ASIA-PACIFIC FISHERY COMMISSION

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Report of the Meeting of the *Ad Hoc* Working Group of Experts in Food Safety

Bangkok, Thailand, 15 – 17 March 1999

APFIC SECRETARIAT
REGIONAL OFFICE FOR ASIA AND THE PACIFIC
FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

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PREPARATION OF THIS REPORT

The report of the meeting was prepared by Mr David G. James, Technical Secretary of the *Ad hoc* Working Group of Experts in Food Safety of APFIC and Senior Fishery Industry Officer (Technology) of the FAO Fisheries Department.

ABSTRACT

The document presents the discussions of the *Ad hoc* Working Group of Experts in Food Safety of APFIC at its First Meeting in Bangkok, Thailand, from 15 to 17 March 1999. The Working Group reviewed emerging food safety hazards from aquaculture products. The problems of food-borne parasites caused by fish consumption, especially on trematodes were discussed. The research priorities for the study on pathogens in the aquatic environment were also given. Subsequently, a regional workshop on research to prevent fish-borne trematode infections was organized and its report was included as Appendix 4 of this report.

Distribution:

Members of the *Ad hoc* Working Group
Members of the Commission
FAO Fisheries Department
Fishery Officers in Regional Offices
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REPORT OF THE MEETING OF THE APFIC
Ad hoc WORKING GROUP OF EXPERTS IN FOOD SAFETY
Bangkok, Thailand, 15 – 17 March 1999

BACKGROUND

The A*d hoc* Working Group of Experts in Food Safety of APFIC met at the FAO Regional Office for Asia and the Pacific, Bangkok, Thailand, from 15-17 March 1999. The agenda and the list of members are attached as Appendices 1 and 2.

The establishment of an *Ad hoc* Working Group was agreed by the Twenty-sixth Session of APFIC, held in Beijing, People's Republic of China, in September 1998. This was in response to the principal recommendation of the APFIC Symposium on Fish Utilization in the Asia-Pacific Region, which was held in conjunction with the Session. The recommendation recognized the urgent need for a regional programme of research to address three serious emerging food safety issues associated with sustainable and safe fish production in the region. These issues are:

- Human parasitic infestations caused by fish consumption
- Pathogenic (disease-causing) bacteria associated with fish products
- Emerging safety hazards in the products of aquaculture as a result of intensification

An outline of the objectives, justification and activities of the Working Group were annexed to the report of the Symposium and are attached to this report as Appendix 3. In brief the Working Group aims to encourage the formulation and conduct of a regional integrated research programme and to report progress to the 27th Session of the Commission. It is understood that the programme will require external funding (for which donor support must be found) and will be designed to:

- Quantify current and potential health hazards and risks associated with consumption of fish products; and
- Develop sustainable strategies to mitigate the effects.

Immediately following the Working Group meeting there was a specific workshop on the issue of parasitic infestations caused by trematodes. The workshop gave the opportunity for a detailed analysis of the problem and the formulation of a proposal for research. Both are included in the report of the workshop attached as Appendix 4. The discussions are summarized below together with consideration of the other issues of pathogenic bacteria and the safety of aquaculture products.

SUMMARY OF DISCUSSION

1. RESEARCH FUNDING AND COORDINATION

It is recognized at the outset that any proposals for research that emanate from the Working Group, either for collective or national activities, will require external funding. While the Fish Utilization and Marketing Service of the FAO Fisheries Department is able and willing to take on the role of coordination the available budget is insufficient to cover research activities. Even in regard to coordination some outside assistance will be necessary to improve networking arrangements. As an introduction to this item the Working Group was addressed by Mr Tim Bostock, Executive Secretary of SIFAR, The Support unit for International Fisheries and Aquatic Research (SIFAR) is an independent, donor funded, unit operating from the Fisheries Department of FAO. It was originally set up in Canada as a response to the recommendations of a major Study of International Fisheries Research, and moved to FAO, in Rome, in 1998. The purpose of the unit is to facilitate communication between donors funding fisheries research and the research community at national and regional levels. The objectives are to achieve more relevant research by providing assistance in the formulation of feasible research proposals, better prioritization of research activities and more effective communication. To promote better communication in the fisheries and aquatic research community SIFAR is creating a novel approach through the Internet called "the Onefish Community Directory". The OneFish Community Directory provides the means for stakeholders in fisheries research to collaboratively organise their own information by creating, categorising, and updating knowledge entries in virtually every useful media format. These knowledge workers can each organise a small portion of OneFish and the larger collection of information on fisheries research available on the internet, and present it back to the rest of the population, culling out the bad and useless and keeping only the best content.

The OneFish community-driven approach can harnesses hundreds of human minds to organise information that small paid editorial staffs and 'search engine robots' cannot possibly handle and it can also filter out irrelevant information. This initiative could be followed up by any institute in the region prepared to take on the task of acting as the editor for a specific topic.

2. TREMATODES

The Working Group was given an overview of the problems of food borne parasites caused by fish consumption, reserving detailed discussion for the Workshop on that topic (see Appendix 4). Of the three types of food-borne parasites, nematodes, cestodes and trematodes, the latter are of greatest significance where fish consumption is concerned. Nematodes or roundworms transmitted by fish cause some problems in Europe, North America and Japan but are relatively easily killed by cooking or freezing. The species most commonly implicated is *Anisakis* found in marine fish of temperate origin. Likewise fish-borne cestode (tapeworm) infections are not common in humans, although one species of *Diphylobothrium,* mainly from cold temperate waters, can be transmitted by freshwater, anadromous and marine fish.

Trematodes or liver flukes, however, are a source of widespread human infection resulting from eating raw or undercooked fish and shellfish. Fish-borne trematode disease is endemic over a large area of the world, including East and Southeast Asia and Russia. WHO indications are that more than 50 million people may be suffering, including more than 10 million each in both China and Thailand. Although the disease is seldom fatal, trematodes can cause serious complications in humans leading to fatalities.

Trematodes are leaf-shaped flatworms (flukes) that have very complex life cycles, generally involving two intermediate hosts, (snails and freshwater fish). Man becomes infected through the ingestion of viable encysted metacercareae of the parasites, which are generally found in the flesh of freshwater fish. The metacercareae can withstand freezing for a period and exposure to elevated temperatures. The life cycle is perpetuated by the constant supply of eggs, shed in human and animal feces, which are taken up by snails. Cercareae released by the snails penetrate the gills and under the scales of fish and encyst in the muscle as metacercareae. The life cycle can be interrupted by not allowing sewage contamination of water bodies and not using nightsoil as fertilizer in aquaculture. Alternatively consumers should avoid eating raw freshwater fish and ensure that what is eaten is properly cooked. While these measures may appear to offer simple solutions it has proved difficult to change ingrained food habits and there is a tendency to rely on controlling the problem with the effective drugs that are available. However, the cost of these drugs puts them beyond the reach of poor consumers. The role of HACCP in controlling the disease was discussed. While this may be possible in the production phase of industrialised aquaculture it would be difficult, if not impossible in integrated aquaculture systems and in the wild. Control of the disease requires more research on the epidemiology of infection, better means of identification of infested fish and better diagnostic methods.

