and inflammatory cells were observed in blood vessels and sinusoids. There was severe mulitfocal hepatitis. Hepatocyte cytoplasm often appeared granular and unhealthy and frequently contained bile pigments indicating bile stasis. Many replete macrophages were observed. Haemorrhages and areas of liquefactive

necrosis were observed throughout the parenchyma. Frequent single cell necrosis was also observed among the hepatocytes. In kidneys from Au affected frogs hyperaemia was again a very prominent feature particularly in the glomeruli. Pigment deposits were observed in the glomeruli which were not observed in clinically normal frogs. Large deposits of eosinophilic material were observed in some tubules. Necrosis of glomeruli and tubules was a frequently finding with swelling of tubular epithelial cells and sloughing into the lumen. In the spleens of Au affected animals there was massive destruction of the haemopoeitic tissue producing a lot cellular debris. The red pulp appeared to be worse affected that the white. Many replete macrophages were observed in the pulp loaded with pigment which had a shiny gold/pink appearance. The hearts and muscle of affected animals showed similar degrees of pathology with myonecrosis and inflammatory infiltrates.

The histological picture of Au septicaemias is therefore typical of a motile aeromonad septiaemia. There is a generalised necrotising septicaemia with focal lesions. Capillary beds are generally hyperaemic, haemorrhagic and contain numerous inflammatory cells. Focal areas of acute liquefactive necrosis are present in many organs and are particularly apparent in the spleen and kidney and liver.

4.3.0 Establishment of a Tropical Vivarium

A tropical vivarium was designed and set-up at the IOA Stirling to house populations of the farmed frogs *Rana rugulosa.* There is very little published information on the long-term maintenance of these animals under artificial conditions therefore initial unforeseen problems arose. Perseverance with essential factors such as adequate water quality, correct dietary adjustment, regulation of constant water temperature, monitoring health and treating disease outbreaks produced a working vivarium.

In this project the animals had to be kept for a protracted period and it was essential for the project that they were in good health. The following criteria of condition had to be met by animals held at IOA. Frogs should have an intact moist skin, clear bright eyes, no external abrasions to the skin, no ulcers and they should continue to grow and exhibit a positive reaction to stimuli. Diseased animals often appear listless, with dull eyes, do not feed and can be caught easily; however it must be noted that it these symptoms are not always readily apparent. Death may occur without any obvious signs.

4.3.1 Stock

South East Asian bullfrogs *Rana rugulosa* were kindly supplied by AAHRI and were transported either by courier or as freight. They were not fed 24 hours prior to transport and were kept in heated holding facilities throughout the journey. The animals usually weighed 10 grammes or less and were packaged in a polystyrene container with some pond weed.

Upon arrival the frogs were placed into propylene or glass reinforced plastic tanks where the water temperature was initially 20^0 C and was raised to an optimal water temperature of 28^0 C over a 24 hour period.

Dead or moribund frogs were immediately removed from the healthy survivors and the animals were closely monitored for an acclimation period of 10-14 days; after this the frogs would begin feeding and stop producing large quantities of mucus. Once settled the animals were observed daily and dead, moribund or diseased frogs were removed and either sampled for bacteria or treated and kept in isolation until recovery. Any animal that did not come on to feed also was put into isolation to encourage eating by minimising competition from the other animals in the tank. If this was unsuccessful and the frog remained thin and did not respond to food it was humanely disposed of following Home Office guidelines.

4.3.2 General Vivarium Maintenance

As these are semi-aquatic animals they spend periods time in and out of the water: therefore water was deep enough to let the frogs swim comfortably but in addition dry areas were provided as environmental enrichment and as hides. The frogs were easily stressed by excessive movement and the hides were used a lot during the aclimation period.

The frog house was kept at the near-optimal temperature (28 °C) and maintained on a 12 hour light: 12 hour dark cycle throughout the holding period.

Water Quality

Information on optimum water quality for maintaining ranid frogs under artificial conditions for long periods of time was scarce. This was a major concern as these animals spent more than 50% of their daily cycle in and surrounded by water. Frogs can suffer from poor water quality diseases: growth deformaties arise from low water pH values and high ammonia levels are toxic. Approximately 70% water changes were carried out daily using water pre-heated to 28° C.

The water quality in each tank was also measured once a week using a Dry-Tab test kit. This included pH, nitrite (ppm), nitrate (ppm) and ammonia (ppm) and results are in Table 2.
Biological filters were tried in an attempt to improve the quality of the aquatic environment. They were established using frog bacteria to produce a micro-organism culture to utilise the unwanted excretory products and reduce the toxic ammonia levels. However there was no change in the measured parameters with or without the biological filters and their use was discontinued as the daily cleaning they needed was causing unnecessary stress to the animals.

Temperature

A constant temperature was essential in helping the animals to settle and reduce stress-associated diseases. Initially it was difficult to maintain an adequate water temperature for the animals using only the normal 150-300 watt water heaters, placed inside the tank. The temperature fluctuated using these heaters and the animals would rest on top of them, causing this delicate skin to burn. This problem was solved by putting heat pads under the tanks.
They generated a reliable constant water temperature. Daily records of tank environment were made and typical data are presented in Table 2.

4.3.3 Nutrition

In most research laboratories, frogs are not held for long periods and therefore sometimes are not fed during the holding period. In this project a correct diet to encourage growth, reduce nutritional malformaties and establish a working population was a prime requirement.
There is, as yet, no scientific understanding of the optimum nutrition for this species and this basic work remains to be done. *R. rugulosa* were fed in Thailand on a commercial pelleted diet from post-metamorphosis onwards. This is a general purpose feed used for many aquatic species, including frogs on farms, where good growth rates have been achieved.

The animals housed IOA did not respond well to this or a similar fish pellet. This may have be due to either the transportation stress, unpalatability or the need for live food. Transportation can be a severe form of stress in sensitive animals and it can sometimes take a long recovery period before they can adapt and begin to eat. Although this may have been true for some of the population, others did appear to be interested in feeding as was demonstrated by attempted cannibalism of other frogs.
Amphibians are motivated to eat by movement and so either the original diet was sub-optimal or else the animals were not receiving the sufficient stimuli to motivate feeding. Several different food sources were tried. These included live threadworms, earthworms and maggots, calf liver and commercial fish pellets and floating fish pellets. The most successful was the floating carp pellets as the live food source did not remain on the food tray long enough to stimulate interest and ended up in the water. However it was interesting to note that throwing the pellets onto a polystyrene food tray did encourage the animals to respond and even floating on the surface of the water appeared to provoke the interest of the frogs to eat the pellets. Latterly crickets, bred from some escapees in the vivarium, were offered. These were well accepted and used as a supplement when available.

The animals were fed once per day *ad libitum* and any waste food was removed during the daily water changes. On this diet the animals grew well and ulcers which had occurred on the legs, eyes and nostrils disappeared.

4.3.4 Mortalities

Mortalities were due to many different factors including stress, dehydration, cannibalism, not feeding, ulcers, rectal prolapse and miscellaneous causes, where there were no obvious signs of disease and which yielded no bacteria upon sampling. Typical data are presented in Table 3. Upon arrival at Stirling there was an immediate loss of approximately 10% of the population from every batch sent from AAHRI. This was attributed to transportation stress. Where post-mortem changes were not too severe, the frog was sampled for bacteria in the organs using standard bacteriological methods and the colonies were identified using primary tests. A range of bacteria including *Pseudomonas* sp., *Enterobacteria* spp., *Aeromonas* spp., *Aeromonas sobria* and *Aeromonas hydrophila* were identified from these frogs.

At the beginning of the project an episode occurred where elevated water temperature caused a bacterial outbreak which spread rapidly throughout the animals housed in one tank.
The frogs also suffered from dehydration, where the skin became brittle and dry and they became too weak to enter into the water. This was attributed to overactive air currents. Therefore correct temperatures were extremely important to reduce bacterial infections and ensure that the animals were able to maintain homeostasis.

In the first two groups of *R. rugulosa* shipped to Stirling, rectal prolapses were observed and successfully treated, however survival rate after re-insertion was poor. There are many different possible explanations for this condition including poor nutrition, parasitic infections and poor water quality. The incidence of this condition was greatly reduced when the water temperature remained constant and the diet improved.

Ulcers of the eyes, mouth and nostril areas were often observed particularly at the beginning of this project. It was not clear why the frogs were getting ulcers and only at these specific areas but it may have been due to damage to sensitive parts of the body when the animals became stressed. This could have occurred during packaging and transportation, during handling procedures when the animals were graded or by knocking against the walls of the tanks. Several treatments were tried without success, however the ulcers disappeared when the frogs were put on the floating carp diet.

Frogs are stimulated to feed by movement and will often try to eat another animal's leg or hand and it is not uncommon to observe a bloated, fat frog which upon dissection may be found to contain two or three smaller animals inside the stomach. To reduce this, grading was done every 4-6 weeks.

Table 2 *Water Quality and Temperature Values For Rana rugulosa*

Tank Number	Number of Frogs	Average weight/tank	pH	Ammonia (ppm)	Nitrite (ppm)	Nitrate (ppm)	Average weekly Water Temperature
1	5	47.8g	7.0	1	0	0	28.8 °C
2	4	37.9g	6.8	1	0	0	29.2 °C
3	3	82.2g	6.8	1	0	0	28.5 °C
4	5	30.9g	6.8	1	0	0	28.2 °C
5	6	14.8g	7.0	1	0	0	29.2 °C

The above values were taken over a 4 week period.

Table 3 *Mortality Rates and Causes for a Population of Rana rugulosa*

Cause of Mortality	Number of Animals over a 4 week period
Dehydration (Transportation Stress)	13
Bacterial Infection*	1
Miscellaneous	1
Ulcerated	2
Regurgitated (attempted cannibalism)	4
Emaciated	4

* = the animal had reddening of the high and abdomen area but bacteriology results produced mixed colony growth.

4.4.0 Innate defence system of *Rana rugulosa*

4.4.1 Components and assays of function

All vertebrate species have a defence system which protects the animal against disease. The more advanced vertebrates, mammals, frogs and fish have evolved independent innate and adaptive immune systems comprising both cellular and humoral components. The non-specific immune system is the primary defence pathway initially engaged in an infectious attack: activation is important therefore in inhibiting pathogenic invasion and establishment of disease. Amphibians , in particular *Xenopus laevis* (the African clawed toad) have proved to be good laboratory models; however most immunological studies with this species have focused on the adaptive immune response, on isolating antibodies and investigating immunological tolerance.

At the start of this project no published information was available on the haematology or immunology of ranids, in particular of tropical farmed ranids. It is generally accepted that such data is useful in defining the health status of animals and so early work was directed at describing the parameters of the blood and innate immune system of the tropical farmed frog *Rana rugulosa* to establish a profile describing a normal healthy population.

A series of assays was adapted from fish and applied to frogs, imported to IOA from AAHRI. These assays then were applied to frogs bred, reared and housed at AAHRI. The Thai frogs were provided as normal; they were in their normal climatic conditions, had not been subject to transportation stress, had no reported disease history and showed no internal or external pathology on visual examination.

Particular attention was concentrated on the functions of phagocytic cells; the tissue dwelling macrophages and the neutrophils, which are usually located in the blood stream. They have a bactericidal function which is very important in primary defence. They are able to recognise, ingest and kill bacteria . Killing is triggered by bactericidal enzyme present in the cells which increases oxygen uptake and activates the membrane bound enzyme nicotinamide adenosine dihydrogen phosphatase (NADP) oxidase to change molecular oxygen to superoxide anions

which are particularly toxic to bacteria. This complex pathway, called the respiratory burst, has been identified in mammals and fish but has not as yet been reported in ranids.

Assays were developed to measure the following parameters:-

- haematocrit values (% packed cell volume)
- total white blood cell counts
- differential white blood cell counts
- serum lysozyme levels
- recovery rate of phagocytes from the spleen
- super-oxide anion concentrations in phagocytes from spleen and blood
- ingestion and killing by spleen phagocytes of challenge bacteria

Protocols for all these procedures applicable to both frogs and fish were gathered in a manual (Appendix 3) which was used in the work shop associated with this project and remains as a resource for more general use in health training programmes in aquaculture.

Fuller details of this work, including methods and results are given in Appendix 4 and summary information was presented in a poster at the World Aquaculture Conference held in January 1996. An outline is given here.

First line defence functions found in other animals, from mammals to fish have been shown to be present in tropical farmed frogs.

The most striking feature of the data generated is the wide variation between individuals (see Table 4). Lysozyme was demonstrated but concentrations were variable and low. Phagocytic cells were found to be present in the blood and internal organs. They were morphologically similar to fish and mammalian phagocytes and, like them able to adhere to glass and plastic surfaces thus allowing separation from other cells and investigation of function. They were able to ingest bacteria and to generate reactive oxygen radicals. Macrophages from fish and animals are able to produce a bactericidal respiratory burst intracellularly and extracellularly. In frogs the former was seen but it has not yet been possible to demonstrate an extracellular respiratory burst in frog macrophages using an assay which had been confirmed in catfish.

Table 4. Non-specific immune parameters in *Rana rugulosa.*

Technique	Number of animals	Average	Standard deviation
Haematocrit (PVC %)	14	25.5	2.9
Intracellular ROS*	23	1.44 mg ml^{-1}	0.59
Total white blood cell count	11	9.6×10^4 ml^{-1}	4.6
No. phagoctyes per speen	15	1.0×10^7 ml^{-1}	0.9

*This is the amount of reactive oxygen species (ROS) which reduced nitroblue tetrazolium (NBT) to formazan per ml of blood.

Assays to measure the final stage in the process of eliminating bacteria, that of actual killing, have been established in catfish cells but it has not yet been possible to demonstrate bactericidal activity in frog cells. This is because it has not been possible to establish cell cultures free of an intrinsic bacterial population (see below). A method of introducing test bacteria which can be distinguished from the carried population now has been developed and by this means it should be possible to investigate the bactericidal capability of cells which are already infected.

4.5.0 Immunostimulation of Frogs

Reports from frog farms tell of heavy losses due to sudden outbreaks of bacterial septicaemia and of the failure of antibacterial chemotherapy in control attempts. Early work in the present project has made it clear that frogs succumb readily to stress-associated bacterial disease. The use of immunostimulation may offer hope in prophylaxis at times of stress and contribute to a reduction in the use of antibiotics where this is ineffective, wasteful and environmentally costly.

Immnuostimulation of the innate defences has been attempted with many cultured species and a range of synthetic and naturally derived chemical stimulators have been found to augment the bactericidal activities of cellular and humoral components of the system. Complex polysaccharides derived from the cell walls of yeasts and fungi have proved particularly effective in fish. No studies on non-specific stimulation of frogs have been published and this aspects was considered to merit investigation.

In initial studies on immunostimulants it is general practice to administer the substance by intraperitoneal injection. This route could not be adopted for field use and although application by immersion is a possibility, the preferred route is peroral, included with the feed. All three methods were attempted. In preliminary i.p.trials, viable yeast cells were observed inside spleen macrophages. This has not been reported previously in studies using i.p. administration of a yeast product but is important in trying to understand mode of action since infection rather than inanimate stimulation cannot be eliminated as a cause of the heightened activity of the innate responses if this happens.

Further treatments in the present project were carried out either using β-glucans which had been autoclaved to kill living cells or by delivering the substance orally by capsule, thus introducing a measured dose directly to the stomach.

4.5.1 Experimental work to investigate functional enhancement of innate defence parameters

In a preliminary study a 1% of a β-glucan (Vetregard, supplied Vetrepharm) was administered to 3 frogs and saline to 3 control frogs. An enhanced respiratory burst, measured by the NBT assay, was seen in the glucan treated frogs after 3 days but this had declined to the control level after 7 days. This indicated that the process was possible and a study was carried out with *Rana rugulosa* to investigate the efficacy of a soluble immunostimulant, laminarin, and an insoluable β-glucan (Macrogard) applied by four different routes. The parameters measured were, packed red cell volume, total white cell count and bactericidal superoxide anion production by macrophages.

Laminarin was applied as a bath at 5 mg l^{-1} for 2 hours. The β-glucan was administered to one group of frogs as a single dose of a 1 % (w/v) by i.p. injection. In another group the same dose was introduced directly into the stomach in a gelatin capsule. To another group it was given surface-coated on the feed pellets at 1 % (w/v) for 2 weeks with a feeding rate of 3 % of body weight per day.

Test and control animals were sacrificed at intervals , the red and white cell counts performed and production of superoxide anion production by separated macrophages measured by the NBT assay. Serum was taken for serum complement analysis and this has yet to be done.

Full details of methods and results are given in Appendix 5a. In summary neither the haematology results nor the macrophage respiratory burst indicated any significant differences between the control and treated groups. There was a lot of individual variation between animals in the same groups but the range of values was similar to data obtained from a population of farmed frogs held under similar conditions and sampled previously (see Appendix 4).

4.6.0 Effects of Stressors on the Innate Immune System of Tropical Farmed Ranids

In aquaculture systems animals are susceptible to many stressors which may leave them more susceptible to bacterial pathogens. It has been shown that one effect of stress on fish is to cause an increase in the level of corticosteroid hormone released into the blood stream. This

has an immediate physiological effect on the animal which ultimately leads to an immunosuppressive state. The stressors faced by farmed aquatic species include overcrowding, poor water quality, temperature fluctuation and inadequate nutrition. Many of these can be reduced by improved animal husbandry but some, such as handling, grading and transportation cannot be avoided.

It has been observed repeatedly by workers in the field that frogs are very susceptible to stress-related outbreaks of disease and so work was conducted to assess the effect of low temperature and poor water quality on selected parameters of the non-specific immune system. Details of the study, methods and results are given in Appendix 6. Frogs either were transferred to a low temperature holding facility (9 °C below ambient) and sampled at 1, 2, 3 and 10 days or else held in tanks without the usual daily water change and sampled at for 2 and 7 days. The capacity of isolated macrophages to produce bactericidal superoxide anion was measured by the NBT assay.

As had been a feature of earlier work the results were low and widely scattered. There was no observable depression of cellular function by cells from the frogs subjected to low temperature or water quality stress: there was an indication that cells from the low temperature group performed better by day 10 and also that the poor water quality group may have performed better.

These findings are unsatisfactory and do not correlate with reports of disease problems following temperature drops and environmental deterioration. It must be re-emphasised that the assays applied are robust and the operators well experienced. Depressed and variable macrophage activity must be explained by other than laboratory technique.

One explanation for the scatter of results on macrophage activation may be that stress is multi-factorial and the negative effect of reduced temperature may be out-weighed by reduced disturbance by locating the tanks in an isolated room. Another explanation may rest in the effects of bacteria carried in the macrophages which have been observed so frequently during this project but at this stage had not been investigated systematically.

During the course of these studies bacteria have often been observed associated with macrophages. They are not easy to see in unstained cells and the finding was unexpected since

the internal organs of healthy animals are usually considered to be free of bacteria. However even after a great deal of very careful work to ensure that this had not arisen from contamination during processing of the cell preparations, the same finding was made repeatedly. It became the cause of major concern.

The implications of these observations with regard to health of the animal are significant. Cells under a continuous bacterial challenge even by organisms of low virulence may be less able to cope with a serious pathogen or even with non-specific stress.

Different degrees of bacterial engagement by these cells may also be a contributor to the wide range of measurements recorded for the non-specific immune functions. It certainly made it impossible to assay, by the conventional means, the final stage in the process of eliminating bacteria, that of actual killing. Effort therefore was re-directed to investigate the frequency and extent of bacterial challenge of macrophages in apparently healthy tropical farmed frogs.