Of the trematode liver flukes, *Clonorchis sinesis* is endemic in China, Japan, Korea and Vietnam, while *Opisthorchis viverrini* infests Southeast Asia and *Opisthorchis felineus* is found in Russia. The flukes attach to the bile ducts and cause damage, gastro-intestinal problems, jaundice and fatigue. There is also an associated increase in liver cancers. Cyprinids, the major source of freshwater fish both from capture fisheries and aquaculture, are very commonly implicated as the intermediate fish host. Lung flukes caused by a number of *Paragonimus* species are found in China, Korea and Vietnam. The second intermediate hosts are freshwater crayfish and crabs that are often eaten raw, lightly cooked or pickled.

The Report of the Workshop (Appendix 4) suggests a work programme for a regional research effort to combat the debilitating effects of the disease and the associated high cost in both social and economic terms.

3. PATHOGENS

Although fish products are not implicated as a major cause of food poisoning outbreaks there is a disturbing trend of increasing incidences of reported cases of food borne illness throughout the world. It is not yet clear whether this is due to better

reporting, globalization of trade or an increase in virulence of the causative organisms. Action is required in the region in order to protect valuable export markets as well as the health of consumers.

Despite the low incidence of food poisoning caused by fish, the safety of fish products carrying bacteria and viruses that are recognized as human pathogens is often questioned. A wide range of organisms has come under suspicion as a result of their isolation by the regulatory authorities of the countries that provide the largest markets for fish products. The list of organisms includes: *Vibrio cholerae, V. parahaemolyticus, V. vulnificus, Salmonella, Shigella, Listeria monocytogenes,* pathogenic varieties of *Escherichia coli, etc.* While the isolation of these organisms in importing countries is considered to be a result of unhygenic handling and processing, some of them may be indigenous in the tropical aquatic environment from which the product came. It is well known that only relatively few strains of many potential pathogens are in fact virulent. To ensure food safety it is important to understand which of the organisms are indigenous and which are contaminants. For example there is a lot of scientific evidence to indicate that *V. cholerae* makes up part of the normal flora of the aquatic environment and can be detected even from waters that are not fecally contaminated. This has been demonstrated in the eastern United States and in the UK, in addition to tropical areas. Only two cholera serotypes (*V. cholerea* 01 and 0139) have been shown to cause the disease and these strains, which produce cholera toxin, are genetically different.

Similarly *V. parahaemolyticus* is part of the flora of the coastal and brackish water environment but only 1 percent of the bacterial population is virulent. Determination of the hazards posed by these organisms requires the detection of toxigenic strains. Polymerase chain reaction (PCR)-based methods have been shown to be useful in detecting the genetic differences between strains.

Salmonella has previously been of greatest concern, particularly to the USFDA which has consistently maintained a zero tolerance for the organism, claiming that its presence is always the result of fecal contamination as salmonella is not indigenous to the aquatic environment. However, there is increasing evidence that, even in catfish ponds in the USA and eel ponds in Japan, salmonella is naturally present in the environment. The presence of salmonella has previously been convincingly demonstrated in shrimp culture ponds in Southeast Asia and the coastal environment in India. However, most of these indigenous aquatic strains belong to the serotype *S weltevreden*, which is not commonly associated with human infections. As further evidence to support the case for removal of the zero tolerance it would be useful to determine whether such aquatic strains possess the potential to cause disease in humans. PCR-based techniques would be helpful in such studies.

While *L. monocytogenes* is a considerable problem in temperate areas it has been implicated very seldom as a causative agent of disease in the tropics. It would be important to establish whether those few serotypes involved in human infections are found in tropical fish products or the processing environment. More information is also

required for *E. coli*. Although pathogenic strains are considered dangerous the role of fish in the transmission of disease has yet to be established.

4. EMERGING FOOD SAFETY HAZARDS FROM AQUACULTURE

Although products from marine capture fisheries in the region have a generally good history of safety there are concerns that with increasing intensification of aquaculture new problems may arise. Asia accounts for the overwhelming proportion (over 90 percent) of world aquaculture production and there are some projections that half of the food fish supply will be produced by culture early in the next century. Assurance of the safety and quality of the products is therefore vital.

Potential problems in the domestic markets could occur through trematode infestation, sustained by the use of human and animal wastes for fertilization and the continuation of traditional food habits. These problems have been reviewed above for the production sector as a whole but they are of particular relevance to the rapidly expanding aquaculture industry.

High value products, such as those for export, are produced under much more controlled conditions but still the problems of the presence of pathogens and their virulence, as outlined above, need to be addressed. Of particular concern is the emergence in recent years of a pattern of resistance to antibiotics by disease causing organisms. Although there is little direct proof, in the minds of many, the increasing frequency of resistance has been associated with the excessive use of antibiotics in intensive aquaculture systems. In the past they were used prophylactically in the feed and, although today use has decreased, large quantities are still employed for the purposes of controlling fish health. The resistance can easily be transferred both to fish pathogens and human pathogens, causing eventual difficulties in chemotherapy. Although it has yet to be established whether the emergence of antibiotic resistance is due to aquaculture practices (there is also widespread misuse of antibiotics in the medical and veterinary fields) the extent of the responsibility of aquaculture needs to be studied.

5. RESEARCH PRIORITIES

As the study of pathogens and the need to combat emerging problems in aquaculture have many common points it was decided to consider them together and to arrange a planning workshop at a later date. This workshop would formulate research proposals for a regional research programme. Such a programme would include the conduct of a series of studies to protect the health of consumers, and to collect and present evidence to the regulatory authorities in importing countries that their standards should be modified to take into account the actualities of the tropical environment.

The first requirement was recognized as a need for better communication at regional and international levels. There are opportunities to achieve this by making use of the SIFAR OneFish Community Directory, currently under development. It was agreed that the Fisheries College, Mangalore, India and the Technical Secretary of the

Working Group would discuss how this could be arranged, with the Fisheries College acting as the editor for this sector through the Internet connection.

Specific research projects to be elaborated at the workshop would include:

- Studies on the serotypes of pathogens in the aquatic environment and development of an understanding of whether they are indigenous to that environment and also possess virulence genes. Techniques of molecular epidemiology would be used to understand and describe the source of pathogens in the aquatic environment.

- Studies on antibiotic resistance of bacteria associated with aquaculture systems. Has the development of resistance been correlated with the use of antibiotics to control fish health?

The possibilities of holding the workshop in Mangalore, India, in December 1999 will be kept under review by the Technical Secretary, but will be subject to the availability of FAO funds.

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APPENDIX 1

AGENDA and TIMETABLE

TIME	AGENDA	SPEAKER
15 March, Monday		
08.30 – 09.00	***Registration***	
09.00 – 09.30	***Background and state of the art: Pathogens***	***I. Karunasagar***
09.30 – 10.00	***Emerging safety hazards in aquaculture***	***P.K. Ben-Embarek***
10.30 – 12.00	***National reviews on current state of knowledge on each of the topics under consideration.***	
13.30 – 15.00	***Indication of current and planned activities, project, etc. being carried out on these topics in each country with comprehensive list of contacts.***	
15.30 – 17.00	***Review of current national policies and/or strategies to overcome the issues and problems in the concerned countries.***	
16 March, Tuesday		
08. 30 – 9.30	***Possible funding support for research.***	***Tim Bostock***
09.30 – 12.00	***Discussion and identification of research required at national and regional levels.***	
13.30 – 15.00	***Prospects for regional collaboration and coordination in research, under the FAO/APFIC umbrella.***	
15.30 – 17.00	***Opportunities for securing research funding for national and collaborative projects.***	
17 March, Wednesday		
08.30-08.45	***Introduction and Welcome address***	***D. James***
08.45-09.30	***International/ regional perspective on the parasites problem***	***Carlos Dos Santos***
09.30-10.00	***Sources of research funding***	***Tim Bostock***
10.30 – 12.00	***Presentation of country papers***	***Dr. Paiboon; Mr. Rasssame; Dr. Praphasri;Dr. Chamnarn***
13.30 – 15.00	***Presentation of country papers***	***Prof. Feng; Prof. Rim; Mr. Tran; Mr. Bounthianne***
15.30 - 17.00	***Building consensus on knowledge gaps and prioritizing research needs to fill these***	

18 March, Thursday	
TIME	**AGENDA**
08.30-10.00	***Stakeholder analysis***
10:30 - 12.00	***Institutional capacities and potential for collaboration***
13.30 – 14.30	***Defining responsibilities for research: National projects.***
15.00 – 17.00	***Defining responsibilities for research:*** ***National projects.*** ***Regional coordination systems.***
19 March, Friday	
08.30 – 10.00	***Continuation of regional coordination systems.***
14.00 - 17.00	***Further action:*** ***- Programme of planning visits.*** ***- Agreement on levels regional and international collaboration.***

APPENDIX 2

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APPENDIX 3

OBJECTIVE, JUSTIFICATION AND ACTIVITIES OF THE AD HOC WORKING GROUP OF EXPERTS IN FOOD SAFETY

Programme Proposal: Emerging Food Safety Issues in the APFIC Region*

Objective: To establish an ad hoc Working Group of Experts in Food Safety, tasked with coordinating a regional programme to address emerging food safety issues associated with fish production in the APFIC region.

Justification:

Aquaculture is currently one of the fastest growing food production systems in the world with production levels increasing at an average rate of 9.6% per year over the past decade. Aquaculture contributes significantly both to the economies and food security of many producing countries. The APFIC region in particular has the longest tradition in aquaculture, producing around 90% of global volume and 82% of value.

While fish from the open ocean are generally considered safe, products from coastal and inland aquaculture are increasingly associated with major food safety concerns. Such concerns are having major impact on the health of millions of people in the region while others threaten the vital international trade in fish products. This programme aims to encourage an integrated approach within the region, which will:

- Quantify current and potential health hazards and risks associated with fish products; and
- Develop sustainable management strategies aimed at mitigation.

Data derived from this programme will be used in the process of risk analysis, which is the basis for the development of food standards that both provide adequate health protection and facilitate intraregional and international trade in food.

Participating countries: Interested APFIC members.

Activities: Three main areas of major interest to the APFIC region are initially proposed:

I. Parasitic infestation:

A large number of fish, particularly freshwater species, can serve as a source of human parasitic infestation. In particular, infestation by the fish-borne trematodes of

* Report of the Twenty-sixth Session of APFIC, Beijing, PRC, 24-30 September 1998. RAP Publication 1998/23, p. 28-31.

prime concern for human health (including *Clonorchis, Opisthorchis* and *Paragonimus*) is recognized as a major burden to public health throughout the world. WHO has recently estimated that the number of people infected by trematodes is 40 million, with over 10% of the world population being at risk. With regard specifically to the APFIC region, a major concern is that trematodes are endemic for many member countries and related impacts on public health are highly significant.

In support of the objectives of the regional programme, it is proposed to carry out the following activities:

- **Determine the sources and relative impacts of infection.** In particular, to establish the relative importance of aquaculture vis-à-vis freshwater capture fisheries as a source of infection. Epidemiological studies would provide quantitative information in support of this activity.

- **Pond management systems.** Working in participation with producers, to identify, test and put into practice, cost effective means of controlling infection through improved management systems. These are likely to be based upon good farming and aquaculture practices.

- **Thermal resistance of parasites.** To carry out resistance trials on fish infested with parasites to determine survival rates under differing processing conditions. The information derived from this work will be used to assist processors in assessing hazards and risks both for local and export markets.

- **Public health policy.** The findings of the above activities will be used to assist partner countries in developing control strategies for prevention. At national level, the results will be used to inform such countries on assessment of risk. At the international level, results would be channelled into the Codex's Codes of Practice relating to products from aquaculture.

II. Disease-causing bacteria (pathogens):

Fish products in particular from aquaculture may be contaminated with pathogenic bacteria such as *Salmonella* and *Vibrio*. These are usually considered as a sign of poor standards of hygiene during handling and processing and are associated with food borne illnesses. However, under particular circumstances, they may survive in warm water climates and become part of the natural environment. It is of great importance to minimize or eliminate their presence in fish products. Studies are required to distinguish between unavoidable contaminants from the environment and pathogens of human origin that may occur in products as a result of poor hygiene during post-harvest handling and processing and which can be avoided.

In support of the objectives of the regional programme, it is proposed to carry out the following activities:

Assess the role of different Salmonella serotypes (in particular *Salmonella weltevreden*) in fish products: In order to better understand and control contamination of fish products, in particular aquaculture products, research activities should:

a) determine and monitor national incidence, prevalence and distribution in humans and in farmed fish;
b) develop outbreak surveillance programmes;
c) conduct transmission studies to understand environmental source and contamination patterns and
d) identify and evaluate control measures.