4.7.0 Micro-organisms in Phagocytes

A fundamental step in diagnosing bacterial infection in fish and frogs is to attempt to isolate the causative agent from the internal organs, often the kidney, of animals displaying symptoms of disease. It is unusual to recover bacteria by this means from healthy animals. Some pathogens, viruses, rickettsiae and some bacteria, are able to survive and even multiply inside macrophage cells but most micro-organisms are killed. Only when they grow to overwhelming numbers are they able to kill the cells and establish systemic infection. A stress test used to detect fish which are symptomless carriers of infections is to depress the immune system by means of an injection of corticosteroid thus allowing any small numbers of bacteria which are present but being held in-check by the first-line defences to grow out. Isolation from the kidney then is easy.

It is common practice in preparing primary macrophage cultures to use media and buffers containing a mixture of penicillin and streptomycin to counteract any bacteria which may be introduced inadvertently to the nutrient rich cell culture system. The bactericidal ability of the

cells, the incorporation of antibiotics and the difficulty of seeing unstained bacteria in living cell preparations all help make it easy to miss bacteria present in isolated macrophages.

Early in this project a high rate of bacterial colonisation of cell cultures was noted. This was a regular finding and could not be ascribed to poor technique. This therefore was investigated systematically to determine the proportion of clinically healthy frogs from which bacteria could be recovered, ease of recovery, the extent of involvement of macrophage cells and the range of bacteria involved. The underlying hypothesis was that continued bacterial challenge of macrophages predisposed animals to stress-related disease such as has been widely reported from the farms and has dogged the transport-stressed frogs used at Stirling in this project.

A scheme was developed to test all groups of frogs as they were encountered in the project and to test the AAHRI population systematically over a season. The majority of non-specific immune function tests in this project had been carried out on the spleen. This organ provided a high yield of macrophages and can be accessed easily with little likelihood of bacterial contamination from the intestine on sampling. It was chosen therefore for routine testing for the presence of bacteria.

In a preliminary study the efficacy of conventional bacteriological sampling of the spleen was compared with spreading a spleen homogenate on solid medium before and after a resuscitation step. It can be seen from Table 5 that best recovery was achieved after culture of the spleen suspension in a nutrient broth (See also Appendix 7).

Table 5 Bacterial recovery from frog spleen with and without a resuscitation step

Method	A1	A2	A3	A4	A5
Conventional loop	no growth	no growth	no growth	no growth	no growth
Piece of spleen streaked onto TSA	no growth	no growth	no growth	8 CFU	1 CFU
Spleen suspension after TSB resuscitation step	no growth	no growth	growth	growth	growth
Spleen suspension directly onto TSA	no growth	no growth	no growth	no growth	no growth

A resuscitation step of macerated spleen or separated macrophages was used in all subsequent tests. Stringent sterility checks were conducted at all stages of these preliminary tests and

subsequently to control against bacteria being introduced during processing. In addition when macrophages isolated for other assays were smeared on to slides, stained and examined, bacteria were observed inside and around the macrophages.

4.7.1 Bacteriological sampling of AAHRI stock frogs over a season

A study was carried out to determine the incidence and magnitude of bacterial carriage by frog spleen macrophages. For a 10 week period at AAHRI, three frogs were sampled weekly. Spleens were removed aseptically, macerated and macrophages separated from the suspension by centrifugation on a differential Percoll gradient. Viable cells were allowed to adhere to glass cover slips, stained with a Romanowsky stain and examined X1000 for bacteria. Aliquots of the suspension were incubated in tryptose soya broth at 22 °C and 28 °C, then plated out on tryptose soya agar and incubated at both these temperatures. Bacteria were seen in all cells examined. They could not always be cultured but the recovery rate was improved when two culture temperatures were used (80% success). Intracellular bacteria were seen in all macrophage preparations and in 69% of samples more than 20% of cells contained bacteria. A wide range of facultative anaerobes were recovered.

These results are being prepared as a paper for presentation at the conference of the European Association of Fish Pathologists to take place in Edinburgh in September 1997. The abstract is contained in Appendix 7.

Three wild frogs were trapped in Thailand and tested similarly; bacteria were recovered from two of the three. Two other wild species were investigated. 20 *Rana pipiens* and 5 *Rana temporaria* were trapped, outside the breeding season, in the Winsconsin area of the US and South West England respectively. Macerated spleen tissue and separated macrophages were examined for bacterial growth after a TSB resuscitation step and for the presence of bacteria after staining the cells. Viable bacteria were recovered from 4 of the 20 *R. pipiens* although no bacteria were seen in stained macrophages. No bacteria were seen and none were recovered from the *R. temporaria*.

Discussion

It was found that there was a heavy bacterial load in the spleens of *Rana rugulosa* held in good environmental conditions in experimental wet laboratories. This challenge was controlled *in vivo* and the animals did not display any symptoms of disease. The wild Thai frogs were two few to provide reliable data but the results from the *Rana pipiens* indicated on-going challenge in some of the population. This was not so in *Rana temporaria* although the number tested also was small.

Frogs are constantly exposed to a high concentration of environmental bacteria in their amphibian habitat. Production of a bactericidal mucus by the skin of some frogs has been reported but there is no published information on this in relation to these farmed frogs nor about the susceptibility of different bacterial species.

Feral species are known to in decline and farmed species prone to sudden severe epizootics with high mortality rates. The non-specific immune systems of these animals may be more continuously engaged and the animals less able to deal with additional stress than other animals. In particular they may be highly susceptible to a high bacteria load in their environment. A small study, arising from this project, is currently underway to investigate the status of phagocytic cells from other cultured aquatic species.

Routine sampling for bacteria using conventional methods may nor reveal the presence of potential pathogens in animals where there are no obvious signs of disease. Concentration of phagocytic cells and further incubation may be needed. However beyond the exceptional circumstances where the bacteria are so numerous as to be detected by simple sampling, or where specific pathogens can be detected after immunosuppression, the clinical significance of viable bacteria in internal organs has not been ascertained.

5.0 Contribution of outputs

This project has contributed greatly to an understanding of bacterial disease in farmed frogs in Thailand. The warm freshwater ponds and corrals, usually with little water movement, in which the frogs live are ideal to support high bacterial loads. By virtue of their amphibian nature and highly permeable skin frogs are constantly exposed to bacterial challenge by a wide range of species. Many facultative anaerobes, including numerous aeromonads, have been

identified in this project and in past AAHRI laboratory records. Virulence to frogs varies. The only method available to demonstrate this at present is experimental challenge to cause mortalities. By this means a highly virulent aeromonad has been identified. It was recovered from several, widely separated, localities in Thailand. It was found associated with diseased frogs only; not with healthy animals and not in the environment. Virulence may be associated in part with the haemolysin produced, and the haemolysin gene certainly provides the basis for making a diagnostic probe should this be considered advisable. More data on incidence is needed before this is pursued.

Other potential pathogens have been found to be less virulent (*A. hydrophila, A. sobria)*, or not virulent (a *Flavobacterium*-like isolate) in laboratory challenges. However bacteria found present in frog phagocytes, but not causing symptoms, can rapidly grow out and result in fatal disease when the animals are stressed. This was seen following transportation stress and heater malfunction and resembles the outbreaks which spread rapidly through farms. The frequent findings of large numbers of bacteria in the macrophages of the great majority of *Rana rugulosa* examined is important. It is considered unusual for animals to be continuously under this degree of challenge and the questions arise as to why this has not been reported before and whether or not frogs are different from other species. Detection of viable bacteria in the organs of frogs is much less likely by conventional bacteriology technique. Routinely this is the only method applied. The observation reported here arose from studies on macrophage isolation and thereafter involved a bacterial resuscitation step. The evidence gathered in this study (see below) does not suggest that the innate immune system of frogs is fundamentally different from other species. The main function of phagocyte cells in all animals is to ingest foreign particles such as bacteria, and it may be possible to demonstrate this with sensitive technique. What is remarkable in frogs are the numbers of bacteria and the proportion of macrophages involved. While knowledge of the situation in other species is scanty it does appear that the finding in frogs is of clinical significance: it is essential to have a clearer understanding of this process and the implications it has for farming of the species.

The innate immune system of these ranids was investigated and humoral (e.g. lysozyme and complement) and cellular (e.g. macrophage bactericidal activity) shown to be present. However, the range of values for all the parameters measured was wider than has been

reported for other animals. It was not possible to demonstrate enhancement of function by immunostimulators applied by any of the several routes using assays and immunostimulation procedures which had been confirmed in fish. These results are consistent with the hypothesis that the cells are constantly stimulated, albeit to different extents in individual animals, so that data on parameters of macrophage activation are scattered and the role of the immunostimulant, pre-empted.

Further support for these findings has been provided by histology reported in this project and in related work (page 5) which found that all the tissue examined showed evidence of pathology. Undetected infection is on-going among farmed frogs.

Husbandry, environmental conditions and diet in frog farming have evolved by trial and error and have been heavily influenced by financial considerations. In the Stirling vivarium health problems such as ulcers and rectal prolapse were common initially but gradually resolved, mainly due, it is believed, to dietary improvement. In 1996 there was concern about wide spread frog losses in Thailand due to introduction of an inappropriate feed (pers. comm.). What is certain is that there is no sure scientific basis of nutrition or husbandry for tropical farmed frogs.

The findings of this project indicate that farmed frogs in Thailand experience an on-going bacterial challenge of a level of severity to engage more than 20% of spleen macrophages. Under these conditions frogs can survive and grow, even to market size. The system however is very vulnerable to breakdown as a result of environmental stress, nutritional disturbances on encountering a pathogen of unusual virulence. It renders the application of non-specific immunostimulants of little or no value and removes vaccination, even against targeted pathogens as a possible prophylactic option.

This project has generated an understanding of the very high degree of sensitivity of farmed frogs in Thailand to stress associated bacterial disease. While this in itself does not immediately provide a control strategy, it lays the essential foundation upon which this can be built and points clearly the most productive ways forward.

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6.0 Follow-up indicated

Development of sustainable frog farming depends on reducing the risk of bacterial disease. Work is needed to determine the conditions under which this can be achieved. This is best begun by a careful survey of frog farming practises in Brazil, Taiwan, Indonesia and Thailand to identify the current most successful management approaches. Frog genetic pools should be screened for resistance and used as the basis of a breeding programme to select animals best suited to intensive cultivation. Nutritional requirements for good health and growth should be determined by systematic study: diets should be evaluated and recommendations made to meet these criteria and the additional requirement of low cost. Stressed frogs are susceptible to a wide range of bacteria. While it is possible to reduce the bacterial load, it is unlikely that all opportunistic pathogens could be excluded. Two approaches are needed to contain this risk to acceptable levels. Parameters of environment and water quality must be set to reduce the bacterial load borne by frog macrophages. Then tank trial are needed to confirm that frogs in these conditions are much less susceptible to stress related disease and this followed by field studies to confirm that the defined environmental conditions can be achieved and sustained. Secondly it is essential to be able to identify particularly virulent pathogens in systems and stocks. The unspeciated aeromonad isolated during this project is one such and merits further investigation. It must be ascertained whether or not this is pathogenic also for fish and then surveys made of fish and frog farms to determine it's incidence and significance. Results presented in this report lay the way clear to develop a DNA probe and a vaccine for this pathogen if the risk is found to great enough to warrant this.

While awaiting scientific progress such as that outlined above, immediate benefit would be gained by wider dissemination of the findings of this report. This would be best achieved by preparation of a handbook based on the manual already produced but also incorporating the main research findings. This then should be used in practical workshops for fisheries officers and field personnel who have direct contact with frog farmers.

Frog farming seemed simple and was set up on a very small knowledge base and on the assumptions that frogs were easy to handle and had no special needs. This had contributed to

the problems facing the industry today. These problems are not insurmountable; they can be overcome and frog farming continued in a successful small scale units.

7.0 Publications

1. 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.

2. Pearson, M.D., Colquhoun, D., Somsiri,T. and Inglis, V. Biochemical characterisation and RAPD analysis of Aeromonas spp. isolated from septicaemic Rana rugulosa (Weigmann) cultured in Thailand. Accepted for publication in the Proceedings of the World Aquaculture Society Symposium held in Bangkok - January 1996.

3. Crumlish, M., Somsiri, T., Chinabut, S. and Inglis,V. 1997. Incidence of bacteria in spleen macrophage cells from apparently healthy farmed tropical frogs. Accepted by European Association of Fish Pathologists Conference on Diseases of Fish and Shellfish to be held in Edinburgh, September 14 -19,1997.

4. Crumlish, M. 1996. Measurement of the non-specific immune system in the farmed frog *Rana rugulosa.* Poster at World Aquaculture Society Conference in Bangkok 1996

   Prepared as a paper for submission to Diseases of Aquatic Organisms.

5. Crumlish, M. and Inglis, V. 1996. Immunomodulation of the innate immune system in the catfish *Clarias gariepinus.* Poster at Symposium on Methodology in Fish Disease Research. Aberdeen, Scotland. September 26 -27.

6. Inglis,V. and Crumlish, M. 1996. Manual of Assays of the Innate Immune System to Indicate the General State of Health of Frogs and Fish. Aquatic Animal Health Research Institute, Kasetsart University, Bangkok

7. Crumlish,M. and Inglis,V. 1995. Studies on the susceptibility of farmed *Rana tigerina* and *Rana rugulosa* to frog septicaemic disease and its control. Aquaculture News, **20**, 25-27.

8. Pearson, M. 1996. Health and disease in laboratory housed tropical frogs. Aquaculture News, **21**, 23-24.

9. Crumlish, M. 1996. The innate defence system in the farmed frog *Rana rugulosa.* Aquaculture News, **21**, 24-25.

10. Pearson, M. 1996. Studies on the role of *Aeromonas* spp. in bacterial septicaemias of cultured frogs. Aquaculture News, **21**, 24.

11. Colquhoun, D. 1995. Randomly amplified polymorphic DNA analysis of motile and non-motile Aeromonads. Dissertation submitted to the University of Stirling in part fulfilment of the degree of MSc.

8.0 Internal reports

Eleven interim reports and three annual reports were submitted to the ODA during the lifetime of this project. These were presented in:

June, September and December 1994 - quarterly reports

December (end) 1994 - first annual report

June, September and November 1995 and February 1996 - quarterly reports

December (end) 1995 - second annual report

June, October, December 1996 and January 1997 - quarterly reports

March 1997 - third annual report.

Appendices

Aquaculture International 4, 43–53 (1996)

Analysis of DNA relationships among *Aeromonas* species by RAPD (randomly amplified polymorphic DNA) typing

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Genetic differences between a collection of aeromonads were studied in two laboratories by analysis of randomly amplified polymorphic DNA (RAPD). A single randomly designed primer, generated reproducible profiles of genomic DNA in both laboratories for *Aeromonas salmonicida* subspecies *salmonicida*, although the profiles differed between laboratories. Analysis of atypical strains of *A. salmonicida* and isolates of the *A. hydrophila* group produced scattered profiles in both laboratories. The uniform fingerprints produced for *A. salmonicida* subspecies *salmonicida* indicate genomic homogeneity. The scattered RAPD profiles of the motile aeromonads demonstrate the genomic diversity of this group. A group of unspeciated motile aeromonads gave uniform fingerprints, suggesting the possibility of a genomically homogeneous species. Although the RAPD technique is susceptible to the effects of minor technical variations, this study has demonstrated that where there is DNA similarity, it can be recognized, and where there is diversity, differentiation can be made. RAPD promises to be useful in epidemiological studies for rapid identification of bacteria where a source of reference DNA is available and may be useful in preliminary investigations of relatedness within groups.

KEYWORDS: *Aeromonas* species, DNA relationships, RAPD analysis

INTRODUCTION

Aeromonads are widely distributed in the aquatic environment and are well-known pathogens of fish, causing significant economic loss in aquaculture facilities throughout the world. In recent years aeromonads have been recognized as primary, opportunistic agents of disease in humans (Joseph and Carnahan, 1994). The correct identification and classification of organisms belonging to the genus is therefore an area of great concern.

The genus *Aeromonas* is broadly divided into two groups: the non-motile psychrophilic organisms and the motile mesophile group. The non-motile group are a homogeneous collection designated *Aeromonas salmonicida* (DNA hybridization group HG 3). This group has been subdivided into three subspecies: the typical strain *A. salmonicida* subspecies *salmonicida* and the atypical strains *A. salmonicida*

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subspecies *achromogenes* and *A. salmonicida* subspecies *masoucida*. Recently the atypical group has been expanding, with reports of isolates which cannot be classified into any of the available strains. The group of motile aeromonads includes three important species: *A. hydrophila, A. sobria* and *A. caviae*. Increasingly it is being recognized as a very heterogeneous collection and is divided into 13 DNA hybridization groups (HG) consisting of nine species and two biovars. Four HGs are still officially unnamed (Joseph and Carnahan, 1994).

The specific identification of bacterial isolates is important in the study of bacterial fish disease for several reasons. Firstly, there is a need to differentiate between like isolates for epidemiological tracing. This is important in understanding the spread of disease and evaluating control measures. It is also important legally, where issues on source of infection and liability for transmission of disease arise. Secondly, precise fingerprinting of pathogens is fundamental in the development of sensitive detection methods.

Traditional methods of bacterial classification by analyses of physical properties and biochemical capabilities are slow, relatively insensitive and rely on phenotypic properties which vary with conditions of growth. The ideal taxonomic tool would not be affected by differing phenotypic expression. One factor which cannot change markedly within a species is the sequencing of its DNA. Progress in molecular biology has been so rapid in the last 25 years (Towner and Cockayne, 1993) that now nucleic acid analysis can be used in the search for relatedness between species.

The aim of this study was to use a recent development in molecular biology, randomly amplified polymorphic DNA analysis (RAPD), to establish DNA fingerprints for aeromonads from geographically diverse areas of the world. RAPD, also known as 'arbitrarily primed PCR' (AP-PCR), was developed by Williams *et al.* (1990). This technique allows the study of DNA polymorphisms between organisms without pre-requisite knowledge of their molecular biology.

An initial study was carried out in Japan on 13 strains of *A. salmonicida* collected from the USA, Scotland and Japan and seven strains of *A. hydrophila* from the USA and Japan. This work was continued in Scotland on a more extensive collection of *A. salmonicida* from the UK and a collection of *A. hydrophila* from the UK and South East Asia. In addition a selection of unspeciated aeromonads isolated from septicaemic frogs in Thailand was analysed, in an effort to establish any relatedness between them and the *A. hydrophila* group.

MATERIALS AND METHODS

Bacterial strains

The strains used in this study are listed in Tables 1 and 2. In the Japanese laboratory the bacterial strains were maintained on Mueller-Hinton (MH) agar (Difco laboratories Co. Ltd, USA) and were incubated at 25 °C for 24 h. In the Scottish laboratory the bacterial strains were maintained on tryptone soya agar (TSA, Oxoid, Hampshire, England). *A. salmonicida, A. hydrophila, A. sobria* strains and the unknown AAHRI 94060 bacterium were maintained at 22 °C while the unknown frog pathogens were maintained at 30 °C.

TABLE 1. Aeromonas strains analysed in Japan

Species	Strain	Host	Source
Aeromonas salmonicida subsp. *salmonicida*	ATCC 14174	Fish	USA
	Ar-620	Fish	USA
	MCK-91-CHS-AJ2	Fish	USA
	MCK-91-CHS-JV	Fish	USA
	B92406 K	Fish	UK
	B92483 B2	Fish	UK
	B93058 (1)	Fish	UK
	B93269	Fish	UK
	B93358	Fish	UK
	CT7401	Fish	Japan
	OT8003	Fish	Japan
	OT8127	Fish	Japan
	619	Fish	Japan
Aeromonas hydrophila	ATCC 7966	Milk	USA
	ATCC 19570	Fish	USA
	A-10	Fish	Japan
	SK-7	Fish	Japan
	28SA	Fish	Japan
	Gifull304	Human	Japan
	Gifull310	Human	Japan

DNA extraction

Bacterial chromosomal DNA was isolated in the Japanese part of the study using a modification of Wilson's method described by Miyata *et al.* (1995). Further studies, in Scotland, used a commercial DNA extraction kit (Flowgen D5500A, Gentra USA) following the manufacturer's instructions.