Understand the presence and importance of pathogenic and non-pathogenic Vibrios in fish products: Not all strains of *Vibrio cholerae* are capable of causing cholera in humans. Since some strains can be part of the natural environment in tropical waters, there is a need to better understand and differentiate between the pathogenic and non-pathogenic strains of these micro-organisms by:

a) Study of the ecology of pathogenic and non-pathogenic strains in aquaculture environment;

b) Better understanding the differences in pathogenic and non-pathogenic strains in terms of their survival capabilities; and

c) Identification and evaluation of appropriate control and management measures.

The information derived from this work will be used to assist processors in assessing hazards and risks related to pathogenic micro-organisms of concern in the products of the region both for local and export markets. It will form the necessary basis for the application of Risk Analysis to fish production, enabling producers, regulatory agencies and exporters to develop effective preventive and control measures, which will promote the supply of safe fish products from the region.

III. Emerging safety hazards in aquaculture products:

Certain types of integrated fish farming systems, where livestock consume feed containing antibiotics, may pose a risk of antimicrobial resistance or unexpected residues in fish. Furthermore, emerging new pathogens of concern associated with intensive livestock production may become potential hazards in integrated fish production systems where such intensive livestock production is part of the system. The health implications of this type of artisanal production, combined with antimicrobial use, are poorly understood and more information is needed before a proper assessment can be made.

In support of the objectives of the regional programme, it is proposed to carry out the following activities:

- **Assess** the impact of the use of livestock wastes in integrated aquaculture on:

  a) antibiotic resistant bacteria in farmed fish;
  b) antibiotic resistant bacteria in the environment;

- **Study and evaluate the potential risk** posed by emerging food home pathogens in the livestock sector to aquaculture products. The data derived from these studies will assist in the identification and prevention of potential emerging hazards in farmed products

Time Frame: The ad hoc Working Group of Experts will report to the 27th Session of APFIC.

Inputs:

- FAO through the Fish Utilization and Marketing Service (FIIU) will provide the coordination.
- WHO will provide technical inputs.
- APFIC will provide funding for the meetings of the ad hoc Working Group.
- National institutions will be expected to contribute technical expertise through national research programmer.

Outputs:

- Mitigation of public health impacts, reducing the burden of fish borne illness.

- Improved safety of fish supply, both for domestic consumption and export.

- Improved management strategies for the production of safe products from aquaculture introduced.
- Disseminated results of the programme through the Internet, particularly FAO/SIFARNET.

Financial Resources:

FAO (through HQ and RAP), together with WHO, will cover the costs of coordination of the programme.

The participating countries will be expected to commit resources to national research programmes and to their participation in this collaborative activity. The Support Unit for International Fisheries and Aquatic Research (SIFAR) will assist, if and when possible, in finding supporting donors.

APPENDIX 4

REPORT OF THE REGIONAL WORKSHOP ON RESEARCH INTO PREVENTION AND CONTROL OF HUMAN FISH-BORNE TREMATODE INFECTIONS
Bangkok, Thailand, 17-19 March 1999

SUMMARY

In support of the work of the APFIC *Ad hoc* Working Group of Experts in Food Safety, FAO and the Support unit for International Fisheries & Aquatic Research (SIFAR) convened a Regional Workshop in Bangkok, Thailand from 17 to 19 March 1999 to address the problem of human parasitic infections (trematodes) due to the consumption of infected fresh water fish in Southeast Asia.

The Workshop reviewed the current situation in the main affected countries of the region and identified knowledge gaps and research needed to assist in the design of national and regional policies to prevent, reduce and control the presence of food-borne trematode (FBT) parasites in freshwater fish. Trematode infections are due to the consumption of infected raw, fermented or otherwise lightly preserved fresh water fish.

The country reviews presented highlighted the regional importance of the problem and the large differences between countries. World wide, it is estimated that 40 million people are infected with most of them being in South and Southeast Asia. Some countries, such as Korea, China and Thailand, have implemented extensive epidemiological surveillance programs for many years which have enabled them to identify endemic areas, populations at risk and sources of infection. In other countries, there is still very few data available and it is therefore difficult to provide a detailed picture of the situation. In countries with good monitoring programs, recent data show that the incidence of trematodiasis is on the rise although, the underlying reasons are different. In some it is due to the migration of population away from traditional endemic areas and into previously unaffected or less affected areas. In some others it is due to an increase in urban migration coupled with increased wealth and a continuation of traditional food habits of eating raw and undercooked fish products.

Based on the reviews presented, knowledge gaps and research needs were identified and prioritised during the Workshop. It was recognized that, so far, the main focus for the control of fish-borne trematode infections in humans has been by the health sector. The magnitude of the problem and the fact that decades of effort by the public health sector have not been able to curb the expansion of the problem call for a more multidisciplinary approach. The prevention and control of FBT infections in fish as food has been neglected by aquaculturists, fisheries departments and national fish inspection services in the region. The need for major changes in the situation was acknowledged and the Workshop agreed to further develop collaborative research proposals aiming at the development of management tools for the prevention and control of FBT in fish as food.

BACKGROUND

Food-borne parasitic diseases are a global public health problem that affect millions of people and cause enormous suffering, particularly in developing countries. The parasites involved in food-borne trematode infections have complex life cycles involving one or two

intermediate hosts, and control strategies are therefore difficult to implement. Cultural habits of fish preparation and consumption play an important role in the transmission of parasitic food-borne disease (Reilly, 1998). A large number of mainly freshwater fish species, are potential sources of these medically important parasitic zoonoses (WHO, 1998). The cause of infection is the ingestion of viable encysted trematode metacercariae, which can be present in the flesh of raw, inadequately cooked or minimally processed freshwater fish. These infections are prevalent in only a few countries in the world and essentially among communities where eating raw, fermented or inadequately cooked fish is a cultural habit (WHO, 1995). WHO estimates show that FBT infections are endemic in about 20 countries and that over 40 million people are infected. It is also estimated that 10% of the world's population are at risk of infections.

The major human trematode infections of aquatic origin are clonorchiasis caused by *Clonorchis sinensis*, opisthorchiasis caused by *Opisthorchis felineus* and *opisthorchis viverrini* and paragonimiasis caused by *Paragonimus westermani* and other *Paragonimus* sp.