Oligonucleotide primers

A randomly designed 12-mer oligonucleotide set was obtained in Japan from Wako Pure Chemical Industries Ltd, Japan. The amplification products of the set of 12 primers were assessed and the three primer sequences giving the clearest patterns were selected for further analyses (Miyata *et al.*, 1995). The three oligonucleotide primer sequences used were: A05 – 'AGCAGCGCCTCA', A07 – 'TGCCTCGCACCA' and A09 – 'CCGCAGTTAGAT'. In Scotland these primers were replicated by Oswell Oligo's, Scotland.

RAPD analysis

In the initial study the PCR was carried out in a final volume of 20 μl containing 20 ng template DNA, 0.4 μM single primer, 0.2 mM (each) 2' deoxynucleoside 5' triphosphates, 10 mM Tris-HCl (pH 8.9), 1.5 mM $MgCl_2$, 80 mM KCl, 0.1% sodium cholate, 0.1% Triton X-100, 50 μg ml^{-1} bovine serum albumin and 0.4 units of Tth DNA polymerase (Toyobo Co. Ltd, Japan). Reactions were performed using the program temperature control system PC-700 (Astec Co. Japan). A total of 40 cycles

TABLE 2. Aeromonas strains analysed in Scotland

Species	Strain	Host	Source
Aeromonas salmonicida subsp. *salmonicida*	NCIMB[a] 1102	Salmon	UK
Aeromonas salmonicida subsp. *salmonicida*	B87183	–	UK
Aeromonas salmonicida subsp. *salmonicida*	B86069	Salmon	UK
Aeromonas salmonicida subsp. *salmonicida*	FCS	Salmon	UK
Aeromonas salmonicida subsp. *salmonicida*	MT423	Salmon	UK
Aeromonas salmonicida subsp. *salmonicida*	B86123[b]	Perch	UK
Aeromonas salmonicida (atypical)	B90226	Salmon	Eire
Aeromonas salmonicida (atypical)	B94230	Goldfish	UK
Aeromonas salmonicida subsp. *achromogenes*		Rudd	UK
Aeromonas hydrophila	NCIMB 1134	Rainbow trout	UK
Aeromonas hydrophila	T4	–	Bangladesh
Aeromonas hydrophila	G49	–	India
Aeromonas hydrophila	UDS	Catfish	India
Aeromonas hydrophila	FIN36	Frog	Thailand
Aeromonas hydrophila	FID75	Frog	Thailand
Aeromonas sobria	T1	–	Bangladesh
Unknown bacteria	AAHRI[c] 94060	–	Thailand
	F3D29	Frog	Thailand
	F4D3	Frog	Thailand
	FID36	Frog	Thailand

[a] NCIMB: National Collection of Industrial and Marine Bacteria Ltd, UK.
[b] Slow pigment producer.
[c] AAHRI: Aquatic Animal Health Research Institute, Thailand.

of thermal cycling with 'hot starting' were run under the following conditions: denaturation at 94 °C for 30 s, annealment at 30 °C for 30 s and extended to 72 °C for 1 min. After thermal cycling, the samples were post heated at 72 °C for 5 min.

Further studies in Scotland were performed using final volumes of 50 μl containing 100 ng of template DNA, 2 μM single primer, 0.2 mM (each) 2' deoxynucleoside 5' triphosphates, 20 mM reaction buffer supplied with DNA polymerase, with resultant reaction conditions of 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl pH 9.0 (at 25 °C), 0.1% w/v Tween, 0.1 units "Red Hot" DNA polymerase (Advanced Biotechnologies, Surrey, UK). Reactions were performed in a Hybaid Omnigene (Middlesex, UK) thermal cycler. In all trials the reaction vessel was heated to 94 °C for 3 min to ensure complete separation of double-stranded DNA before cycling commenced. A total of 40 cycles were run under the following conditions: denaturation at 94 °C for 30 s, annealment at 30 °C for 30 s and extension (action of DNA polymerase) at 72 °C for 1 min. After completion of 40 cycles the samples were maintained at 72 °C for 5 min to allow all amplified products to be fully extended.

Assessment of PCR products

Initially amplified DNA fragments were electrophoresed in a 2.0% agarose gel in 1 × TBE (89 mM Tris-borate, 2 mM EDTA), the gel stained in 1 × TBE containing ethidium bromide (0.5 mg ml^{-1}) for 20 min and patterns of DNA fragments compared

with each strain using UV light. For later studies in Scotland the PCR products were initially run on a 2% agarose gel (Ultra-pure, Gibco, Paisley, Scotland) minigel (Bio-Rad, 77 ml volume) in Tris-acetate (TAE) buffer prestained with ethidium bromide (0.5 mg l^{-1}), then visualized by UV light. If amplification was satisfactory, the products were then run on a maxi-gel (Pharmacia, Sweden, 200 ml volume) in Tris-borate (TBE) buffer to obtain better resolution of the bands. The maxi-gel was stained in ethidium bromide (0.5 mg l^{-1}) for about 20 min after completion of electrophoresis and then photographed (Polaroid DS 34) to give a permanent record.

RESULTS

Initially RAPD analysis of 14 strains of *A. salmonicida* and seven strains of *A. hydrophila* were performed using each of the three primers. All the profiles of the *A. salmonicida* subspecies *salmonicida* strains were identical (Fig. 1). The common profiles generated using the primers A05, A07 and A09 contained five, four and two fragments respectively with a few other faint products. The products of primer A05

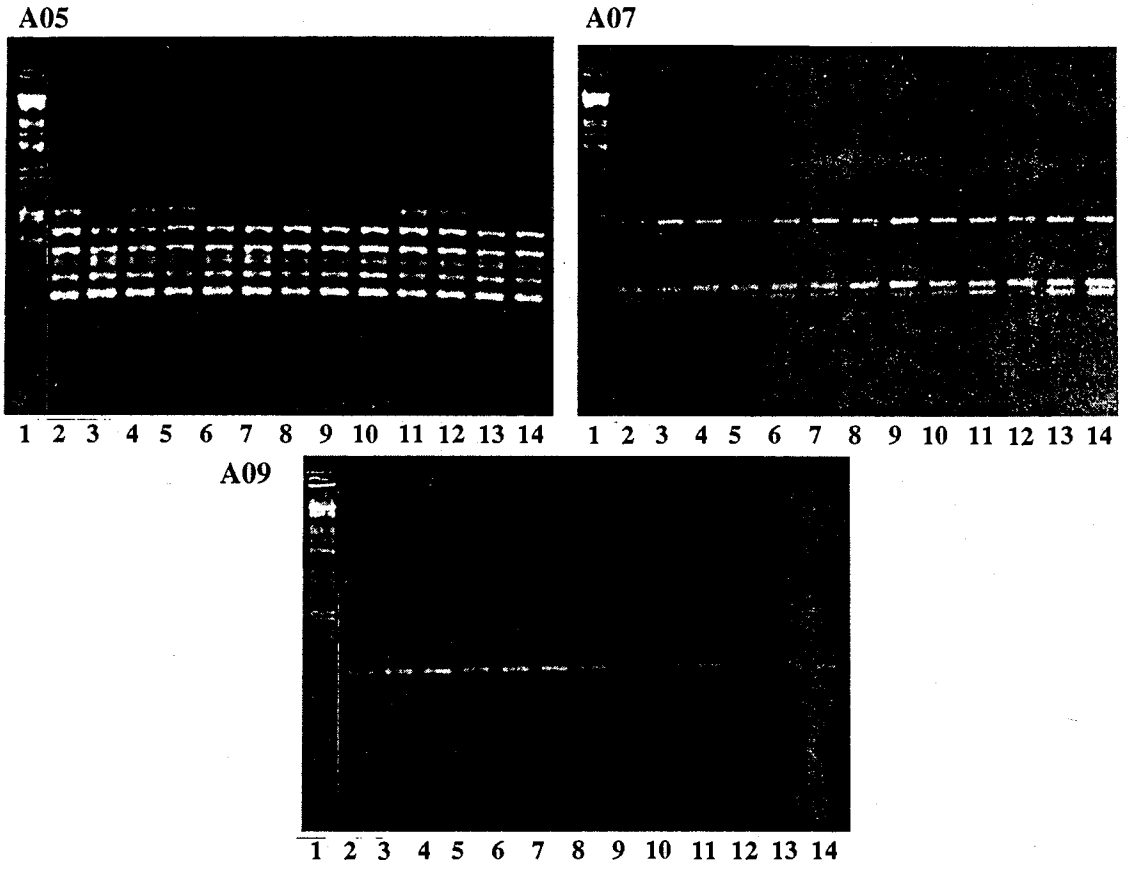

FIG. 1. RAPD profiles of *Aeromonas salmonicida* subspecies *salmonicida*, using three different primers: A05, A07 and A09. Lane 1, mixture of the *Eco*RI-*Hind*III double digested λ-DNA and *Hin*fI digested pUC as DNA ladder; lane 2, ATCC 14174; lane 3, Ar-620; lane 4, MCK-91-CHS-AJ2; lane 5, MCK-91-CHS-JV; lane 6, B92406 K; lane 7, B92483 B2; lane 8, B93058(1); lane 9, B93269; lane 10, B93358; lane 11, C T7401; lane 12, OT8003; lane 13, OT8127; lane 14, 619.

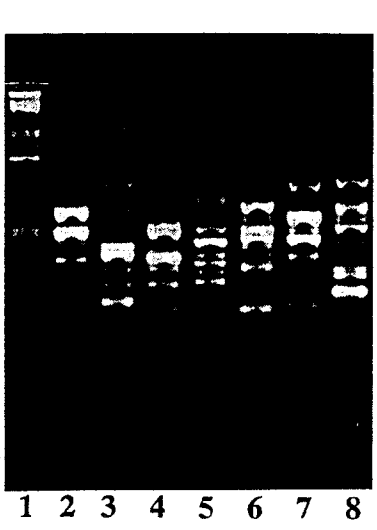

FIG. 2. RAPD profiles of *Aeromonas hydrophila*. Lane 1, mixture of the *Eco*RI-*Hind*III double digested λ-DNA and *Hin*fI digested pUC as DNA ladder; lane 2, ATCC 7966; lane 3, ATCC 19570; lane 4, A-10; lane 5, SK-7; lane 6, 28SA; lane 7, Gifull304; lane 8, Gifull310.

were 0.45, 0.28, 0.25, 0.22 and 0.19 kilobase (kb) in length. The four products of A07 were 0.54, 0.23, 0.22 and 0.19 kb in length and the two products of A09 were 0.23 and 0.18 kb in length. In contrast the RAPD profiles of the seven strains of *A. hydrophila* were scattered. No species-specific fragment was evident (Fig. 2).

Further studies undertaken in Scotland produced RAPD fingerprints with only one of the three primers on all species of bacteria tested. With primer A05 (Fig. 3), all of the subspecies *salmonicida* with the exception of B86123 produced an almost identical fingerprint with five very clear bands of approximately 2.10, 1.95, 1.215, 0.74 and 0.63 kb. Strain B86123 (subspecies *salmonicida*) produced a fingerprint with only two of those common bands (1.95 and 0.74 kb) faintly amplified and a smaller fragment approximately 350 base pairs (bp) strongly amplified. Of the atypical strains, B90226 produced an amplification profile identical to that of the majority of subspecies *salmonicida* strains, while subspecies *achromogenes* produced a fingerprint containing 0.63 and 0.74 kb in common with the typical strains and a 0.97 kb fragment in common with B94230, which in turn produced a distinctive three-banded profile (0.97, 0.92 and 0.84 kb). With primers A07 and A09, amplification was extremely poor.

RAPD analysis of *A. hydrophila* strains FID 75, FIN 36, NCIMB 1134, T4, G49 and UDS and *A. sobria* strain Tl (Fig. 4) revealed distinctive reproducible fingerprints for each strain but no common amplification products between all the strains. There were, however, bands that were common to more than one strain. In contrast the unspeciated aeromonads from Thailand, AARHI 94060, FID 36, F4D3 and F3D29, produced indistinguishable fingerprints comprising two main bands of around 0.61 and 0.98 kb.

DISCUSSION

The initial phase of this work, carried out in Japan, suggested that RAPD profiles were highly reproducible in repeated analyses. The profiles for *A. salmonicida* subspecies *salmonicida* were identical, indicating a genomic homogeneity in line with the findings of previous studies (Boyd *et al.*, 1994; Nielsen *et al.*, 1994).

In the continuation of this work in Scotland, the only oligonucleotide to produce satisfactory fingerprints was A05. However, fingerprints generated with this oligonucleotide were remarkably uniform. The differing yields from the same primers found in the two components of this study are consistent with the variability of RAPD analyses found by other workers. Concern on this issue has been expressed by

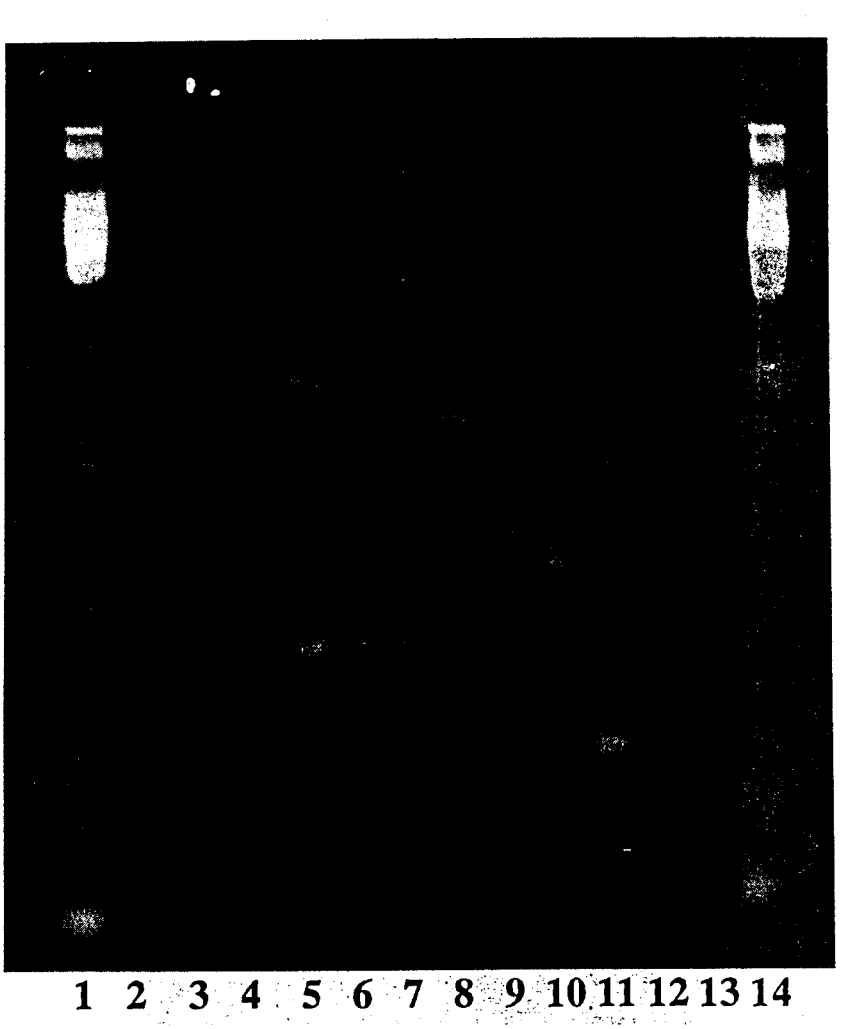

FIG. 3. RAPD profiles of *Aeromonas salmonicida* subspecies *salmonicida*, subspecies *achromogenes* and atypical strains. Lanes 1 and 14, DNA ladder, 123 to 4182 bp in increments of 123 bp; lane 2, control relevant to reactions in lanes 3 and 4; lane 3, subspecies *salmonicida* FCS; lane 4, subspecies *salmonicida* MT423; lane 5, subspecies *salmonicida* B86069; lane 6, subspecies *salmonicida* NCIMB 1102; lane 7, subspecies *salmonicida* B90226; lane 8, atypical B90226; lane 9, subspecies *achromogenes*; lane 10, atypical B94230; lane 11, subspecies *salmonicida* B86123; lane 12 control relevant to reactions in lanes 5, 6, 8, 9, 10 and 11; lane 13, control relevant to reaction in lane 7.

Ellsworth *et al.* (1993) and Penner *et al.* (1993), who established that even minor variations in reaction conditions could lead to differing results.

In the present work, different DNA polymerase enzymes were used in the two laboratories. It is well documented that DNA polymerase enzymes isolated from different species of bacteria produce different amplification profiles (Fekete *et al.*, 1992; Meunier and Grimont, 1993; Schierwater and Ender, 1993). Each enzyme must also be used with an optimal concentration of $MgCl_2$, any variation in which can cause considerable differences in amplification profile.

Thermal cycler variation has been named as one of the main causes of interlaboratory variation (Penner *et al.*, 1993). In this study, two different thermocyclers were used, resulting almost certainly in a different temperature profile, due to differences in rates of heating and cooling (even if reaction conditions are programmed uniformly). Another factor which may affect the amplification profiles is primer variability. Farber and Addison (1994) discovered that primers synthesized and purified by different means yielded slightly different banding profiles.

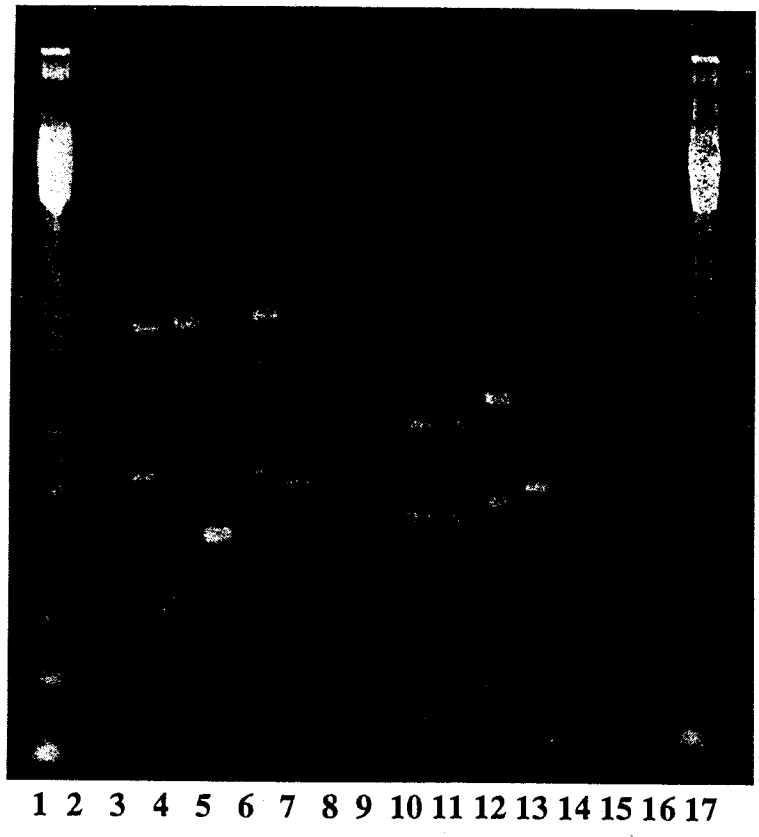

FIG. 4. RAPD profiles of *Aeromonas hydrophila*, *A. sobria* and unspeciated aeromonads. Lanes 1 and 17, DNA ladder, 123 to 4182 bp in increments of 123 bp; lane 3, *A. hydrophila* UDS; lane 4, *A. hydrophila* G49; lane 5, *A. hydrophila* T4; lane 6, *A. hydrophila* NCIMB 1134; lane 7, *A. sobria* TI; lanes 8–11, unspeciated aeromonads AAHRI 94060, FID36, F4D3 and F3D29 respectively; lane 12, *A. hydrophila* FID75; lane 13, *A. hydrophila* FIN36, lanes 14 and 15, controls.

Inconstant primer:template ratios have been cited by several authors as a possible source of product variation (Mazurier *et al.*, 1992; Ellsworth *et al.*, 1993; Stephan *et al.*, 1993; Neiderhauser *et al.*, 1994) and the importance of spectrophotometric analysis to standardize the DNA concentration in the template solution is stressed. In contrast, McCourt *et al.* (1992) and Fukatsu and Ishikawa (1994) found that concentration of template DNA (5–800 ng) had little effect on banding patterns. Levels of RNA within the extracted nucleic acids may differ depending on method of extraction and this too can interfere with the spectrophotometric quantification and cause artefacts (Ellsworth *et al.*, 1993; Micheli *et al.*, 1994).

The annealing temperature may also have a significant effect on amplified products and the number of bands produced (Muralidharan and Wakeland, 1993; Penner *et al.*, 1993). A difference of only 1 °C in annealing temperature can lead to qualitatively different results (Penner *et al.*, 1993). In stringent PCR, the use of high annealing and extension temperatures, combined with very specific primers, normally prevents the extension of any contaminant DNA. In RAPD, however, the use of low annealing temperatures and subsequent match/mismatching of the very short arbitrary oligonucleotides to almost any DNA fragment may make contamination a more serious problem. Neiderhauser *et al.* (1994) reported faint banding among some negative controls (no template DNA) in an RAPD experiment. This was attributed to contamination by extraneous DNA, or possibly DNA contamination of the T*aq* polymerase used, a possibility already demonstrated (Schmidt *et al.*, 1991).

The RAPD technique, therefore, is as yet very susceptible to the effects of minor technical variations. While this makes it difficult to reproduce profiles exactly under different conditions, this study has shown that where there is DNA similarity it can be recognized and where there is diversity, differentiation can be made.

The *A. salmonicida* subspecies *salmonicida* isolates gave uniform fingerprints in the first part of the study and another uniform set in later work. Strain B86123 was designated typical on the basis of its biochemical reactions; however, it had been observed to produce pigment at a much slower rate than the other subspecies *salmonicida*. On RAPD analysis it produced a different profile but this did contain two common fragments, although they were poorly amplified. The scattered RAPD profiles of *A. hydrophila* found consistently, demonstrated the genomic diversity of the motile aeromonads, correlating with other studies reporting antigenic and genetic heterogeneity (MacInnes *et al.*, 1979; Popoff *et al.*, 1981; Kuijper *et al.*, 1989).

Fingerprints produced for the unspeciated aeromonad from Thailand AAHRI 94060, and the Thai frog pathogens FID36, F4D3 and F3D29 were identical. This suggests that these bacteria are at least very closely related. These results support earlier analysis of the bacteria (Pearson M.D., unpublished data) which revealed identical biochemical profiles for each isolate but showed that they differed from *A. hydrophila* and *A. sobria*. These bacteria were isolated from geographically diverse areas of Thailand. This evidence, though limited, may point towards a species of bacteria pathogenic to frogs in SE Asia which has a remarkably conserved genome, paralleling that of *A. salmonicida* subspecies *salmonicida* in salmonids in the northern Atlantic and Europe.

CONCLUSIONS

1. RAPD analysis has proved useful in demonstrating the similarity of isolates of *A. salmonicida* subspecies *salmonicida* from widely diverse geographical origins.
2. The technique allows discrimination of atypical strains and demonstration of like isolates within the heterogenous hydrophila-complex.
3. RAPD promises to be useful in epidemiological studies for rapid identification of bacteria for which a source of reference DNA is available and may be useful in preliminary investigations of relatedness within groups.
4. The limitations of the method in comparative studies between systems must be borne in mind, at least within the current technical constraints. It should not be regarded as a method of providing a data base of bacterial DNA but rather as a productive screening method to identify species and genomic fragments for further, more detailed investigation.

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BIOCHEMICAL CHARACTERISATION AND RAPD ANALYSIS OF *Aeromonas* sp ISOLATED FROM SEPTICAEMIC *Rana rugulosa* (Weigmann) CULTURED IN THAILAND.

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Abstract

An investigation into the role of aeromonads in bacterial diseases of farmed frogs was undertaken. Five field trips were made to farms in different geographical locations in Thailand. On each farm bacteriological samples were taken from 10 diseased frogs and 10 clinically normal frogs. Classification of isolates with the API 20 E identification kit (BioMerieux, France) revealed that suspected aeromonads from the internal organs of the diseased frogs consistently produced acid from glucose and mannitol but did not utilise any other sugars. Negative reaction for sucrose fermentation is considered atypical for the *Aeromonas hydrophila* complex. Bacteria conforming to the sugar fermentation patterns of *A. hydrophila* and *A. sobria* were isolated from the skin and intestine of diseased and clinically normal frogs but none were found internally in adults. The DNA relatedness of the collection of aeromonads was studied by analysis of randomly amplified polymorphic DNA (RAPD). A single randomly designed primer produced identical fingerprints for the sucrose negative isolates. In contrast RAPD profiles of *A hydrophila* revealed distinctive reproducible fingerprints for each isolate but no common amplification products between the isolates. This work indicates 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. The consistency in phenotypic characteristics is reflected by a homology of the genome unusual among geographically diverse isolates of motile aeromonads.

Introduction

Although mankind has been hunting and consuming frogs for thousands of years successful intensive culture is a very recent development. In Thailand frog culture has been practised for more than 10 years but commercial success was only attained in the 1990's (Somsiri 1994). Most farms are family owned, broodstock, nursery and growout operations are normally performed within a single small scale "backyard" operation. Farms vary in size from a few concrete corrals to large farms covering 0.5 hectare.

The rapid expansion of the industry coupled with the inexperience of most farmers has led to an equally rapid rise in the outbreak of stress related diseases. Poor husbandry adds to continual disease problems and epizootics of bacterial diseases can lead to huge financial losses for farmers.

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

Aeromonads are frequently recorded as the cause of disease in amphibians, reptiles and fish. (Roberts, 1993). *Aeromonas hydrophila* infections (also known as red-leg) are the

most commonly identified cause of mortality in laboratory frog populations (Gibbs 1973) and have frequently been reported as the cause of epizootic bacterial septicaemia's in wild populations of frogs (Dusi, 1949, Nyman 1986, Bradford 1991).

Most of the published literature on bacterial septicaemia of frogs is however based on laboratory and wild populations. The environmental conditions found on frog farms are very different to those found in laboratories or under natural conditions. Information on bacterial septicaemic disease in farmed frogs is limited although outbreaks can kill 80-90% of tadpoles and growout frogs (Somsiri pers comm). The objective of this study was to investigate the role of aeromonads in bacterial diseases of farmed frogs.

Materials and Method

Collection

Five field trips were made to farms in different geographical locations in Thailand; farm 1 in Kam Pam Petch, farm 2 in Nakon Rachisima, farm 3 in Petchburi, farm 4 in Pathun Thani and farm 5 in Chanburi. On each farm bacteriological and histological samples were taken from 10 diseased frogs and 10 clinically normal frogs.

Bacterial sampling

Bacterial samples were taken from frog: inner thigh skin, lesions, thigh muscle, heart, blood, kidney, liver, spleen and intestinal contents. Aseptic procedures were followed; instruments were cleaned, dipped in 95% ethyl alcohol and flamed between each sample from each animal.

Samples were streaked directly onto tryptone soya agar (TSA, Oxoid) Pseudomonas selective media (Oxoid) and Aeromonas selective media (Oxoid) and incubated at 30°C. Suspected pathogens were subcultured on TSA and stored on TSA slopes and in stab cultures.

Biochemical characteristics of bacterial isolates

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 the ability of the isolates to produce oxidase and to metabolise glucose fermentatively or oxidatively. Isolates were classified to species level on the basis of their biochemical reactions in the API 20 E enterobacteriacae system (BioMerieux, France). Further biochemical tests were carried out following an identification system (Aerokey II) described by Joseph and Carnahan (1994). 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 Bauer-Kirby disc diffusion method. All biochemical tests were carried out at 30°C.

RAPD Analysis

Bacterial chromosomal DNA was prepared from cultures grown overnight in tryptone soya broth. The test strains are listed in Table 1 *A. hydrophila* and *A. sobria* were

incubated at 22°C while the unknown frog isolates were incubated at 30°C. DNA was isolated using a commercial DNA extraction kit (Flowgen D5500A, Gentra USA) following the manufacturers instructions. A 12-mer primer A05 - 'AGCAGCGCCTCA' which had been used successfully in Japan (Miyata *et al.* 1995) to give clear reproducible patterns for a number of aeromonads was selected and replicated by Oswell's Oligo's Scotland. Fifty µl reactions were prepared, each containing 100ng of template DNA, 2µM single primer, 0.2mM (each) 2' deoxynucleoside 5' triphosphates, 20mM reaction buffer supplied with DNA Polymerase, with resultant reaction conditions of 20mM (NH4)2SO4, 75mM Tris-HCl H 9.0 (at 25°C), 0.1% w/v Tween, 0.1 units "Red Hot" DNA polymerase (Advanced Biotechnologies, Surrey, UK.). Reactions were performed in a Hybaid Omnigene (Middlesex, UK) thermal cycler. In all trials the reaction vessel was heated to 94°C for 3 minutes to ensure complete separation of double stranded DNA before cycling commenced. A total of forty cycles were run under the following conditions : denaturation at 94°C for 30 seconds, annealment at 30°C for 30 seconds and extension (action of DNA polymerase) at 72°C for 1 minute. After completion of 40 cycles the samples were maintained at 72°C for 5 minutes to allow all amplified products to be fully extended. Amplification products were analysed on an agarose gel (2%), stained with ethidium bromide and visualised by U-V light.

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Results

Biochemical Characterisation

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 2. 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* unknown (Au).

Aeromonas unknown was isolated from the internal organs of frogs in all five of the regions visited. It was isolated from one skin sample only 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 three isolates of *A. sobria* and two isolates of *A. hydrophila* were obtained from the internal organs of septicaemic tadpoles. The distribution of the *Aeromonas* isolates is summarised in Graphs 1 and 2.

Further identification tests using the Aerokey II (Joseph and Carnahan, 1994) allowed division of the initial two *A. hydrophila* and *A. sobria* like groups into six species. There is 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 see Table 3.

RAPD analysis

The RAPD profiles generated for the five *A. hydrophila* strains and one *A. sobria* strain from S. E. Asia (Figure 1.), were strikingly different to the profiles generated for the unspeciated aeromonads from Thailand. Although distinctive reproducible fingerprints were generated for *A. hydrophila* and *A. sobria*, the profiles were scattered with no common amplification products between all the strains. In contrast all the RAPD profiles of the unspeciated aeromonads from Thailand were identical. The common profile of *Aeromonas* unknown contained two main fragments of 0.61Kb and 0.98Kb. Neither of these bands were found in any of the *A. hydrophila* or *A.. sobria* profiles, despite the fact that two of the *A hydrophila* strains, F1N36 and F1D75 were isolated on the same farm, at the same time, as the *Aeromonas* unknown isolate F1D36.

Discussion

The identification of the motile mesophilic aeromonads is complex, there is considerable genetic diversity within the genus, and poor correlation between genotype and phenotype (Janda and Duffy, 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 different hybridisation groups which are biochemically indistinguishable from each other (Kuijper *et al*, 1989). The taxonomy of the genus Aeromonas has therefore undergone major revisions. There are at least 13 DNA hybridisation groups within the genus and currently the following twelve species are recognised: A. *caviae*, *A. eteropelogenes*, *A. eurenophila*, *A. hydrophila*, *A. ichthiosmia*, *A. jandaei*, *A. media*, *A.salmonicida*, *A. schubertii*, *A.sobria*, *A. trota* and *A. veronii* (Martinez-Murcia *et al*, 1992).

Phenotypic characteristics used to identify *Aeromonas* strains are not however sufficiently specific and are subject to variation with changes in e.g. incubation temperature and composition of the test medium (Hanninen, 1994). Bacteria of similar phenotypic expression may belong to different genospecies (Kuijper *et al*, 1989).

Studies on DNA homology of motile aeromonads have demonstrated the genetic diversity of this group of organisms (MacInnes and Trust, 1979, Miyata *et al* 1995).

The scattered RAPD profiles of *A. hydrophila* isolates generated in this work correlate with the findings of genetic heterogeneity in the previous studies. However the identical fingerprints produced for the sucrose negative isolates suggest that a group of motile aeromonads exists in geographically diverse areas of Thailand which are at least very closely related.

It is important to identify isolates completely, to determine if different Aeromonas spp. are associated with specific disease processes (Joseph and Carnahan, 1994). To some extent this work has been carried out for aeromonads involved in human diseases for example *Aeromonas veronii* biovar *sobria* associated with bacteraemia, *A. schubertii* associated with traumatic wound infections and *A. caviae* associated with paediatric gastro-enteritis (Carnahan *et al* 1991). Bacterial septicaemia in frogs caused by *Aeromonas* spp. other than by *A. hydrophila* have not so far been documented.

This work indicates 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. This consistency in phenotypic characteristics is reflected by a homology of the genome unusual among geographically diverse isolates of motile aeromonads. Further work is needed to completely characterise the sucrose negative isolates and achieve an understanding of the epidemiology and pathogenesis of these isolates in frog septicaemic disease.

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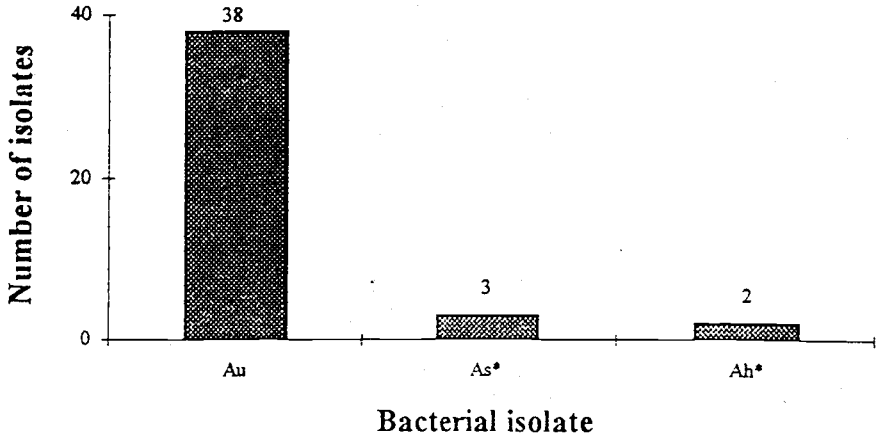
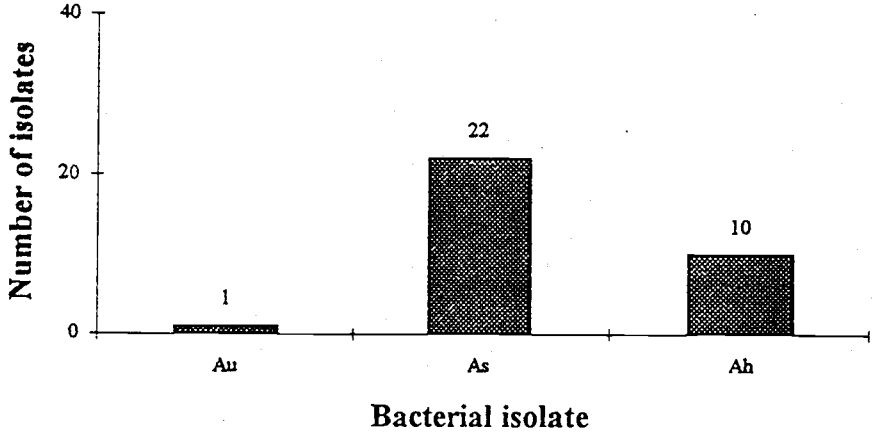
Graph 1. Total number of isolates from internal organs



Graph 2. Total number of isolates from intestine and skin



Au = unspeciated aeromonad

As = *Aeromonas sobria*

Ah = *Aeromonas hydrophila*

*tadpoles only

Table 1. Origin of Aeromonas spp. used in RAPD analysis

Species	Strain	Host	Source
Aeromonas hydrophila	NCIMB[a] 1134	R. Trout	UK
Aeromonas hydrophila	T4	--	Bangladesh
Aeromonas hydrophila	G49	--	India
Aeromonas hydrophila	UDS	Catfish	India
Aeromonas hydrophila	FIN36	Frog	Thailand
Aeromonas hydrophila	FID75	Frog	Thailand
Aeromonas sobria	T1	--	Bangladesh
Aeromonas unknown	94060	Frog	Thailand
	F3D29	Frog	Thailand
	F4D3	Frog	Thailand
	FID36	Frog	Thailand

a NCIMB: National Collection of Industrial and Marine Bacteria Ltd, UK

Table 2. Percentage of isolates giving positive reactions on API 20 E tests

Test	A. u n = 23	A. s n = 27	A. h n = 14	ref. A. s. ncimb37	ref. A h. ncimb89	ref. A. j atcc49568
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	-	+	-
Arabinose	0	0	100	+	+	-

Au = *Aeromonas* unknown
As = *Aeromonas sobria*
Ah = *Aeromonas hydrophila*
Aj = *Aeromonas jandaei*
ref. = reference strains

Table 3. Percentage of isolates giving positive reactions in Aerokey II

Test	Au n=10	Ah n=12	Ac n=5	Av bv sob n=11	Av bv ver n=3	A sch n=1	ref As ncimb 37	ref Ah ncimb 89	ref Aj ncimb 49568
AES	100	100	100	0	100	-	-	+	-
TSI	90	100	0	82	100	+	+	+	+
ARA	0	83	60	0	0	-	+	+	-
CEPH	100	83	100	9	0	S	S	R	R
IND	100	92	100	100	100	-	+	+	+
VP	80	92	80	100	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
As	=*Aeromonas sobria*
Ah	=*Aeromonas hydrophila*
Ac	=*Aeromonas caviae*
Av bv sob	=*Aeromonas veronii* biovar *sobria*
Av bv ver	=*Aeromonas veronii* biovar *veronii*
Asch	=*Aeromonas schubertii*
ref.	=reference strain

A05

lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

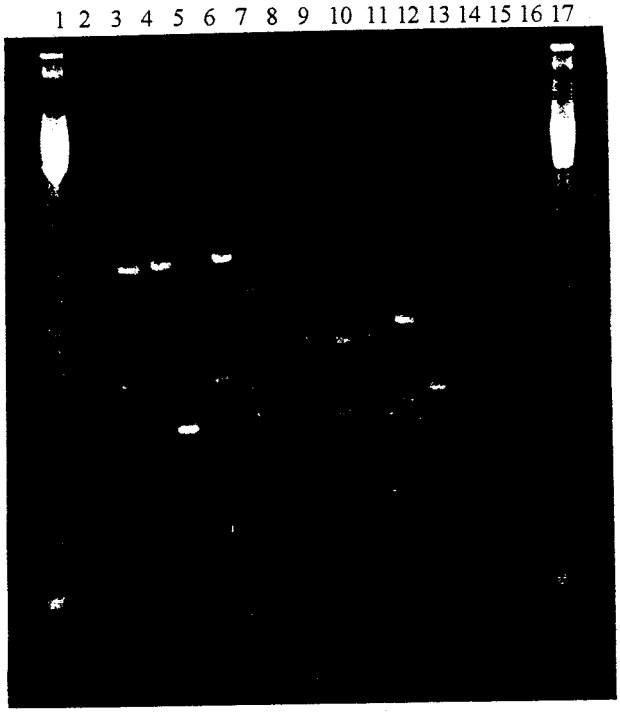


FIGURE 1. RAPD profiles of *Aeromonas hydrophila*, *Aeromonas sobria* and *Aeromonas* unknown

Lanes 1 and 17, DNA ladder, 123 to 4182 bp in increments of 123 bp; lane 3, *A hydrophila* UDS; lane 4, *A hydrophila* G49; lane 5, *A hydrophila* T4; lane 6, *A hydrophila* NCIMB 1134; lane 7, *A sobria* T1; lanes 8-11, *Aeromonas* unknown 94060, FID36, F4D3 and F3D29 respectively; lane 12, A hydrophila FID75; lane 13, *A hydrophila* FIN36, lanes 14 and 15 controls.