Clonorchiasis is endemic in China, the Republic of Korea, Japan, Hong Kong SAR, Viet Nam and the Russian Federation. About 7 million people are estimated to be infected, more than half in China (WHO, 1995). A total of 113 fish species, mainly Cyprinidae, are known to be potential hosts. Opistorchiasis due to *O. viverrini* is endemic in Thailand and Laos and opistorchiasis due to *O. felineus* is endemic in at least Kazakhstan, Russia and Ukraine. Ten million persons are estimated to be infected, 7 million in Thailand alone. Here also a number of Cyprinidae are known to be potential hosts. Paragonimiasis affects 22 million persons in at least 20 countries. In Asia, endemic regions cover China, the Republic of Korea, Laos, Philippines and Thailand. *Paragonimus* sp. are ingested through raw or inadequately cooked crabs or crayfish.

While FBT infections are a major public health problem that has largely gone unrecognized by the health sector and the food inspection services in recent years, the relative contributions of farmed fish and wild caught fish to the burden of the disease is unclear.

INTRODUCTION

A Regional Workshop on Prevention and Control of Fish-borne Trematode Infections was convened at the FAO Regional Office for Asia and Pacific in Bangkok, Thailand, from 17th to 19th March 1999 concurrently with the first meeting of the APFIC *Ad hoc* Working Group of Experts in Food Safety, which was held from 15th to 17th March 1999. Twenty-two participants from six countries of the region as well as five FAO staff members attended the Workshop.

The Workshop was opened and the reason for convening it were outlined. It was stressed that FAO has limited own resources and it is necessary to look for external sources of funding in particular for research projects.

The role of SIFAR in facilitating this process was presented by Tim Bostock, Executive Secretary of SIFAR, who described the need to address both the regional and international perspective on the parasite problem. Mr Bostock explained that SIFAR is an independent, donor funded, unit operating from the Fisheries Department of FAO and was originally established in Canada in response to the recommendations of the Study of International Fisheries Research. The Unit had moved to FAO, Rome, in March 1998 and its purpose is to

facilitate communication between donors funding fisheries research and the research community at national and regional levels. The objectives are to achieve more relevant research by providing assistance in the formulation of feasible research proposals, better prioritization of research activities and more effective communication.

With regard to the latter objective, SIFAR is about to create an innovative Internet approach to knowledge sharing called *OneFish Community Directory*. OneFish will provide the means for "stakeholders" in fisheries research to collaboratively organise their own information by creating, categorising, and updating "knowledge objects" in virtually every useful media format. These stakeholders will be able each to organise a small portion of OneFish under specific areas of interest. Mr Bostock pointed out that OneFish would be deployed as the networking, coordination and dissemination tool for this project allowing the formation of an "expert trematodes virtual working group".

Emphasis was put on the fact that it was necessary to address the problem in fish as food. While a lot of work has been done on treating infected populations and on education, a more multidisciplinary preventive approach is needed in order to reduce the prevalence of infected fish. This should involve participation of fish technologists, aquaculturists, and health professionals.

WORKSHOP OBJECTIVES

The following objectives were agreed by all participants.

1. Identify gaps in current knowledge that require immediate research inputs. Multi-sectoral/multi-disciplinary perspective will be required.

2. Prioritise the areas of research. Criteria for prioritisation to be defined by the group.

3. Identify a range of institutions in the region that would be willing to participate in nationally-based research programmes which would contribute to a regional effort aimed at mitigation (respective to capacities and comparative advantages)

4. Identify institutions in the region that would be willing to coordinate the future research programme with technical support from FAO/SIFAR/WHO as appropriate.

NATIONAL REVIEWS

Following the introduction, the Workshop went on to consider the country reviews. Invited experts from Thailand, China, the Republic of Korea, Vietnam and Laos reviewed the situation in their respective countries and highlighted new information and changes that have occurred in recent years. These presentations were a welcome and very useful addition to the existing material available from the previous international conferences.

Thailand :

In his presentation, Dr. Paiboon Sithithaworn, Khon Kaen University in Northeast Thailand, gave an update of the situation in this region. The main trematode of concern there is *O. viverrini* infection with which has been linked to liver cancer. The incidence of liver cancer in Khon Kaen is in the range of 90-100 cases per 100 000 inhabitants while the incidence in developed countries or in non-endemic regions is less than 2 per

100 000 inhabitants. In Northern Thailand, opisthorchiasis infections are linked to consumption of traditional raw freshwater fish (*Koi pla*) dishes or fermented fish products made with fish captured from the main lakes around Khon Kaen. The work presented has been based on wild captured fish and Dr. Paiboon indicated that there are no figures available on the infection rate or the role of farmed fish in the transmission of FBT in NorthEast Thailand. Improved isolation and identification techniques have enabled his group to show that often, several types of parasites are present in infected fish. Minute intestinal flukes are very often isolated from the fish harbouring *O. viverrini*. These parasites do not trigger any particular external symptom in humans but provoke minor intestinal irritation.

Initial studies in Khon Kaen on the survival of *O. viverrini*, show that the parasite can survive more than a month at 4°C and can survive 30 min at 70°C and 15 min at 80°C. Thus indicating a relatively high resistance to refrigeration temperature and cooking time/temperatures used for fish. However, these initial results warrant further studies.

Mr. Rassamee Keawvichit, Chiangmai University, presented an update of an extensive study of school children in Chiangmai province. About 50% of them are infected with parasites and 38% are infected with *O. viverrini*. The main cause of opisthorchiasis infections has been identified as resulting from the consumption of *Plaa raa* and *Plaa som*, two traditional low salt content fermented fish products. Demonstration and educational campaigns have been run for children and in villages but a high rate of re-infection of children after treatment show that the food habits of their parents are difficult to change.

Dr. Praphasri Jongsuksuntikul, Department of Communicable Disease Control, Ministry of Public Health, and Dr. Swangjai Pungpak, Mahidol University, Bangkok, gave a history of the control of opisthorchiasis in Thailand. There have been 3 phases:

I. 1950-1968: where pilot control programs were conducted mainly using chloroquine for treatment of patients.

II. 1968-1986: where emphasis was given to health education programs and the drug Praziquantel was introduced from 1980.

III. 1987-today: where a national control program has been included under the 6th National Public Health Development plan. First in the Northeast region from 1987 and expanded to the North and the Central regions in 1992.

Over the years the strategies developed have centred around 3 main focii: 1) systematic stool examination and treatment; 2) health education and promotion of better cooking practices for fish; and 3) promotion of good hygienic practices.

The distribution of the prevalence of opisthorchiasis in Thailand shows the differences in regions. The North–East, which has seen much of the control efforts over the years actually shows a decrease in the prevalence while for the whole country and for the north in particular, the prevalence is actually increasing (Table 1).

Table 1: Distribution of prevalence of Opisthorchiasis in Thailand by region.