Innate Immune System
Assays to Indicate General State of Health of Frogs and Fish

Tuesday 24 Sept - Friday 4 Oct 1996.

Aquatic Animal Health Research Institute
Bangkok
Thailand

ACKNOWLEDGEMENT

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Project Manager	Dr. V. Inglis	
Research Staff	Ms. M. Crumlish	(PhD student ODA)
	Ms. M. Pearson	(Veterinary Research Fellow - BSRC)

ASSESSMENT OF THE NON-SPECIFIC DEFENCE MECHANISMS IN THE FISH AND FROG.

All animals including fish and amphibians have both innate and adaptive immune responses. These have both humoral and cellular components. Humoral or non-cellular defences include antibodies, which are specific, and non-specific antimicrobial chemicals and enzymes such as lysozome. Cellular functions include the specific immune response of antigen uptake and triggering of antibody production and the non-specific function of the phagocytes (cells that eat) which are able to ingest and kill micro-organisms. In both processes the cells involved are the white blood cells, LEUKOCYTES. These cells originate in the bone marrow, where many also mature. They then migrate to patrol the tissues and circulate in the blood and lymphatic system. The cells of the adaptive immune response are the lymphocytes, which provide long lasting immunity to a disease after a previous exposure. Cells important in the innate defences are the phagocytosing cells.

Phagocytosing cells are found circulating in the blood (predominantly neutrophils and monocytes) and fixed in the tissues (macrophage cells) of the circulatory and lymphatic system.

The specific immune response is activated by exposure to a pathogen with subsequent recovery, or to a vaccine: on subsequent exposure a specific response is mounted which is directed, very closely, to the antigens which primed the response. Some fish vaccines have been prepared, found to be effective and are now commercially available. However against many microbial diseases and for many species of aquatic animal there are, as yet, no vaccines.

On first exposure to a pathogen the outcome depends on interaction with the non-specific defences. The state of activation of this system varies. It is depressed in adverse conditions and may be heightened by use of general immunostimulants. Between these extremes measurement of the parameters of the non-specific defences can give an indication of the general state of health of the animal, act as an early warning of incipient problems and provide a foundation for evaluating substances and procedures aimed at improving disease resistance status.

Blood analysis and determination of the activity of phagocytosing cells provide a foundation for these studies.

COURSE OBJECTIVES

The objectives of this course are to gain an understanding of the components of the innate immune system and experience of the following techniques:-

- Separation of phagocytic cells from the blood and spleen (by density gradient centrifugation) and determination of their level of activation.
- Cell counting and culturing *in vitro*.
- Morphological indicators of activation.
- Bacterial phagocytosis by blood neutrophils and spleen macrophages.
- Macrophage production of reactive oxygen species (lethal for bacteria), nitroblue tetrazolium and ferricytochrome c reduction.
- Bacterial killing by macrophages.
- Lysozyme levels in the blood and tissue fluids.
- Red and white blood cell numbers.

TIMETABLE

Day 1-Mon 30 Sept.

Frog dissection-demonstration.

Removing spleen aseptically and separating macrophages using Percoll gradient.

Preparing coverslip cultures of macrophages.

Day 2-Tues 1 Oct.

Staining cover slip cultures.

Preparing solutions for cell separation.

Bleeding frog.

Making macrophage monolayers.

Day 3-Wed 2 Oct.

Counting cells adherent in cultures.

Preparing solutions and do assay of intracellular respiratory burst (NBT).

Separating serum from blood.

Examining stained macrophage coverslip cultures.

Day 4-Thurs 3 Oct.

Lysozyme assay (spectrophotometric method).

Collecting blood to:

- measure haematocrit
- do total white cell counts
- prepare smears for differential blood cell counts.
- start phagocytosis assay
- measure intracellular respiratory burst in blood phagocytes.

Day 5-Fri 4 Oct.

Staining and examining blood smears and phagocytosis assays.

Demonstration of assay of macrophage killing potential.

Recapitulation and talk session about possible participant projects.

DISSECTION GUIDE

Fish or frogs should be killed immediately before examination either by severance of the spinal cord, anaesthetic overdose or a sharp blow to the head (see Frerichs & Millar, 1995 pp7-9). Before examining the animal internally, the external body surfaces should be examined and any abnormalities recorded. Attention should be given to the appearance of the eyes, bone deformities, external lesions, skin colour and state of nutrition. This information together with the size, weight and any other relevant data should be recorded.

When the external examination is complete, the surface of the animal should be cleaned by spraying with ethanol and opened to exposure the internal organs. It is important to observe aseptic technique throughout microbiological examination and when removing organs for tissue culture.

The internal organs, the liver, heart, kidney and spleen, should be examined for colour, size and presence of abnormalities. The quantity of body fat and any other feature of interest should be recorded. Care must be taken not to puncture the intestinal tract and expose the internal organs to bacterial contamination. For bacteriological examination individual organs should be seared with a hot scalpel blade and then sampled by inserting a sterile loop and plating out onto growth medium. In the absence of any internal lesions the kidney is sampled; but it may also be of value to sample the liver heart and spleen.

An autopsy record should be completed for every animal examined.

For macrophage isolation, it is equally important to maintain a high level of asepsis. Macrophages can be isolated from a variety of sources; from the blood, lymphoid organs and peritoneal cavity. In fish, the head kidney is a good source while in the frog, the spleen is preferred because of accessibility. This tissue should be collected early in the examination to minimise opportunities for bacterial contamination.

SPECIMEN AUTOPSY RECORD

Autopsy Data

Date:

Tank:

Animal: weight: age:

1. External Observations

Eyes opaque:		Y/N.
Bone deformities:		Y/N
Ulcers present:	Head	Y/N
	Forelimbs:	Y/N
	Hind limbs:	Y/N
	Body :	Y/N

Skin colour:

Overall appearance:

2. Internal Observations

Organs	**Colour**	**Size**	**Comments**
Liver			
Heart			
Spleen			
Kidney			
Body fat			
Other			

3. Bacteriology Completed

Organs	**Bacteriology Results**
Liver	Y/N
Heart	Y/N
Spleen	Y/N
Kidney	Y/N
Others	Y/N

BLOOD ANALYSIS

Collect blood from stunned or anaesthetised animal, either by cardiac puncture for fish or frogs or from the caudal vessel for fish. If serum is required, no anticoagulant should be used, however blood from tropical aquatic animals is prone to rapid clotting and flushing the needle with heparin may prove beneficial in extending clotting time.

Differential Blood Cell Counts

Rowely. A.F. 1990. Techniques in Fish Immunology.

Blood smears are stained and examined to enable recognition of the different cell types and to give an indication of the health status of the animal. Elevated phagocytic cells may be a sign of septicaemia: depressed white cell counts may also be a sign of ill health.

Equipment:	**Reagents:**
Cleaned glass slides	95% methanol
Coplin jars	Sterile saline (Ringers solution)
Coverslips	Soresen's Buffer (0.1M pH6.8)
Filter paper (Whatman 1/2)	Giemsa stain
	Rapi-Diff II Stain

Method:

1. Place a drop of approximately 5 µl blood on to an alcohol-cleaned microscope slide.
2. Position another cleaned microscope slide at a 45^0 angle on the drop of blood and allow the blood to flow along the edge of the angled slide.
3. Push the angled slide away from the drop, creating a blood smear. Allow to air dry.
4. Once the smear has dried the cells can be fixed and stained using 2 different methods.

Method 1.

1. Fix smear in 95% methanol for 2 minutes, dry and stain in Giemsa for 25 minutes. Rinse slides in buffer, dry and view cells at 100x magnification under oil immersion.

Giemsa stain

Dilute filtered stock Giemsa 1 in 10 with Sorensen's buffer (pH 6.8), filter and store in a Coplin jar.

Method 2.

1. The air dried smears can be fixed and stained using Rapi-Diff II, which contains 3 different solutions:

Solution A: Fixing Solution (Methyl alcohol) 30 seconds
Solution B: Acid dye (Eosin) 30 seconds
Solution C: Basic dye (Methylene blue polychrome) 30 seconds

1. The slides are then rinsed in water, dried and examined at 100x magnification under oil immersion.

Results:

Count the total number of different WBC in approximately 200 cells per slide. From this the percentage different cells types can be calculated.

TOTAL WHITE BLOOD CELL COUNTS

As in the determination of differential blood cell counts, the total number of white blood cells per ml is an indication of the general health and a useful marker in investigating the effects of immunostimulants.

Equipment:

Haemocytometer

Microscope

Microtitre pipettes

Reagents:

Sterile saline (Ringer solution)

Manual Counts

Method:

1. Remove blood from a stunned or anaesthetised animal using 27 gauge needle and a 1 ml syringe, flushed with heparin, and transfer to an Eppendorff.
2. The blood can then be diluted (1:20) with sterile saline and left at room temperature until ready to count the cells.
3. A small volume of the cell suspension is placed under the "heavy" coverslip of the haemocytometer and the white cells in the 4 large corner squares counted once the flow has ceased.
4. This figure is then averaged and multiplied by the dilution factor [x20 for the above dilution and $x10^4$ (to correct for the volume under the cover slip)] to obtain the number of white blood cells per ml. The dilution will depend on the species and the physiological state of the animals in question and should be appropriately adjusted.
5. Care should be taken when distinguishing the different cell types present.

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USING THE COULTER COUNTER

Equipment:

Coulter counter

Pipettes 100 µl - 1000 µl

Reagents:

Isoton II

Saponin

Sterile saline (Ringers' solution)

Method:

1. Add 100 µl of fresh blood to 20 mls of Isoton II and add 100 µl Saponin.
2. Need 20 ml of Isoton II as a blank.
3. Take duplicate readings per sample from the Coulter counter.

Results:

The Coulter counter only measures 0.5 ml in a sample so, reading x 2000 x 2 to give volume of WBC ml^{-1}.

HAEMATOCRIT VALUES

The percentage volume of red blood cells is a general indicator of health status and is a recommended parameter to measure after chemical administration or change in diet.

Equipment:

Haematocrit centrifuge

Haematocrit tubes

Sealant

Slide reader

Method:

1. Take blood samples from anaesthetised animal.
2. Let blood flow up the pre-heparinised haematocrit tubes to 3/4, wipe and seal. Prepare duplicate sample.
3. Place in the haematocrit centrifuge at 10,000rpm for 1 minute.
4. Place in haematocrit slide reader.

ADHERENCE AND ACTIVATION OF NEUTROPHILS AND MONOCYTES.

Adherence to glass and increased oxygen radical production as shown by the reduction of nitroblue tetrazolium to formazan are indicators of activation of phagocytic cells.

Equipment:

Coverslips

Slides

Petri dishes

Humid chamber

Microscope

Reagents:

0.2% nitroblue tetrazolium (NBT)

Sterile saline (Ringer solution)

Method:

1. Withdraw blood and place drop onto coverslip in Petri dish. Place in the humid chamber and incubate for 30-60 minutes at room temperature.
2. Wash the coverslip with saline to remove non-adherent cells.
3. Add a drop of NBT onto the coverslip and incubate in the humid chamber for 20-30 minutes at room temperature.
4. Wash off the NBT with saline and place a drop of NBT onto the slide.
5. Invert the coverslip with the adherent cells onto the slide and count the cells using a normal microscope at 40x magnification.

Results:

Count the number of positively blue cells in approximately 100 cells.

PRODUCTION OF REACTIVE OXYGEN SPECIES BY NEUTROPHILS AND MONOCYTES

Production of bactericidal anions by circulatory phagocytes can be seen by the ability to reduce yellow nitroblue tetrazolium to blue formazan and quantified by spectrophotometric analyses of the blue formazan after it is solubilized in dimethyl formamide.

Equipment:

Spectrophotometer
Microtitre plates
Microtitre pipettes
Glass test tubes
Glass microvolume cuvettes (1 ml)

Reagents:

Heparin
0.2% nitroblue tetrazolium (NBT) solution
Sterile saline (Ringer solution)
N-dimethyl formamide (DMF)

Method:

1. Withdraw blood from anaesthetised frog or fish using a heparinised (150 U ml^{-1}) syringe.
2. Place 100 µl of blood into a microtitre plate well and add 100 µl of 0.2% NBT solution.
3. Incubate at room temperature for 30 minutes.
4. After incubation period, remove 50 µl of the NBT/blood solution and add to a **GLASS** test tube which contains 1 ml of DMF (N.B. DMF degrades plastic)
5. Centrifuge for 5 minutes at 3000g
6. Remove supernatant and read in a spectrophotometer at 540nm in a 1 ml **GLASS** cuvette.

Results:

Reading value x4 = mg NBT formazan /ml blood

PHAGOCYTIC INDEX READING FROM BLOOD

Circulatory phagocytic cells are able to internalise and destroy invading micro-organisms. These cells are predominantly neutrophils and monocytes and coating the bacteria with serum (opsonising) increases the attraction of the bacteria to phagocytic cells.

Equipment:

Microtitre well plate
Microscope slides
Microscope
Microtitre pipette

Reagents:

Heparin
Bacterial suspension
95% Alcohol
7% Giemsa or Rapi-Diff II Stain

Method:

1. Remove blood from an anaesthetised animal via cardiac puncture using a heparinised needle.
2. Place 100µl of blood with 100µl of bacterial suspension into a microtitre well.
3. Mix with a pipette and incubate for 30 minutes at 22-28°C.
4. Remove 5µl and place on an alcohol cleaned slide and make a smear.
5. Fix with 95% Alcohol for 5 minutes.
6. Stain with 7% Giemsa for 10 minutes, or Rapi-Diff II for recommended time.
7. Examine 200 cells and record the number with engulfed bacteria.

Bacterial Suspensions

1. Obtain bacteria at a concentration of 1×10^8 ml^{-1}*
2. Opsonise with serum.
3. Dilute serially to a range from 10^8 - 10^3 cfu ml^{-1}

* = OD of 0.4 for *Aeromonas hydrophila* at absorbance of 610nm.

Calculations

Count 200 cells and record the number with ingested bacteria.

The phagocyte index is:

$$\frac{\text{Number of cells with ingested bacteria}}{\text{Number of cell counted}} \times 100$$

BLOOD RESULT SHEET 1

Experiment No: **Average Weight:** **g**

Animal	Date	PCV Average			Observations
		1	2	%	

DIFFERENTIAL WHITE BLOOD CELL COUNTS

Animal	Date Fixed	Date Stained	Cell counts (100 cells counted in total)				
			Neut %	Mono %	Eosin %	Baso %	Lymph %

TOTAL WHITE BLOOD CELL COUNTS

Animal	Date	Square				Average per square	Value per ml
		1	2	3	4		

LYSOZYME ASSAY (TURBIDIMETRIC ANALYSIS)

Ellis. A.E. 1990. Techniques in Fish Immunology.

Lysozyme is an enzyme present in the blood and tissues which cause bacterial lysis. The concentration can be assayed by measuring lysis of the highly susceptible bacterium *M. luteus* (*lysodiekticus*)

Equipment:

Centrifuge
Eppendorf
Pipette
Universals
Cuvettes
Spectrophotometer (540 nm)

Reagents:

Bacteria (*M. luteus*)
0.04M PBS pH 5.5

Method:

1. Collect serum sample (either fresh or frozen)
2. Make the bacterial suspension so that the Transmission = 40% at λ 540nm
3. Cuvette 1 = blank (PBS only)

Cuvette 2 = Bacterial suspension + serum sample

4. Dilute serum sample in the bacterial suspension 1 in 5. e.g. 0.4 ml serum to 2 ml bacterial suspension.
5. Place cuvette 2 in the spectrophotometer and record reading after 0, 1, 2, 3, 4 and 5 minutes at 540nm on Transmission.

Results.

An increase in Transmission of 0.001/minute is defined as a unit of lysozyme activity.

LYSOZYME LYSOPLATE ASSAY

Ellis. A.E. 1990. Techniques in Fish Immunology.

Equipment:

10 x 10 cm Glass plates

Punch

Microvolume pipettes

Filter papers

1 kg weight

Reagents:

PBS buffer (0.07M, pH 6.2)

15 ml of 1% agarose gel

Suspension of *Micrococcus luteus*

Distilled water

1.25% Methyl violet solution

Lugol's iodine

Absolute alcohol

Hen egg white lysozyme

1% bovine serum albumin (BSA)

Method:

1. Prepare 15ml of 1% (w/v) agarose gel in 0.07M pH6.2 PBS buffer, containing 50 µg/ml *M.luteus*
2. Pour onto 10 x 10 cm cleaned glass plates on a flat surface
3. Punch rows of 3 mm depth holes along the agar gel plate
4. As standards add 2 fold serial dilutions of hen egg white lysozyme (original conc. 1.6µg/ml) in 1 % bovine serum albumin (stabiliser) in PBS, and add to wells.
5. Add samples of serum, plasma, or mucus are added to the wells in 9 µl volumes and store the plates overnight at 20°C for a minimum of 17 hours
6. Measure the diameter of the zones of clearing.

Staining:

1. Wash the plate in distilled water for 30 minutes.
2. Cover the gel with 1 wet and 7 dry filter papers, and a glass plate on the top. Add pressure with a 1kg weight and press for 10 minutes.
3. Remove the filter papers and dry the gel plate using hot air.

4. Place the gel plate in solution of 1.25% methyl violet for 1 minute.
5. Transfer Lugol's iodine solution for 15 seconds.
6. De-stain in absolute alcohol until the clear zones appear.
7. Air dry and measure the diameter of the zones of clearance.

Results:

Concentration of lysozyme in the sample is read against a standard graph of the hen egg white lysozyme. Diameter of the cleared zones are proportional to the $\log_2$ of lysozyme concentration between 1.6-1.0 ∝g/ml.

MACROPHAGE SEPARATION

Secombes. C.J. 1990. Techniques in Fish Immunology.

In fish and frogs the anterior kidney and spleen, respectively, are the preferred organs for isolation and separation of the tissue dwelling phagocytic cells.

Equipment:	**Reagents:**
Dissection kit	Percoll
100μm nylon mesh	Hanks Balanced Salt Solution (HBSS)
Petri dishes	L-15 medium
Universals	Foetal Calf Serum (FCS)
Centrifuge	Penicillin and Streptomycin
Haemocytometer	Heparin
	Minimal Essential Medium Eaglex10 (MEM)

Solution A:

L-15, 2% FCS, heparin ($10uml^{-1}$)

Solution B:

L-15, 0.1% FCS, penicillin and streptomycin ($100uml^{-1}$)

Solution C:

L-15, 5% FCS

Percoll Gradient:	**51%**	**34%**
Sterile distilled water	3.9 ml	5.6 ml
Percoll	5.1 ml	3.4 ml
MEM	1 ml	1 ml

Transfer 10ml of 51% Percoll to a Universal . Mark the interface. Gently layer 5 ml of 34% percoll on top. If using a continuous Percoll gradient then place 6 mls of the Percoll into a universal, mark the interface and gently layer the cell suspension on top.

SEPARATION OF MACROPHAGES FROM SPLEEN OR HEAD KIDNEY.

Method:

1. Either overdose the animal with an alcohol solution of benzocaine in water or stun by a blow to the head.
2. Blood may be taken at this stage via **cardiac puncture** with or without anticoagulant.
3. Dissect out spleen or head kidney using aseptic technique and place individual organs into **5mls** of solution **A.**
4. Transfer the organs and solution to a sterile Petri dish and macerate by pushing through a sterile 100 μm mesh using the end of a sterile syringe. Replace in the original container.
5. Carefully layer the 5mls of spleen suspension onto **Percoll Gradient** and centrifuge at 400g (2000rpm) for 25 minutes at 4°C.
6. Remove the cells at the marked interface and place into 3 mls of solution **B.** Centrifuge at 1100g (3400rpm) at 4°C and for 15 minutes to allow the cells to pellet and increase the numbers of cells recovered.
7. Re-suspend the pellet with 1 ml of solution **B**.
8. Remove 20μl of the cell suspension add to 20μl of 0.1% Trypan Blue, and count the viable cells (NOT BLUE), using a haemocytometer.
9. The concentration of cells in the suspension is given by average in 4 large cells x 2 x 10^4 = number of cells ml^{-1}. It may be necessary to adjust the cell concentration at this stage.

These cell suspensions can be used on coverslips for phagocytosis assays or in microtitre plates for assays of reactive oxygen species or bacterial killing capacity.

PHAGOCYTOSIS ASSAY USING TISSUE MACROPHAGES.

Secombes. C.J. 1990. Techniques in Fish Immunology.

Macrophages are able to recognise, internalise and destroy micro-organisms by a process called phagocytosis. Activated macrophage cells engulf bacteria and produce bactericidal anions which destroy the internalised organism.

Equipment:

Coverslips

Petri dishes

Haemocytometer

Microscope

Reagents:

L-15 medium

Foetal Calf Serum (FCS)

Rapi -Diff II stain

Hanks Buffered Salt Solution (HBSS)

Lysis Buffer(0.1M Citric acid,
1% Tween20 and 0.05% crystal violet)

Method:

1. Harvest macrophages from spleen or head kidney, count viable cells and adjust the concentration of viable cells per ml in L-15 containing 5% FCS, if required.
2. Place 100 µl on to a coverslip in a Petri dish and incubate for 2 hours at 22°C.
3. Wash off the non-adherent cell with sterile HBSS and feed the macrophage monolayer with 100 µl L-15 + 5% FCS, and maintain at 22°C.
4. To estimate the number of macrophages which have adhered, lyse the cells of a representative culture and count the nuclei. This is done by draining the selected culture and treating with 100 µl of lysis buffer. Gently scrape the cells off the coverslip with the pipette tip, transfer to a counting chamber and count the number of nuclei.
5. Prepare an opsonised bacterial suspension containing 10^8 bacteria cells per ml. Make serial dilutions and add 100 µl to each monolayer to give a bacteria to cell ratio of 10:1 - 5:1.
6. Incubate at 22°C for 1 hour.

7. Wash cell macrophages three times with HBSS.
8. Fix cells in 95% alcohol for 5 minutes.
9. Drain, air dry and stain with Rapi-Dif II stain for recommended time period.
10. Rinse thoroughly dry and examine under oil immersion at x 100 magnification.
11. Count 200 macrophages and record the number containing ingested bacteria.

MEASURING RESPIRATORY BURST FROM TISSUE MACROPHAGE CELLS

Phagocytic cells possess a unique membrane bound enzyme called NADPH oxidase, capable of reducing molecular oxygen to superoxide anions. This anion has bactericidal function and the whole process is called the Respiratory Burst (RB).
The superoxide anion (O_2^-) is the first product to be released from the RB and production of this anion can be enhanced after *in vitro* membrane stimulation using a chemical stimulator phorbal myristate acetate (PMA). Measurement of this anion is a reliable method of quantifying the RB produced by activated macrophage cells.

MEASUREMENT OF THE INTRACELLULAR RESPIRATORY BURST

This method measures the ability of the activated macrophage cells to produce reactive oxygen species (O_2^-) intracellularly, which is released into the culture medium after cell membrane rupture.

Equipment:

Multi-scan spectrophotometer
at 620 nm
0.22μm porosity disc filter

Reagents:

nitroblue tetrazolium (NBT)
phorbol myristate acetate (PMA)
Absolute alcohol
70% alcohol
2 M Potassium hydroxide (KOH)
Dimethyl sulphoxide (DMSO)
Hanks Balanced Salt Solution (HBSS)

Method:

1. Prepare macrophage monolayers as before.
2. Nitroblue tetrazolium (NBT) is dissolved at 1 mg ml $^{-1}$ HBBS. This is then filter sterilised using a 0.22 μm porosity disc filter. Aliquot some NBT in a bijou and to this add PMA at 1 μg/ml^{-1}, from a stock solution of 1 mg ml $^{-1}$ in ethanol, stored at -20^{0}C.

3. Wash the monolayers twice with filter sterilised HBSS and then add 100 µl of either NBT or NBT + PMA to the appropriate cells.
4. Incubate the cells and solution at the required temperature for 60 minutes*, after which the reaction is stopped by the removal of the medium and addition of absolute alcohol, for 2 minutes. The cells are now fixed and need to be washed three times with 70% alcohol and left to air dry.
5. The formazan produced by the cells is then dissolved by adding 120 µl of 2 M KOH and 140 µl of DMSO. This should be done in a vented hood as the DMSO is very toxic. The solutions are mixed together and produce a blue colour.
6. The optical density (O.D) of the blue colour can then be read in a multi-scan spectrophotometer at 620nm.
7. Blanks should contain NBT+PMA only.

* The incubation time may need to be adjusted to suit the experimental purpose

MEASUREMENT OF EXTRACELLULAR RESPIRATORY BURST

This method measures the ability of the activated macrophage cells to produce reactive oxygen species (O_2^-) extracellularly. This technique also enables repeated measurements to be made over a time period as the cells do not have to be fixed to release the O_2^- into the culture medium.

Equipment:

Multi-scan spectrophotometer

0.22µm porosity disc filter

Reagents:

Hanks Balanced Salt Solution (HBSS)

Distilled Water

phorbol myristate acetate (PMA)

Ferricytochrome c

REFERENCES

The following methods can be found in Anderson.D.P and Siwicki.A.K. 1993. Immunostimulation in Fish: Measuring the Effects of Stimulants by Serological and Immunological Methods.

- Total white blood cell counts (Manual)
- Haematocrit values
- NBT adherence
- NBT spectrophotometer
- Circulatory Phagocytic Index

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- Isolation of macrophage cells
- Intracellular respiratory burst
- Extracellular respiratory burst.

The Non-Specific Immune System of Farmed *Rana rugulosa*

Introduction

Frogs have been extensively farmed over the last 10 years in South East Asia, where farming has been kept as a profitable small-scale industry. Initial problems associated with the culture of these semi-aquatic animals were identified as poor husbandry, inadequate nutrition and sub-optimal water quality and attempts have been made to highlight, to the farmers, the importance of correct environmental conditions to produce healthy stock. One of the major problems in amphibian culture is bacterial disease, particularly septicaemia and large losses of stock due to this infection, commonly referred to as 'red-leg' have occurred. The symptoms of this condition include oedema, lethargy, haemorragic lesions on the thigh and abdomen and eventually death, but diagnosis is difficult and often there are no signs prior to death.

A single causative agent has not yet been identified but a range of bacteria have been recovered from septicaemic frogs, although the opportunistic micro-organism *Aeromonas hydrophila,* ubiquitous in the aquatic environment has been suggested to be the main pathogen associated with 'red-leg'. On the farms prophylactic use of antibiotics has been used in attempts to control this disease, making future treatments even moredifficult due to increased antibiotic resistance.
Stress is a major concern in aquaculture and factors of importance may include poor water quality, high stocking densities, inadequate nutrition, grading, handling and transportation. Although some of these can be controlled, they cannot be eliminated especially when taken in consideration with commercial pressures.

All vertebrate species have a defence system which protects the animals against disease. The more advanced vertebrates (fish, frogs and mammals) have evolved independent innate and adaptive immune systems comprising of cellular and humoral factors. The non-specific immune system is the primary defence pathway that initially encounters a infectious attack and therefore activation is important to inhibit pathogenic invasion and establishment of disease. It is known that when many species of animal are stressed an immunocompromised state may result and thereby increased susceptibility to specific and opportunistic pathogens in the environment. This may also happen with farmed frogs.

Amphibians have proved good laboratory models, in particular, *Xenopus laevis* (the african clawed toad) however most immunological studies with this specieshave focused on the adaptive immune response isolating antibodies and investigating immunological tolerance. There was very little information on the innate immune system in ranid species and this data was fundamental before further work investigating the effect of immunostimulant and stress on the non-specific immune system of frogs could be completed.

The study was carried out to investigate and measure the red and white blood cell function and morphology and evaluate serum lysozyme activity in the tropical farmed frog *Rana rugulosa.*

Animals

A population of *Rana rugulosa* were bred and housed in the wet laboratory in the Aquatic Animal Health Research Institute (AAHRI) Bangkok, Thailand. They were maintained under laboratory management in conditions similar to the farms. Frogs were given 100% daily water changes and feed once per day at approximately 3% body weight on a pelleted diet. All animals were kept in glass tanks which comfortably held 20 animals with an average weight of 60-80g and the ambient temperature was 29 ± 2^0 C.

Experimental Procedures

At the appropriate sample time five animals were removed from their tanks and stunned by administering a sharp blow to the head. They then were bled by cardiac puncture using a 27 gauge needle, coated with heparin for whole blood analysis or without anticoagulant for serum collection. Approximately 0.5 - 1 ml of blood was removed from each animal and immediately transferred to an Eppendorff tube.
The haematocrit or packed cell volume (PCV) of the red blood cells was determined by allowing the blood to flow up haematocrit capillary tubes, sealing with a commercial sealant and then centrifuging in a haematocrit centrifuge at 10,000 rpm for
1 minute. Duplicates were completed per animal and the the percentage PCV read using a haemotocrit slide reader.
To calculate the total white blood cell counts, a volume of blood was diluted using sterile amphibian saline and the white cells counted using a haemacytometer and phase contrast microscope at x 20 magnification. Counts for each animal.

The reduction of nitroblue tetrazolium (NBT) to formazan by oxygen radicals from peripheral blood phagocytes was measured using the spectrophotometric method.
One hundred microliters of blood was placed into a microtitre well plate and 100µl of a 0.2% NBT solution was added to the wells and left at room temperature for 30 minutes. Then 50µl of the blood-NBT suspension was transferred to a glass test tube containing 1 ml of N,N-dimethyl formamide (DMF) and centrifuged at 300g for 5 minutes. The supernatant was removed and absorption read in a spectrophotometer at 540 nm in a 1 ml cuvette. The average value per animal was taken from duplicate samples and the result was expressed as optical density per ml of whole blood.

If serum was required the remaining blood was allowed to clot at room temperature for 4 hours and then centrifuged at 13,000 rpm for 5 minutes. The clear supernatant was collected and frozen at -20^0 C until required.
The serum from individual animals was pooled because the volumes recovered were small. The turbidimetric method was used to measure the serum lysozyme activity: briefly, serum was added to a suspension of *Micrococcus lysodeikticus* in 0.04M phosphate buffered saline at pH 5.5. This was placed into a cuvette in a spectrophotometer and the transmission at 450nm was recorded every minute for a total of 5 minutes. A unit of lysozyme activity was defined as the amount of sample causing a decrease in transmission of 0.001 per minute.

After exsanguination, the spinal cord of the frogs was severed to ensure the animal was dead and the spleen was removed using aseptic technique . On removal of the ventral skin and muscle the internal body cavity were sprayed with 70% alcohol and the instruments were kept in 70% alcohol during the procedure. Whilst removing the spleen, the frogs were examined for any external or internal signs of pathological abnormalities and results were noted.
Once the spleen was removed it was transferred immediately into 5 mls of solution A (L-15 medium, 2% FCS and 10 units ml^{-1} heparin). The organ was macerated using a sterile 100μm mesh and a syringe to produce a spleen cell suspension. At this stage there is a mixed cell population and so 5 mls was carefully placed onto a prepared 34:51% density percoll gradient and centrifuged at 400g for 25 minutes at 4^0 C. Cells were collected from the marked interface the and placed in 3 mls of solution B (L-15 medium, 0.1% FCS and 100 units ml^{-1} penicillin/streptomycin) and centrifuged again at 1100 g for 15 minutes at 4^0 C. This produced a concentrated cell pellet which was resuspended in 1 ml of solution B. An aliquot of this was added to 0.1% trypan blue solution and the number of viable cells was counted using a haemacytometer and a phase contrast microscope x 20 magnification.

Results

The average PCV value for control *R. rugulosa* was recorded as 25.5 ± 2.9% (n=14) with a range of 19-29%. The average total white blood cell counts for control animals was $7.6 \pm 3.6 \times 10^7$ ml^{-1} (n=11) with a range of 3.2×10^7 - 1.6×10^8 cells per ml.
The sera had been pooled in groups chronologically as the blood was taken where there were 9 samples of pooled sera, each sample representing 3-5 animals. This allowed duplicate measurements to be recorded. No lysozyme activity was found in 4 of the pooled samples and the remaining 5 samples gave an activity range of 50-500 units per ml and this represented 56% of the frog population sampled.

The NBT assay results were expressed as optical density ml^{-1} of whole blood and gave an average value of 19.3 ± 6.7 (n=19) and this included a range of values from 11.6 to 30.4.
The number of spleen macrophage cells recovered per animal gave an average value of $1 \pm 0.9 \times 10^7$ ml^{-1} (n=15) with a close range of 1.6×10^6 to 3.2×10^7 cells per ml.

Discussion

Data on the afore mentioned innate parameters was not available for the farmed tropical frog *Rana rugulosa*, prior to this study. There was a lot of individual variation as indicated by the high standard deviations; this was particularly true for the whole blood and serum lysozyme activities. The values for the PCV and white blood cell counts are similar to ranges in fish.
The blood NBT results are interesting as this clearly demonstrates the ability of frog blood phagocytic cells to produce the intracellular respiratory burst anion (O_2^-).
The number of spleen macrophage results is slightly lower than has been found from fish anterior kidney but this may be because initial tissues extracted as less.

However the results did provide a base-line range of values for a single population of frogs held under the described conditions and could be used to compare with other normal, stimulated and stressed animals.

Administration of Immunostimulation *in vivo* to farmed ranid species.

Introduction

Immunomodulation of the innate defence system has been attempted for many cultured aquatic species using synthetically and naturally derived substances. Administration of chemical stimulators to fish has augmented the bactericidal activities of the cellular and humoral parameters of the innate defence system, and previous work available in the literature has highlighted a specific enhancement of the phagocytic cell functions by increasing ingestion and killing of pathogens. In more recent studies naturally derived compounds called glucans (in particular β-1, 3 -1,6 linked polysaccharides derived from yeast and fungi) have been shown to be particularly effective in fish.

Application of β-glucans to animals in culture systems may prove beneficial by enhancing the primary defence pathway thereby increasing the animals resistance to specific and opportunistic pathogens and reduce the need for antibacterial prophylaxis and therapy. Beta-glucans may be particularly useful at specific stressful periods to reduce the impact of immunosuppression and stress-associated mortalities.

Although frogs are regarded as robust creatures adapting to varied conditions and environments, they are nevertheless extremely susceptible to stress: this can result in large-scale losses of stock due to stress-associated diseases.
The aim of this study was to investigate the effect of β-glucans administered by different routes, on the innate defence parameters of tropical frogs previously identified.

Animals

A population of *Rana rugulosa* were bred and housed in the wet laboratories of the Aquatic Animal Health Research Institute (AAHRI) Bangkok, Thailand. They were maintained under laboratory management in conditions similar to the farms. Frogs were given 100% daily water changes and feed once per day at approximately 3% body weight on a pelleted diet. All animals were kept in glass tanks which comfortably held 20 animals with an average weight of 60-80g and the ambient temperature was 29 ± 2^0 C.

Immunostimulants

There were 2 different β-1,3 - 1,6 linked glucans administered to the frogs. The insoluble compound Macrogard (Vetrepharm) and the soluble glucan Laminaran (Sigma).

1. Administration by Bath

Frogs were removed from their original tank and placed into glass tanks with 5 animals in each. There were 3 experimental groups and 1 control group.
The β-glucan Laminarin was administered at 5mg/L water for 2 hours to 3 treatment groups after which the animals were removed and placed into another tank and held within appropriate conditions. The control animals received no treatments. Frogs were observed daily for any adverse behaviour or signs of illhealth. At 3, 7 and 12 days post-treatment 5 animals were sampled to measure the selected innate defence parameters.

2. Administration by Capsule

Frogs were given β-glucan Macrogard orally by capsule. Fifteen frogs were given the glucan suspension in sterile amphibian saline in a gelatine capsule. Each capsule contained 200µl of a 1% (w/v) suspension. The control animals received nothing. At 5, 10 and 15 days post-treatment 5 animals were sampled to measure the selected innate defence parameters.

3. Administration by Intraperitoneal injection (i.p)

Frogs were administered the β-glucan Macrogard by i.p injection. Fifteen frogs were given 200µl of a glucan suspension at 1% (w/v) in sterile amphibian saline. The control animals were injected with a similar volume of sterile saline. At 3, 7 and 15 days post-treatment 5 animals were sampled and selected parameters of the innate defence system were measured.

4. Administration of glucan orally in the feed

Fifteen frogs were fed β-glucan (Macrogard) coated pellets at 1% (w/w) for 2 weeks once daily at 3% bodyweight. The control animals received the normal diet at the same rate. After the 2 weeks, 5 animals were sampled once a week to measure the innate defence system parameters.

NBT Assay

Upon activation of macrophage cell membrane there is increased uptake of molecular oxygen by the cell which is then reduced to toxic anion (O_2^-) by a membrane-bound enzyme cascade. The NBT assay measures the ability of the macrophage cells to produce the bactericidal anion.
The wells containing the macrophages were washed twice with sterile hanks buffered salt solution (HBSS) and then 100µl of either NBT alone or NBT+PMA (phorbal myristate acetate is a chemical stimulator frequently in fish used to induce the respiratory burst activity of phagocytes). The cells and solutions were incubated at 22^0 C for 1 hour after which the reaction was stopped by addition of 100% alcohol to the wells, before carefully washing with 70% alcohol to remove any remaining NBT solution. The cells were left to air dry, 2M potassium hydroxide and

dimethylsulphoxide (DMSO) added which solubilises the released product and results were read in a multiscan spectrophotometer at wavelength 610nm.

Experimental Procedures

At the appropriate sample time 5 animals were removed from their tanks and administered a sharp blow to the head to stun the frogs. The animals were then bled by cardiac puncture using a 27 gauge needle coated with heparin for whole blood analysis or without anticoagulant for serum collection. Approximately 0.5 - 1 ml of blood was removed per animal and immediately transferred into an Eppendorff tube.
The haematocrit or packed cell volume (PCV) of the red blood cells was determined by allowing the blood to flow up haematocrit capillary tubes, sealing with commercial sealant and then centrifuged in a haematocrit centrifuging at 10,000 rpm for 1 minute. Duplicates were completed per animal and the results were read using a haemotocrit slide reader to determine the percentage PCV.
Approximately 20µl of whole blood was placed onto a cleaned microscope slide and a blood smear prepared: this was done in duplicated per animal. The smears were allowed to air dry, fixed in alcohol and stained using an acidic and basic staining kit (Rapidiff II) according to the manufacturesers instructions. The slides were left to air dry, examined under oil immersion at 100x magnification and the percentage differential cell counts were determined by counting 100 cells per slide.
To calculate the total white blood cell counts, a volume of blood was diluted using sterile amphibians saline and the number of white blood cells were counted using a haemacytometer and a phase contrast microscope at x 20 magnification. The total number of white blood cells per ml was recorded..
If serum was required the remaining blood was allowed to clot at room temperature for 4 hours and then centrifuged at 13,000 rpm for 5 minutes. The clear supernatant was collected and frozen at -20^0 C until required.
The haematology results are presented in Table.1.