	Country	Northeast	Central	North	South
1981	14.7%	34.6%	6.3%	5.6%	0.01%
1991	15.2%	24.0%	7.3%	22.9%	0.3%
1996	16.0%	15.3%	7.9%	32.6%	0.1%

The reasons behind these differences and trends are not fully understood but infection rate of fish, culinary habits and migration are all important factors. Following the recent economic crisis, there is a risk for a further increase in the infection rate due to a million laid-off workers returning to their home provinces in the North-East from the main economic centres. These groups have probably never been examined or treated and have probably not been reached by public awareness campaigns run in the target provinces in the Northeast.

The fact that seasonal and climatic variation also plays a role is illustrated by the results of a recent study presented by Dr. Swangjai Pungpak on the prevalence of parasites in freshwater fish from the North. Out of 361 samples, representing 6 fish species, 281 were positive for intestinal flukes but none were positive for *O. viverrini*. At the time of the study, the water level was very low due to draught. Similar results were obtained from another reservoir at the same time. In this study and the other recent studies presented above, it was noted that the use of praziquantel in combination with a laxative gave much better results than previous techniques. This method allowed a good recovery of adult parasites (worms) in the stool, thus facilitating isolation and identification.

Dr. Chumnarn Pongsri, Inland Fisheries Division, Department of Fisheries, Bangkok noted in his presentation that in Thailand, there was a very low profitability from freshwater fish aquaculture and therefore introduction of HACCP and other measures would have to be done in a cost-effective way in order to be accepted and successful. New exploratory ways included the development of techniques to culture snail eating fish spp. like *C. enophos* which could assist in breaking the reproductive cycle of trematodes in aquaculture ponds.

China :

Prof. Zheng gave an update on the trematode situation in P.R. China. Clonorchiasis, due to *Clonorchis sinensis* is one of the most severe food-borne parasitic diseases in China with 27 provinces affected and 10 million people infected. As many as 572 million people live in the endemic provinces and are at risk. One of the most affected provinces is Guanxi where 48 out of 82 cities and counties are endemic. Endemic areas tends to expand together with the expansion of the live fish market. In China, infected animals and humans contribute to the contamination of waterways and in particular small aquaculture ponds where latrines are traditionally built nearby or directly on top of the ponds. Contaminated aquacultured fish are, in return, a major source of infection and re-infection for humans. Nine species of snails can act as first intermediate host and 84 species of fish (mainly cyprinids) are known to act as second intermediate host. In Guanxi, the surface area used for aquaculture is about 170 000 ha with a production estimated at 700 000 t/year. The production has multiplied 5-6 times in recent years. The culinary habit of eating raw fish and undercooked fish dishes is on the rise in particular in cities where the increase in wealth in recent years has prompted a renewed interest in these relatively expensive and socially prestigious raw fish dishes. This is illustrated through one study done with two villages of similar size. One is a middle income village where people eat raw fish occasionally and the other is a richer village where people

consume raw fish 1 to 3 times a day. In the first village, the incidence rate is 25.9 % while in the second village, the incidence rate is 40.2 %. It was noted that in China, *Clonorchis* is mainly transmitted through aquacultured fish. In terms of international trade, a potential source of concern is the fact that fresh water fish are exported to the EU (Netherlands, live fresh water fish), the USA, Australia and Japan. However, it seems that exported fish are produced under more controlled conditions and are less likely to be infested.

Republic of Korea:

Prof. Rim, in his presentation, gave an update of the situation in Korea. *Clonorchis sinensis* is also here the main trematode of concern. More than 30 species of *C. sinensis* are involved. Over the years, there has been a change in the pattern of chlonorchiasis. Now, it is a problem mostly affecting men in rural areas. Since 1971, 5 yearly surveys have been conducted and the infection rate at national level is now at 2.5 %. It is estimated that 1 million people are infected. The infection rate in a common infected fish species (*Pseudorasbora parva*) in one area has declined from 1312 metacercariae per fish in 1972 to 12.3 metacercariae per fish in 1993. This reduction has been attributed to the increased industrialisation and pollution which has in turn reduced the number of snails which serve as intermediate hosts. Another parasite of concern has been *Paragonimus* which use crabs as secondary host. The infection rate in crabs is high but a large survey involving 10 million school children has shown a low incidence rate. In the past eating raw or undercooked crabs was popular among children but this habit seems to be on the decline. It is estimated that about 1000 people are infected. Metagonimus is another trematode infection affecting about 500 000 people in coastal regions and involving mainly sweet fish which is highly infected (often with several thousand metacercariae per fish). This is an expensive fish which people like to eat raw. Prof. Rim presented some preliminary results on the resistance of *Clonorchis* to preservative parameters. In vinegar, it died after 6 days; in a mix of vinegar and soybean sauce, it survives for 5 hours; in wine it died after 3 days; it also died after 2 days in saturated saline solution; it was dead after 35 h at –12°C and did not survive 30 seconds in liquid nitrogen. It was still alive in large fish cooked for 1 h at less than 80°C.

Vietnam:

Mr. Son, Seaprodex, Ho Chi Minh City, gave an update on the situation in Vietnam. There are only few surveys done in Vietnam but the results available indicate that trematode infections are a serious problem, in particular in the North. *Clonorchis* seems to be the most important FBT infection. Surveys in the north indicate an infection rate in humans of 19% while fresh water fish have an infection rate of 93%. *Opisthorchis* has been found in surveys in the central part of the country. There, people have a habit of consuming raw fish. Studies in central provinces show an infection rate in fresh water fish of 40% and of 39% in humans. Mr. Son noted that fresh water fish, in particular catfish are exported and thus may be of concern in regard to trematodes for export markets. However, catfish fillets are mainly exported frozen reducing the likelihood of surviving trematodes.

Lao PDR:

Mr. Bounthiane Somthaboun, Department of Agriculture and Forestry, Savannakhet, presented an overview of the situation in Laos. Estimates of the contribution of fish to the Lao diet vary from 7 to 35 kg per capita per year but it is clear that fish and aquatic products are the most important source of animal protein. In addition the Lao tradition of preparing

fish and aquatic products tends to avoid direct cooking. Fish are traditionally eaten raw, fermented or otherwise lightly preserved. These facts combined with a general poor level of hygiene and a lack of public awareness on health issues and in particular on trematodes could explain the suggested high infection rate in human from the few surveys conducted in Laos. Incidence rate has been reported between 40 and 90% of the population.

Following these national reviews, the Workshop went on to identify knowledge gaps and discuss research needs and their prioritisation. It was also agreed to draft collaborative research proposals based on the identified research needs. The Workshop agreed unanimously that there is an urgent need for action in the region and that the problem of FBT has not been given the appropriate attention by all concerned agencies and organizations so far.