After exsanguination, the spinal cord of the frogs was severed to ensure the animal was dead and the spleen was removed aseptically. Whilst removing the spleen, the frogs were examined for any external or internal signs of pathological abnormalities and results were noted.
On removal of the ventral skin and muscle the internal body cavity was sprayed with 70% alcohol and the instruments were kept in alcohol during the procedure. Once the spleen was removed it was transferred immediately into 5 mls of solution A (which contained L-15 medium, 2% FCS and 10 units ml $^{-1}$ heparin). The organ was macerated using a sterile 100µm mesh and a syringe to isolate the spleen cell suspension. Macrophages were separated from the mixed cell population by differential centrifugation. Fine mls of cell suspension was carefully placed onto 6mls of a prepared continous density Percoll gradient and centrifuged at 400g for 25 minutes at 4^0 C. The cells at the interface were collected and placed into 3 mls of solution B (which contained L-15 medium, 0.1% FCS and 100 units ml^{-1} penicillin/streptomycin) and centrifuged again at 1100g for 15 minutes at 4^0 C. This produced a concentrated cell pellet which was resuspended in 1 ml of solution B. An

aliquot of this was added to 0.1% trypan blue solution and the number of viable cells were counted using a haemacytometer and a phase contrast microscope x 20 magnification.

One hundred microliters volumes of the cell suspension were placed into a sterile 96 well plate which was incubated at 22^0 C for 2 hours to let the macrophage cells to adhere to the plastic surface in a monolayer. After incubation the wells were washed twice with sterile amphibian saline and 100µl of solution B added to each well. The cells were then used immediately or left overnight at 22^0 C before measuring production of the intracellular respiratory burst using the nitroblue tetrazolium (NBT) assay. The number of macrophage cells adherent in a representative well was estimated by counting the number of nuclei released from the cells after addition of 100µl of a lysis buffer (0.1M citric acid, 1% Tween 20 and 0.05% crystal violet).

Superoxide anion production

An NBT assay was carried out on th macrophage cultures and the optical densities read after reduction of NBT to reduced formazan. This assay measures the colour change from blue nitroblue tetrazolium to reduced fromazan, where optical densities are directly related to the amount of O_2^- released from the macrophages.

The results were then adjusted to read for 1×10^5 cells ml^{-1} and the average value for 5 animals was calculated and presented in Table 2.

Results

Table.1. Haematology results from control and immunostimulated frogs

Experiment	Average PCV ± s.d (%)	Average TWBC ± s.d (ml^{-1})
Bath control (n=3)	32 ± 7	1.1 ± 0.3 x 10^8
Bath Day 3 (n=4)	33 ± 5	0.9 ± 0.6 x 10^8
Bath Day 7 (n=5)	29 ± 7	8.0 ± 1.0 x 10^8
Bath Day 12 (n=5)	30 ± 5	6.0 ± 1.0 x 10^7
Capsule control (n=5)	26 ± 6	4.0 ± 2.0 x 10^7
Capsule Day 5 (n=5)	30 ± 3	6.0 ± 2.0 x 10^7
Capsule Day 10 (n=5)	34 ± 4	5.0 ± 2.0 x 10^7
Capsule Day 15 (n=2)	29 ± 0	7.0 ± 3.0 x 10^7
I.P control (n=5)	22 ± 11	4.0 ± 2.0 x 10^7
I.P Day 3 (n=5)	24 ± 5	
I.P Day 7 (n=5)	19 ± 15	9.5 ± 3.0 x 10^7
I.P Day 15 (n=4)	39 ± 3	7.0 ± 0.6 x 10^7
Oral Control (n=5)	35 ± 5	7.4 ± 1.6 x 10^7
Oral Week.1 (n=5)	27 ± 7	5.0 ± 3.0 x 10^7
Oral Week.2 (n=5)	25 ± 6	7.0 ± 4.0 x 10^7

The serum from different animals was pooled together and frozen at -20°C, where the haemolytic activity of complement in the sera is yet to be analysed. The values for differential white blood cell counts have still to be completed.

Table.2. NBT spleen macrophage results

Experimental Group	**NBT+PMA ($1x10^4$cells per well)**	**NBT only ($1x10^4$cells per well)**
Bath Control	0.015 ± 0.007	0.011 ± 0.005
Bath Day 7	0.005 ± 0.003	0.001 ± 0.0009
Bath Day 12	0.014 ± 0.014	0.014 ± 0.015
Control Capsule	0.032 ± 0.019	0.032 ± 0.014
Capsule Day 5	0.023 ± 0.013	0.020 ± 0.016
Capsule Day 10	0.011 ± 0.003	0.013 ± 0.004
Capsule Day 15	0.001 ± 0.0009	0.003 ± 0.001
I.P Control	0.027 ± 0.014	0.046 ± 0.027
I.P Day 3	0.013 ± 0.009	0.023 ± 0.019
I.P Day 7	0.021 ± 0.025	0.035 ± 0.025
I.P Day 15	0.009 ±0.006	0.010 ± 0.005
Oral Control	0.041 ±0.014	0.023 ± 0.021
Oral Week.1	0.002 ± 0.0008	0.002 ± 0.0008
Oral Week.2	0.004 ± 0.002	0.005 ±0.002
Oral Week.3	0.001 ± 0.000	0.002 ± 0.000

The results above include the optical density adjusted for 1 x 10^5 cells ml^{-1} ± the standard deviation.

Discussion

The haematology results did not indicate any significant differences between the control and glucan-treated groups. There was a lot of individual variation between animals in the sample groups. The range of values for the controls was similar to data from previously sampled frogs held under the same conditions (see Appendix 4). The serum and differential white blood cell values have still to be calculated and are therefore not included in this discussion.

The chemical stimulator PMA did not appear to enhance the production of intracellular O_2^- as measured by the NBT assay in either the stimulated or the control frogs except for the bath control and oral (feed) control animals where a greater amount of measurable O_2^- was produced compared with the NBT only cells.
In general there was not a great difference between the treated and control animals. The process of administering the glucans may in itself be stressful to the frogs. Injection and capsule administration in particular are invasive and in some cases treated animals did succumb to bacterial disease.

When isolating the macrophage cells, bacteria were routinely observed during the counting stage and were visible in the microtitre well plates. Antibiotics were included in the maintenance media (solution B) and this appear to inhibit further growth of the bacteria in the plates so innate parameters could be measured.
This finding initially was unexpected but already been noted as a phenomenon of farmed frogs early in the project. This fact must be taken into consideration when analysing the results since the frogs are under constant bacterial challenge so that the innate immune system is working to high capacity and may not be capable of further enhancment. This may also explain why administering the glucans to the animals sometimes resulted in mortalities: it may have been that any beneficial protection given by the glucan was outweighed by outgrowth of carried bacteria when the innate immune system was immunocompromised by to stress.

Effect of bacterial challenge in frogs administered the β-glucan Macrogard.

Introduction

It has been shown that administering a β-1,3 and -1,6 linked glucan derived from the cell walls of the yeast *Saccharomyces cerivisea* in fish has enhanced protection of the animal against bacterial challenge. This has been demonstrated for many fish species where the glucan has been administered either by intraperitoneal injection (i.p) or orally after coating onto the food.
Therefore both oral and i.p routes of administration were attempted in a population of ranid frogs given a bacterial pathogen and survival of the animals measured over time and the number of bacterial colonies recovered recorded.

Oral administration

A population of *Rana rugulosa* (average weight = 40g) bred and housed at the wet laboratory at Aquatic Animal Health Research Institute (AAHRI), Thailand were fed the β-glucan Vetregard (kindly supplied by Vetrepharm) at 0.1% (w/w) for 14 days prior to bacterial challenge.
The frogs were placed into control and experimental tanks with 6 animals per tank and the control animals received either nothing, bacterial injection alone or a combination of immunostimulant and bacterial challenge.
The bacterial pathogen was kindly supplied by M.Pearson identified as F2D8 which had been isolated from a diseased animal on a frog farm the previous year. A pure culture of the bacteria grown onto a tryptone soya agar plate (TSA) was measured spectrophotomically to produce 1 x 10^8 colonies ml^{-1} and then serially diluted in sterile saline to 1 x 10^3 and 1 x 10^4 colonies ml^{-1}.
Control and glucan-treated frogs received 0.1ml of each bacterial concentration. The animals were then replaced into their original tanks and 6 hours after bacterial administration 3 animals per group were killed and their spleens removed and macerated in 2 mls of tryptone soya broth (TSB). The original sample and a 10^{-1} dilution of the macerated organ was plated onto TSA plates and incubated overnight at 28^0 C. The following day the colony forming units were counted on the TSA plates.

This was completed again in 24 hours post-injection of the bacteria and the bacterial colonies recovered were compared for the control and stimulated animals.

Results

There were no mortalities in either the control or stimulated groups over the allocated time. However this was thought to be due to a low bacterial concentration administered to the frogs. There was a similar number of bacterial colonies grown on the TSA plates for both the control and experimental groups. This made quantification very difficult and suggested the need to repeat this work, however qualitatively there were fewer colonies in the glucan-treated frogs compared with the bacterial challenge group only.

Intraperitoneal administration

A population of *Rana rugulosa* (average weight = 40g) were bred and house in glass tanks in the wet laboratory at AAHRI, Thailand. The animals were separated into 4 groups of 6 animals per group : control (given no treatment), control (administered the bacterial concentration only) and 2 treated groups (received glucan and bacterial injection). Each of the groups receiving the β-glucan (Vetregard) were administered 0.2mls of a 1% (w/w) suspension in sterile saline. After 3 days the appropriate groups were given a bacterial injection by i.p with animals receiving 10^5 or 10^6 bacteria ml^{-1}. Administration of the bacteria was as described above and 6 hours and 24 hours 3 animals per group were sacrificed to remove the spleens. The organs were macerated in 2 mls of TSB and drop counts (20μl) were plated onto TSA plates as either neat, 10^{-1}, 10^{-2} and 10^{-3} dilution's where appropriate. The plates were incubated overnight at 28^0 C and the colonies counted the following day.

Results

For frogs given bacterial concentration of 10^6 bugs$\cdot ml^{-1}$ in both the control (bacteria only) and the treated (bacteria + glucan) groups there were a similar number of mortalities and there were no mortalities in the 10^5 bacterial ml^{-1} group.
Observations of the colony growth on the agar plates gave confluent growth for both bacterial concentrations even with extra dilution's completed.

Discussion

It was difficult to assess any protective effect that the glucan may have had as the numbers of colonies recovered for both studies were similar.
It should also be noted that there were no spontaneous mortalities except in the 10^6 bacterial ml^{-1} group administered either glucan + bacteria or bacteria only. It may be that the glucan gave no protection to the animals against a virulent challenge or that the bacterial concentration was too severe for the animals to cope with therefore not allowing the glucan to have a chance to exert any protection.
In the i.p glucan treated groups for both bacterial concentration there were no colony forming units (cfu) for the controls and only cfu for the glucan treated animals. This could be due to the samples being mixed together or it may be that there were more bacteria introduced to the animals that originally intended, as preliminary work on immunostimulants at Stirling produced live yeast cells and bacteria recovered from spleen cell suspensions of animals administered the same glucan.
It may be that the glucan treated animals had a deliberate and incidental bacterial infection which may have resulted in overwhelming the innate parameters of the frogs. hence mass colony growth from the glucan + bacterial animals and mortalities produced from the high bacterial concentration only. Therefore in future glucan work, a sterilisation step of the compound where applicable was done.

Effect of immunomodulation in tropical frogs with bacterial challenge.

Introduction

In following previous work on the effect of chemical stimulation by administering a β-glucan compound *in vivo* and investigating the effect of common environmental stressors on the cellular and humoral aspects of the frogs innate defence system, the animals were subjected to a bacterial challenge.
This was to observe any protective effect of the glucan and to see any detrimental effect of the temperature stress against survival rates of the frogs after receiving a bacterial pathogen.

Animals

A laboratory population of farmed ranid species were bred and housed at the Aquatic Animal Health Research Institute (AAHRI), Bangkok Thailand. They were fed once daily at 3 % body weight with pellets and given 100% water changes daily. The animals were kept inside the wet laboratory with approximately 20 animals per glass tank. The frogs used in this experiment had an average weight of 120g and all were regarded as clinically healthy. The criteria for this was: good appetite, increased body weight, intact, moist skin, clear eyes and active. Animals were monitored twice daily and any frogs with signs of disease were removed and isolated immediately.
For experimental purposes the animals were separated into groups of 5.

Experimental Procedure

The experimental groups were as follows: control group (saline), bacteria only, temperature stress and bacteria, glucan administration and bacteria and combination of glucan, temperature stress and bacteria.
The glucan treated animals were given 0.2mls of a 1% (w/v) intraperitoneal injection (i.p) of the glucan Macrogard suspended in sterile amphibian saline. The groups under temperature stress were maintained in a constant temperature room at
20 ± 2^0 C. The control group received an i.p injection of 0.2mls saline only.
All experimental groups were separated into their tanks and administered glucan or kept at 20^0C for 24 hours prior to injecting the bacteria. This was to monitor for any abnormal behaviour or impact of disease due to stress.

The animals were observed twice daily for 7 days and mortalities counted. At the end of the 7 days the surviving animals were sacrificed and bacterial recovery from the spleen recorded.

Bacteria

Animals were intraperitoneally injected with 0.1 mls of bacterial suspension in sterile saline per 100g body weight.
The bacteria was kindly supplied by Dr Temdoung Somsiri at AAHRI and had originally been recovered from a diseased frog and previously identified as

Aeromonas hydrophila before being kept on tryptone soya agar (TSA) slopes. The bacterium was resuspended in tryptone soya broth (TSB) and incubated at 28^0C for 24 hours before being streaked onto a TSA plate to check for pure growth.
A suspension of bacteria was obtained to give 4 x 10^7 CFU ml^{-1} and was suspended in sterile saline and injected into the animals.
The dose and route of administration of the bacteria was recommended by Dr. Somsiri.

Results

Table.1 Mortality Rates over Time

Time	Control (saline)	Bacteria only	Cold and Bacteria	Glucan and Bacteria	Combination and Bacteria
Day 1	no mortalities	3 mortalities	2 mortalities	no mortalties	1 mortality 2 diseased
Day 2	no mortalities	no mortalities	3 mortalities	1 mortality 1 diseased	4 mortalities
Day 3	no mortalities	no mortalities		1 mortality 1 diseased	
Day 4	no mortalities	2 diseased		2 mortalities	
Day 5	no mortalities	2 diseased		1 diseased	
Day 6	no mortalities	2 diseased		1 mortality	
Day 7	no mortalities	2 diseased			

At the end of the experimental period in total there were 0% mortality in the control (saline) group, 60% mortality in the bacteria only group and 100% mortality in all other groups.

In both the cold and bacteria and combination and bacteria groups there were 100% mortality by day 2.

Bacteriology results

The surviving (diseased) animals in the bacteria only group were sacrificed and the liver and spleen sampled for bacteria. There was heavy bacterial growth in the spleen of animal 1 (A1) compared to little growth from the liver and heavy growth in the liver of animal 2 (A2) compared with the spleen. Samples of pure growth were therefore taken from spleen of A1 and the liver of A2 and put onto TSA slopes for identification at IoA, Stirling.
The control (saline) animals had no mortalities over the 7 days and all animals were sacrificed at the end of the experimental period and again the liver and spleens were

sampled for bacteria. There was no growth on the plates for any of the saline treated animals except for animal 5 (A5) which gave heavy pure growth from the spleen. This was also preserved and identified at IoA, Stirling.

Table.2 Identification of bacteria using primary tests

Sample	CFU	Gram	Motility	O/F	Oxidase
A1 Bacteria spleen	Small cream	Negative SFR*	Yes	F	Positive
A2 Bacteria liver	Small cream	Negative SFR*	Yes	F	Positive
A5 saline spleen	Small white	Positive coccus	No	F	Negative
Original bacteria	Small cream	Negative SFR*	Yes	F	Positive

* SFR = short fat rod.

Discussion

There were no mortalities from the saline treated animals over the 7 days and no bacteria could be recovered from the liver or spleen using conventional methods. Administration of bacteria alone resulted in 60% mortality in 24 hours. The remaining animals survived for the duration of the experiment however by day 4 both had sever signs of disease recognised in the frogs as mass oedema of the abdomen, drying of the skin, pale and lethargic.
There was 100% mortality in both the cold and bacteria and the combination group within 48 hours. It was interesting that in the combination group 2 animals had rectal prolapse prior to other symptoms before death. It was suggested that this may have been due to internal parasites rather than an effect from the bacterial injection.
In all of the groups the mortalities were not sampled for bacteria due to post-mortem changes which may have affected results.

Results would suggest that the glucan alone did not give more protection against bacterial challenge compared to the other groups. The mortalities were more rapid in the cold+bacteria and the combination groups, suggesting that reduced temperatures exacerbated the disease status of frogs compared to other groups in the experiment.
The finding of reduced temperature as a form of stress to frogs has been investigated by Dr. Temdoung Somsiri, based at AAHRI, where the effect of reduced water temperatures on tadpoles given a bacterial challenge were investigated.

Effects of Two Stressors on the Innate Immune System of Farmed ranid species.

Introduction

In aquatic culture systems animals encounter many different forms of stress which can leave the animals immunocompromised and susceptible to aquatic pathogens. Previous work on the effect of stress in fish available in the literature provided information on in increased levels of corticosteriod hormone released into the blood stream. This compound has an immediate physiological effect on the animal and ultimately produces immunosuppressive state.
The stressors farmed aquatic species face include, overcrowding, poor water quality, sub-optimal temperatures and inadequate nutrition. The impact of many of these can be reduced by improved animal husbandry; however regular handling, grading and transportation stressors cannot be avoided and this can lead to stress-associated mortalities.
Frogs are very susceptible to stress and in particular to fluctuating temperatures, poor water quality and transportation which increase the number of disease outbreaks. The aim of this study was to investigate the effects over time of two selected stressors on the parameters of the non-specific immune system of frogs.

Animals

A population of *Rana rugulosa* were bred and housed in the wet laboratory in the Aquatic Animal Health Research Institute (AAHRI) Bangkok, Thailand. They were maintained under laboratory management in conditions similar to the farms. Frogs were given 100% daily water changes and fed once per day at approximately 3% body weight on a pelleted diet. All animals were kept in glass tanks which comfortably held 20 animals with an average weight of 60-80g and the ambient temperature was 29 ± 2^0 C.

Experimental Protocol

1. Temperature stress

Fifteen frogs were removed from their original tank and placed in three glass tanks with 5 animals per tank. These animals were housed in a temperature controlled room, with an average daily water and ambient air temperature was 20 ± 2^0 C.
All animals were fed once a day at 3% body weight with pellets and given 100% water changes each day with pre-cooled water.
The control group (n=5) were placed into a glass tank kept in the wet laboratory and fed with pellets once daily at 3% body weight and given 100% water changes daily. The average ambient temperature was 29 ± 2^0 C.
The frogs were sampled 24, 48 and 72 hours after being placed and maintained at 20^0C. The control animals were sampled after 96 hours.

The experiment was repeated with another 15 frogs which were sampled after 24, 72 and 240 hours at 20 ± 2^0 C. Macrophage cell function was measured using the NBT assay and serum samples were taken.

2. Poor Water Quality

It was suggested that frogs became susceptible to opportunistic pathogens in the environment if the water was not changed daily. This therefore was selected as a stressor to measure the effect on the innate immune system.
Ten frogs were selected randomly from the wet laboratory and transferred to an experimental tank. They were fed at 3% bodyweight with commercial fish pellets and given no water changes. After 48 and 168 hours, 5 animals were removed and sampled to measure the cellular and humoral aspects of the non-specific immune system. The animals were sampled for macrophage superoxide anion production using NBT assay and serum samples were taken.
The controls were kept in a different glass tank and given daily water changes and fed once a day at 3% body weight with commercial pellets.

Material and Methods

At each sample time fiveanimals were selected, stunned by a sharp blow to the head; blood was taken from three and the serum separated and the haematocrit, total white blood cell count and differential white blood cell counts were measured for the remaining two. Spleen weight, number of macrophage cells recovered and adherent ml^{-1}, and the NBT response *in vitro* was recorded for all five animals in the group.