IDENTIFICATION OF KNOWLEDGE GAPS AND RESEARCH NEEDS

A number of important areas where crucial knowledge is missing were identified. These areas were roughly divided into 4 main research objectives which are reflected in the logical framework (see next section):

Research Objective 1 is related to the sources of the problem and on the socio-economic impact of FBT. Research programs within this area would assist in providing a better picture of the situation in the different countries and regions affected. Information on the influence of environmental differences would also be provided. The work would concentrate on fish species of commercial importance and on the endemic areas. An important factor to be studied would be to assess the role of aquaculture versus wild capture in FBTs. Consumption surveys and cost analysis of different control systems would also provide necessary information for decision makers.

Research Objective 2 will cover management and control. Options. Research programs under this objective, will study the efficacy of efficiency of pond management options and in particular, HACCP based preventive approaches will be introduced in pilot trials. Control options for the intermediary hosts (snails) would also be envisaged.

Research Objective 3 will cover projects to study means to control and inactivate the parasites in foods. The resistance of the parasites to a number of preservative parameters will be studied as well as their survival capabilities in traditional lightly preserved fish products.

Research Objective 4 would examine options for public health policy. In other words, the findings of the above activities will be used to assist partner countries in developing control strategies for prevention. At national level., the results will be used to inform such countries on assessment of risk.

In addition, it was agreed that the development of rapid identification methods was necessary in order to support the above mentioned research areas. Therefore a number of institutes would develop a particular research proposal to address this topic.

An important aspect of this regional approach would be to ensure the exchange of information in an interactive way and in this regard, SIFAR and FAO would provide an appropriate forum for the FBT research institutes through the OneFish Community Directory internet site.

Based on the discussion, a logical framework approach was adopted to define, prioritize and organize the research needs in the region. A draft logical framework plan was developed (Table 2).

Table 2: Incidence and control of human, food-borne trematode[1] infections from fresh-water fish - draft logical framework

Goal	Indicator	Assessment of indicator	Assumptions
Public health burden from fish-borne parasitic infections reduced in the SE Asian region and consumer health in endemic areas significantly improved	Significant decline in trematode infection in endemic regions; Corresponding improvements in health indices.	National health statistics Local health records Test surveys	
Purpose			
National food safety and health policies aimed at reducing trematode infection are revised and informed by research outputs.	Collaboration between fisheries and health sectors and stakeholders in policy development.	National health and food safety policy instruments. Subsequent field verification and product testing.	Policies adopted. Major impact is on rural poor. Project complements medical control efforts. Methods are cost effective and efficacious.
Outputs			
1.Sources and characterisation of principal impacts of infections determined	Improved knowledge informs good production practices	New policy instruments Reports	Multi-sectoral approach adopted (health / fisheries / food & agriculture)
2. Sustainable systems for effective management and control recommended.	Package of cost effective and efficacious measures recommended and applicable to producers.	Reports Field surveys	Participatory, community-based approach to pilot activities adopted
3. Parasite survival conditions during food processing better understood.	Critical limits developed for destruction or inactivation of parasites during fish processing ops.	Recommended values for preserving parameters available. Standards	Consumer behaviour in fish preparation largely maintains current characteristics
4. Rapid diagnostic tools for assessment of metacercaria viability developed	Test kits available for rapid detection of viable metacercaria.	Various test kit formats	Kits are made available in support of general programme (Possible) private sector sponsorship
5. Effective regional internet-based knowledge network established	SIFAR *OneFish Community Directory* online with locally managed research categories.	Relevant *OneFish* sites	

[1] Trematodes of principal public health concern and of relevance to this study include those of the genera *Clonorchis* (China, Vietnam), *Opisthorchis* (Thailand, Lao) and *Paragonimus*

Activities			
1.1 Research into relative importance of aquaculture *vs.* capture fisheries as source of infection in endemic areas.	Comparative studies of species and production systems; consumption surveys.	Reports Peer reviewed papers Survey reports	Close collaboration with fish producers (farmers and productive sector in general).
1.2 Participatory research into social, economic and health impact of current levels of infection.	Studies on economic cost of current (medical) disease control methods; Quantification of health burden by country/region	Reports Peer reviewed papers Info on website	
2.1 Community-based pilot action research to validate 'good aquaculture practices' and preventive safety assurance principles.	Pilots operating in at least 2 endemic locations in the region Net costs and benefits analysed.	Reports	Research scope largely informed by activity 1.
2.2 Community-based pilot action research into parasite control systems from capture fishery	Pilots established in at least 2 endemic location in the region ; Overall requirements costs and benefits established.	Reports Info on website	Research scope largely informed by activity 1. Community training and awareness requirements addressed
3.1 Processing trials carried out to determine metacercaria survival rates under differing conditions	Trials carried out on chilling, freezing, heating, pH, and traditional processing	Reports and peer reviewed papers Info on website	
3.2 Restaurant surveys carried out to assess parasite loading in range of local dishes	Survey work completed in x countries by …	Survey reports Info on website	Consistent experimental methodology ensures comparability of results
4.1 Research into rapid field tests for viable metacercaria	Metacercaria antibody assays completed by year 2 DNA probes or PCR tests completed Year 3	Peer reviewed papers Info on website	Results able to inform development of test kits
5.1 Set up regional knowledge network for project-related research information exchange, discussion and dissemination	Capacity and awareness building in oneFish Community Directory	Website online	OneFish is used for research dissemination and discussion

The involvement of the participants in the different research areas was also discussed and each country's level of participation based on interest and means was tentatively assessed (Table 3). For each area, a leading institute was identified to coordinate future research program development and serve as main focal point for the particular area.

Table 3: Stakeholders: proposed indicative levels of country participation in the project.

Country	Output 1	Output 2	Output 3	Output 4	Output 5
Thailand	✓✓✓	✓✓✓	✓✓✓	✓	✓
India				✓✓✓	✓
Korea	✓			✓✓✓	✓
Vietnam	✓✓	✓✓		✓	✓
Lao			✓		✓
China	✓✓	✓	✓✓	✓✓	✓

✓✓✓ = research leader
✓✓ = research partner / studies
✓ = uptake / collaboration / website editors

DRAFT PROJECT PROPOSALS

From each country, the following non-exhaustive list of potential collaborative institutes was drawn up.

Thailand: The Department of Fisheries (DOF), The institute of clinical tropical medicine at Mahidol University, the Department of Communicable Disease Control of the Ministry of Public Health, Chiangmai University, Khon Kaen University, Kasetsart University, Chulalongkorn University.

China: IPD, China Academic of Preventive Medicine (Institute of Parasitic Medicine), Guangdong IPD, Gauangdong AES (food control), Guangxi IPD, Guangxi AES.