At each sampling time the animals were removed from their tanks and administered a sharp blow to the head. The stuned animals were then bled by cardiac puncture using a 27 gauge needle coated with heparin for whole blood analysis or without anticoagulant for serum collection. Approximately 0.5 - 1 ml of blood was removed per animal and immediately transferred to an Eppendorff.
The haematocrit or packed cell volume (PCV) of the red blood cells was determined by allowing the blood to flow up haematocrit cappliary tubes, sealed using commercial sealant and then centrifuged in a haematocrit centrifuge at 10,000 rpm forl minute. Duplicates were completed per animal and the results were read using a haemotocrit slide reader to determine the percentage PCV.
Approximately 20µl of whole blood was placed onto a cleaned microscope slide and a blood smear was completed in duplicated per animal. The smears were allowed to air dry and then fixed in alcohol and stained using an acidic and basic stainig kit (Rapidiff II). The slides were left to air dry before being examined under oil immersion at 100x magnification and the percentage differential cell counts were completed by counting 100 cells per slide.
To calculate the total white blood cell counts, a volume of blood was diluted using sterile amphibian saline and then the number of white blood cells counted using a

haemacytometer and a phase constrast microscope at x 20 magnification. The total number of white blood cells per animal was recorded.
The remaining sample of blood without anticoagulant was left to clot at room temperature for 4 hours and then centrifuged at 13,000rpm for 5 minutes to collect the serum. The clear supernatant was then removed and pooled together and stored at -20^0 C. These samples will be used to assess the glucose level in the serum using a kit (Sigma).

After exsanguination, the spinal cord of the frogs was severed to ensure the animal was dead and the spleen was removed aseptically. Whilst removing the spleen, the frogs were examined for any external or internal signs of pathological abnormalities and results were noted.
On removal of the ventral skin and muscle the internal body cavity was sprayed with 70% alcohol and the instuments were kept in alcohol during the proceedure. Once the spleen was removed it was transferred immediately into 5 mls of solution A (which contained L-15 medium, 2% FCS and 10 units per ml heparin). The organ was macerated using a sterile 100µm mesh and a syringe to isolate the spleen cell suspension. At this stage there is a mixed cell population and so 5 mls was carefully placed onto 6mls of a prepared single density percoll gradient and centrifuged at 400g for 25 minutes at 4^0 C. The cells at the interface were collected and placed in 3 mls of solution B (which contained L-15 medium, 0.1% FCS and 100 units per ml penicillin/streptomycin) and centrifuged again at 1100 g for 15 minutes at 4^0 C. This produced a concentrated cell pellet which was resuspended in 1 ml of solution B. An aliquote was added to 0.1% trypan blue solution and the number of viable cells was counted using a haemacytometer and a phase contrast microscope x 20 magnification.

One hundred microliters aliquots of the cell suspension were dispensed in sterile 96 well plate and incubated at 22^0 C for 2 hours allowing the macrophage cells to adhere to the plastic surface in a monolayer. The wells then were washed twice using sterile amphibian saline and 100µl of solution B wasded. The cells could then be used immediately or left overnight at 22 ^{0} C before measuring production of the intracellular respiratory burst using the nitroblue tetrazolium (NBT) assay. The number of macrophage cells in a representative well estimated by counting the number of nuclei released from the cells after addition of 100µl of a lysis buffer (0.1M citric acid, 1% Tween 20 and 0.05% crystal violet). This released the nuclei which could be seen and counted to give the total number per well.

Upon membrane activation of macrophage cells there is increased uptake of molecular oxygen which is reduced to toxic anion (O_2^-) by membrane-bound enzyme cascade. The NBT assay measures the ability of the macrophage cells to produce the bactericidal anion.
The wells were washed twice with sterile hanks buffered salt solution (HBSS) and then 100µl of either NBT alone or NBT+PMA (phorbal myristate acetate is a chemical stimulator frequently in fish used to induce the respiratory burst activity of phagocytes). The cells and solutions were incubated at 22^0 C for 1 hour, the reaction was stopped by addition of 100% alcohol to the wells and the wells carefully washed with 70% alcohol to remove any remaining NBT solution. The cells were left to air dry and 2M potasium hydroxide and dimethylsulphoxide (DMSO), added to solublise

the released product, which was then read in a multiscan spectrophotometer at 610nm wavelength.
The results were then adjusted to read for 1 x 10^5 cells ml^{-1} and the average value for 5 animals was calculated and presented in Table.1.

Results

Haematology results for control and stressed farmed frogs.

Results of whole blood analysis in stressed frogs have not been included. This was because when blood was sampled from the stressed groups, it clotted very quickly and so serum was isolated and stored for analysis of the stress levels in frogs by measuring the glucose in the sera. This assay is waiting to be completed. Moreover as there did not appear to be a significant difference between control and experimental animals on initial studies and there were large individual variations, making conclusions as to the effect of stimulation and stress difficulat to assess it was thought more useful to modify procedure and save sera for glucose analysis.

Table.1 NBT Spleen Macrophage Results for Temperature and Water Quality.

Experimental proceedure	NBT+PMA at 1x10^4Cells $well^{-1}$ (O.D)	NBT at 1x10^4Cells $well^{-1}$ (O.D)
Temperature control (Exp.1)	0.002 ± 0.0005	0.001 ± 0.001
Temperature stress 24 hours (Exp.1)	0.002 ± 0.001	0.003 ± 0.0009
Tempaerature Stress 48 hours (Exp.1)	0.004 ± 0.003	0.002 ± 0.002
Temperature Stress 72 hours (Exp.1)	0.002 ± 0.001	0.002 ± 0.0009
Temperature Control (Exp.2)	0.002 ± 0.0005	0.001 ± 0.001
Temperature Stress 24 hours (Exp.2)	0.012 ± 0.009	0.010 ± 0.008
Temperature Stress 72 hours (Exp.2)	0.015 ± 0.005	0.012 ± 0.005
Temperature Stress 240 hours (Exp.2)	0.025 ± 0.007	0.032 ± 0.016
Water Quality Control	0.002 ± 0.003	0.003 ± 0.002
Water Quality 48 hours	0.015 ± 0.008	0.009 ± 0.006
Water Quality 168 hours	0.012 ± 0.010	0.010 ± 0.009

Exp.1 and Exp.2 = experiment 1 and experiment 2. There were 5 animals sampled per group except for Temperature stress day 240 which had only 4 animals left.

Discussion

The control animal NBT results were low for both the NBT+PMA and the NBT only groups.
There was a slight increase in the NBT+PMA and NBT only groups for the second temperature stress groups by day 10. This may have been due to the reduced impact of surrounding stressors such as movement in the wet laboratory. The animals tend to sit at the bottom of the tanks and so ar not disturbed so frequently.

In the water quality groups the NBT response for both NBT+PMA and NBT only was greater for the stressed compared to the control groups. This may also have been due to reduced impact of surrounding stressors such as during water changes.

It may be that there is a cummulative stress effect on the immune system of frogs held under these conditions and a single factor may not be responsible but a combination of stressors commonly applied to the animals on a daily basis.

Appendix 7a

Isolation and recovery of bacteria from spleen macrophages in clinically healthy tropical frogs.

Introduction

As a general rule healthy animals do not harbour bacteria in their organs. However, during experimental work to isolate tissue-dwelling macrophage cells from healthy frogs, bacteria were observed routinely whilst counting the number of viable macrophage cells recovered. The animals on which these observations were made had been maintained in optimal environmental conditions and had no external or internal signs of disease. Furthermore the criteria used to asses a healthy frog namely, intact moist skin, clear bright eyes, good appetite with increased growth over time and positive reaction to stimuli were present in all animals sampled.
Although bacteria were visible in the cell cultures they were difficult to grow and using normal bacteriological methods.
The continued recovery of the bacteria was initially thought to be due to contamination of the reagents or due to poor technique, however each step of the procedure was carefully controlled and the possibility of introduction of bacteria from without was discounted.
A systematic study was started to investigate the frequency of this occurrence.

Material and Methods

As each animal was sampled for other experimental reasons a of the macerated spleen cell suspension was put into tryptone soya broth (TSB) and incubated for 24 hours at 22° before streaking onto tryptone soya agar plates (TSA) and incubating at 22^0 C for another 24-48 hours. The growth on the plates was observed and a pure culture of the dominant colony type was produced to investigate the bacterial species using primary and secondary tests.

Results

Recovery was successful from all the frogs tested in this pilot study and the results indicated a range of bacteria were involved, mostly identified as enterobacteria.

Discussion

It was not completely unexpected to detect bacteria in macrophage cells; however it was unusual to recover bacteria so easily from spleen suspensions of animals which had shown no clinical signs of disease.
Further work was needed to investigate the efficacy of different methods of recovering bacteria from spleens of clinically healthy frogs.

Appendix 7b

Bacterial Recovery from Clinically Healthy *Rana* spp. : A Comparative Study of Routine and Novel Methods.

Work has shown that bacteria are present in the spleen of clinically healthy frogs in high numbers but are difficult to recover using conventional bacteriology methods. A brief study using 5 apparently healthy frogs was completed at AAHRI, where bacterial recovery by conventional and novel methods were compared.

Five animals with no external signs of ill-health were sampled. The criteria to define the health status included intact, moist skin with no external lesions or ulcers present, clear eyes, an increase in body weight and the animals reacted to stimuli. The frogs were randomly selected from a single tank housed in the wet laboratory at AAHRI where animals were given daily water changes and fed once a day with commercial pellets at 3% body weight. The average weight of the animals was 80g.

The frogs were stunned by a sharp blow to the head and bled by cardiac puncture. They were then sacrificed by severing the spinal cord. The skin and muscle was removed to reveal the internal organs which were immediately sprayed with 70% alcohol before being placed to the side to allow the spleen to be collected. There appeared to be no visible abnormalities of any of the internal organs. Aseptic technique was used throughout.
A normal bacteriology loop was flamed, cooled and inserted into the spleen and this was immediately streaked onto a TSA plate. A small piece of the spleen was also put onto a TSA plate and streaked. These were incubated at 28^0 C for 24 hours.
The remaining spleen was put into 2 mls of L-15 medium supplemented with foetal calf serum and heparin and the organ was macerated through a sterile 100µm mesh to produce a spleen cell suspension. Five hundred microliters of the cell suspension was placed into 2 mls of TSB and incubated at 28^0 C for 24 hours prior to streaking onto a TSA plate. A loop of the remaining suspension was streaked directly onto the TSA plate.

Results

Method	A1	A2	A3	A4	A5
Conventional loop	no growth	no growth	no growth	no growth	no growth
Piece of spleen streaked onto TSA	no growth	no growth	no growth	8 CFU	1 CFU
Spleen suspension after TSB resuscitation step	no growth	no growth	growth	growth	growth
Spleen suspension directly onto TSA	no growth	no growth	no growth	no growth	no growth

Discussion

Bacteria were present in the spleens of clinically healthy frogs, however it was not always possible to recover the bacteria using the conventional bacteriology loop technique.

When counting the number of cells recovered from the spleen for other tests, motile bacteria have often been seen and yet unless the animal was compromised in some way or in an obvious diseased state, recovering the bacteria without the TSB resuscitation step often failed.

RESULTS

Macrophage Separation From Spleen Tissue

Body weights of the frogs sampled ranged from 41 to 133g (n = 32, mean = 76.4 ± 24.2 g) and weights of excised spleens ranged from 0.025g to 0.134g (n = 27, mean = 0.054 ± 0.027g) see table.1.
There was no direct association of spleen size and body weight: large spleens did not necessarily originate from large frogs. Spleen weight, taken as a fraction of body weight varied from 1:33 to 1:206 (n= 27, mean = 1:70 ± 42). The number of viable macrophages recovered per µg of spleen (see table.2) also varied widely with a range of 15 to 314 (n= 27, mean = 145 ± 72).

Extent of macrophage activation and bacterial ingestion

Ingested bacteria were seen in cells from all frogs examined. The percentage of cells in each sample with internalised bacteria varied from 5 to 63% (see table 3). Activated macrophages with spreading pseudopodia were seen in many samples even when 40 to 60% of the cells contained bacteria.
The number of macrophages recovered per unit weight of spleen was considered in relation to the population with ingested bacteria (see table 4) since a high level of bacterial challenge may result in cell killing and low macrophage yields. Visual examination of the distribution of the cells with internalised bacteria did not indicate that this was so. The highest cell recovery rate was associated with 56% infection rate and low infection rates were seen at low, medium and high cell recovery.

Recovery of bacteria from spleen

Bacteria was recovered from spleen tissue which had been incubated in TSB for 24 hours and then plated onto TSA in 24 of the 29 frogs tested (see table 5). Eight samples gave growth at both 22 and 28^0 C; seven gave growth at 22^0 C only and nine at 28^0 C only.
The bacteria recovered were identified using primary and secondary bacteriology tests and results are awaiting verification.
Intracellular bacteria were also seen in samples of the same cell preparations, fixed and stained on coverslips (see table 1). All of the samples, including those from which no bacteria had been recovered by culture, had cells with intracellular bacteria. Extracellular bacteria were observed in all but one specimen; and that one had yielded growth at 28^0 C. Usually the extracellular bacteria were numerous.
The proportion of cells containing bacteria in a preparation ranged from 5 to 63% but most (28 out of 32) had 12 to 49%.

Table 1. Number of spleen macrophage cells recovered and incidence of bacteria in stained macrophage preparations.

Week N°	Animal N°	Body weight (g)	Spleen weight (g)	N° cell recovered ml^{-1} per spleen*	Bacteria inside cell (%)	Extracellular bacteria visible.
1	1	52g	N/A	N/A	5	few
	2	43g	N/A	N/A	22	few
	3	47g	N/A	N/A	49	few
	4	41g	N/A	N/A	18.5	few
	5	44g	N/A	N/A	15	few
2	1	63g	0.051g	1.5×10^6	6	none
	2	53g	0.031g	2.1×10^6	29	few
	3	90g	0.034g	3.8×10^6	14	few
3	1	59g	0.044g	2.1×10^6	49	few
	2	67g	0.036g	2.6×10^6	13	few
	3	71g	0.025g	3.9×10^5	12	few
4	1	92g	0.031g	3.8×10^6	19	few
	2	74g	0.027g	2.2×10^6	17	few
	3	91g	0.032g	2.3×10^5	22	few
5	1	79g	0.071g	6.9×10^6	41	many
	2	59g	0.051g	8.0×10^6	56	many
	3	79g	0.059g	$4.4.x\ 10^6$	46	few
6	1	68g	0.042g	1.7×10^6	32	few
	2	107g	0.070g	5.2×10^6	39	few
	3	117g	0.069g	2.5×10^6	30	many
7	1	133g	0.076g	6.6×10^6	43	many
	2	86g	0.070g	3.2×10^6	31	many
	3	133g	0.056g	2.7×10^6	36	many
8	1	58g	0.113g	3.0×10^6	12	many
	2	99g	0.047g	4.0×10^6	24	many
	3	81g	0.037g	2.7×10^6	20	many
9	1	89g	0.057g	7.7×10^6	33	many
	2	88g	0.029g	2.9×10^6	27	few
	3	84g	0.061g	5.2×10^6	43	few
10	1	82g	0.089g	8.7×10^6	44	many
	2	65g	0.134g	7.7×10^6	27	few
	3	50g	0.029g	2.3×10^6	63	few

N/A = data not available

This table also includes the whole body weight and spleen weight per animal.

* This number was estimated from number of cells per ml contained in half of the spleen.

Table 2 Spleen to body weight ratios and number of viable phagocytic cells recovered from frogs.

Week number and Animal number	Spleen : body weight ratio ($\times 10^{-5}$)	Number of viable cells per µg spleen
A1Week.2	81	59
A2Week.2	58	135
A3Week.2	38	224
A1Week.3	75	96
A2Week.3	54	145
A3Week.3	35	31
A1Week.4	34	245
A2Week.4	36	163
A3Week.4	35	15
A1Week.5	90	195
A2Week.5	86	314
A3Week.5	75	149
A1Week.6	62	81
A2Week.6	65	149
A3Week.6	59	73
A1Week.7	57	174
A2Week.7	81	92
A3Week.7	42	97
A1Week.8	195	45
A2Week.8	47	170
A3Week.8	46	146
A1Week.9	64	270
A2Week.9	33	200
A3Week.9	73	171
A1Week.10	109	196
A2Week.10	206	115
A3Week.10	58	159

Table.3 Percentage of macrophage cells with ingested bacteria present.

Cells with bacteria ingested(%)	1-9	10-19	20-29	30-39	40-49	50-59	60-69
Number of Frogs in each group	2	8	7	6	7	1	1

The total number of frogs sampled were 32.

Table 4 Relationship between the number of cells recovered per microgram of tissue and percentage of cells containing bacteria.

Cells per ug spleen	0 - 49	50 - 99	100-149	150-199	200-249	250-299	300-349
N° frogs represented	3	6	6	7	3	1	1
% Phagocytic Index*	12, 22, 12	6,49,32 30,31, 36	29, 13, 46, 39, 20, 27	17, 41, 43, 24, 43, 44, 63	14, 19, 27	33	56
N° frogs represented	3	6	6	7	3	1	1

* the phagocytic index is the number of cells with ingested bacteria displayed as a percentage of the total number of cells counted.

Table 5 Recovery of bacteria from spleen cell suspensions after enrichment step grown at 22 and 28^0 C.

Week and Animal Number	Growth at 22^0 C	Growth at 28^0 C
Week.1 A1	+	-
Week.1 A2	-	+
Week.1 A3	-	+
Week.1 A4	+	+
Week.1 A5	+*	+*
Week.2 A1	-	+
Week.2 A2	+	+
Week.2 A3	+*	+*
Week.3 A1	-	+
Week.3 A2	+	-
Week.3 A3	-	+
Week.4 A1	+	-
Week.4 A2	+	+
Week.4 A3	-	-
Week.5 A1	+	-
Week.5 A2	-	-
Week.5 A3	+	-
Week.6 A1	-	-
Week.6 A2	-	+
Week.6 A3	+*	+*
Week.7 A1	N/A	N/A
Week.7 A2	N/A	N/A
Week.7 A3	N/A	N/A
Week.8 A1	+*	+*
Week.8 A2	+	-
Week.8 A3	+	-
Week.9 A1	-	-
Week.9 A2	+	+
Week.9 A3	-	-
Week.10 A1	-	+
Week.10 A2	-	+
Week.10 A3	-	+

* denotes colony type at 22^0 C were different from those at 28^{0}C
N/A = data not available

Discussion

Differences in spleen size per animal were obvious with the naked eye however frog size was not an indicator of spleen size, although errors could have been associated with dividing the organ into 2 sections. It was reasonable to think that macrophage recovery would be associated with spleen size however this was not applicable. It was not because poor cell recovery was associated with high levels of infection., which may have been killing the cells. Our findings suggest that a high level of challenge by assorted bacteria is continuously associated with phagocytosis cells. The cells appear to remain functional when the level of infection is high. It was not unusual for 30-40% of cells to be engaged and have internalised numerous bacteria. Many of the cells were regarded as activated in this situation and this is consistent with the variable results we have obtained when attempting chemical immunostimulation (data not presented).
Bacteria were seen in all samples: they could not always be cultured and recovery rate was improved when two temperatures were used. A range of bacteria were recovered. It may be that the bacteria observed but not recovered were damaged.
Although this was all from the same population of animals there were large variation in weight between individuals but there does not appear to be a direct relation between animals weight and spleen size. This also had large individual variation.

In all cases macrophage cells were recovered and adhered to the coverslip. There was always bacteria present but again this was variable.
The activity of the macrophage cells which was measured morphologically does not appear to be related to the size of the animals, size of the spleen, or the number of cells recovered and is most likely to be in relation to the bacterial presence.
This study gave an indication, due to the state of activity of the cells attached to the coverslip that the cells are actively phagocytosis the bacteria *in vivo* The cells were large, spread with pseudopodia present and reaching for the bacteria.