Laos: LARRI, Department of Fisheries, Food Control (DDFC), IMPE, NIHE,.

Vietnam: SEAPODEX, NAFICACEN, Agriculture Institute.

Rep. of Korea: Korean Association of Health, SN University, National Fishery University.

India: College of Fisheries Mangalore, CIFT,

International Organizations: FAO, NACA, INFOFISH, ICLARM, SEAFDEC, WHO.

CONCLUSION AND FOLLOW UP

It was agreed as a follow up to the Workshop to prepare draft research proposals in the coming months which could be further developed and presented to potential donors with the assistance of SIFAR. Preliminary drafts are included in the annexes.

Annex 1

AGENDA

Regional Workshop on Research into Incidence and Control of Human, Food-borne Trematode Infections from Freshwater Fish

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Date: 19th-21st March 1999
Location: *FAO-RAP, Bangkok, Thailand*
Sectretariat: FIIU/RAP
Rapporteur: P.K. Ben Embarek, FIIU, FAO
Facilitators: D. James, C.L. Dos Santos and T. Bostock, FAO

Wednesday, 19 March 1999	
TIME	**AGENDA**
09:00-09:45	*Introduction* *International/ regional perspective on the parasites problem*
15:15-17:00	*Building consensus on knowledge gaps and prioritising research needs*
Thursday, 20 March 1999	
09:00-17:00	*Stakeholder analysis*
	Institutional capacities and potential for collaboration
	Defining responsibilities for research: National projects *Regional coordination systems*
Friday, 21 March 1999	
09:00-17:00	*Regional coordination systems*
	Further action: *- Programme of planning visits* *- Agreement on levels regional and international collaboration*

Annex 2

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Annex 3

DRAFT PROJECT PROPOSALS

1. Improved Rapid Detection Methods

Title : Development of rapid and sensitive methods for detection of trematode metacercaria in edible fish species.

Background

Fish borne trematodes (commonly called fluke) infections are a serious public health problems in several parts of the world. It is estimated that about 40 million people are affected world wide (WHO, 1995). Considering the number of people affected, the most important parasites are those belonging to genera *Clonorchis*, *Opisthorchis* and *Paragonimus* (Howgate, 1998). All the species have similar life cycles involving a definitive host (man, dog, cat, etc.) and two intermediate hosts, snail and fish. Consumption of raw or minimally cooked fish causes infection in man. A wide range of freshwater fish including the cyprinid fish which are most commonly cultured in Asia can act as intermediate host (WHO, 1995; Rim, 1998).

Clonorchiasis, the disease caused by *Clonorchis sinensis* is endemic in East Asia, viz China, Hong Kong, Macao, Republic of Korea, Laos and Vietnam (Malek, 1980; WHO, 1995) while Opisthorchiasis, caused by *Opisthorchis viverrini* and *O. felineus* is endemic in Thailand, Laos and the Russian Federation, Ukraine and Kazakhstan (Peng *et al.*, 1993).

The aquaculture and fishery industry is seriously threatened by the public health concerns due to trematode infections. The burden of producing fish which is safe for human consumption is on the fish producers and technologists due to the preventable nature of the problem. The eggs of these trematodes enter the aquatic system through contamination by excreta of man and animals. If this can be prevented, the fish would be free from the trematodes. Nevertheless, the absence will have to be demonstrated by fish inspecting agencies.

Determining the safety of fish with respect to trematode infections currently involves detailed physical examination of fish for metacercaria of trematodes. This is a rather tedious procedure and requires considerable taxonomic expertise.

Objectives

The objective of this project is to develop a rapid and sensitive method for detection of trematode metacercaria in the edible fish species. Presently, the detection methods involve elaborate procedures of digestion of fish flesh, excystation of larvae and identification based on detailed morphological criteria. Antibody and nucleic acid based diagnostic methods have found wide applications in a number of diseases due to their sensitivity and rapidity. Antibody based methods have been developed and applied for diagnosing human infections by trematodes. However, these antibodies are directed against antigens of the adult stage of the parasite and are not suitable for the metacercarial stage, which is found in fish. The objective of this project is to develop such antibody or DNA based methods for rapid detection of

trematode metacercaria in fish tissue. Such tests will be very helpful for fish inspectors and public health workers to determine the safety of fish for human consumption.

Justification

The countries participating in this project are Korea, India, China and Thailand. These countries fall in the region most affected by the problem. The participating institutions from Korea have a very long-standing experience of conducting field investigations on trematode infections. There is a very high level of expertise in parasitology (Rim *et al.,* 1986; 1996; Rim, 1998), which is essential for development of such rapid diagnostic methods. The participating institution from India has considerable experience in development of molecular diagnostics for fish associated pathogens (Karunasagar *et al.,*1995; 1996; 1997). The institutions from China have significant experience in field investigations and parasitology and the participating institutions from Thailand have experience in field investigations, parasitology and diagnostic development.

Korea, China, and Thailand are the countries where the infection is endemic. An estimated number of 1 million people are affected in Korea, and the figure could be as high as 10 million each for Thailand and China. For India, figures are not available, but the potential for problem exists due to the practice of sewage fed fish culture in some areas.

Methodology

The project would begin with the analysis of various surface antigens associated with free swimming, and encysted stages of metacercaria of *C. sinensis* and *O. viverrini*. Monoclonal and polyclonal antibodies will be raised against these antigens and the antibodies will be screened for specificity using metacercaria from various other trematodes. Antibodies specific for the target species will be chosen for further development of test kits based on immunodot, coagglutination or ELISA formats.

Similarly, a gene library of metacercaria would be made and gene fragments specific to target species would be identified by cross hybridisation with various other metacercarial DNA. Specific regions will be cloned, sequenced and used for development of DNA probes or polymerase chain reaction (PCR) primers. Suitable test kits based on these will be prepared.

Expected Outputs

The outputs expected out of this project are test kits which are simple to use, sensitive and provide rapid results for detection of fish carrying trematode metacercariae. Such test kits will be highly useful for fish inspectors to determine the safety of fish for human consumption.

Workplan

Year I

The major surface associated proteins of metacercaria of *C. sinensis* and *O viverrini* will be analysed and polyclonal antibodies will be raised against them.

A gene library of metacercarial DNA will be prepared. Gene library will be screened for fragments specific for the target species.

Year II

The various antibodies will be screened for cross reaction with antigens from metacercaria of other trematodes. Antigens specific for *C. sinensis* and *O. viverrini* will be identified. Monoclonal antibodies will be raised against the antigens.

Gene fragments specific for target species will be sequenced. Based on the sequences, DNA probes and PCR primers will be developed.

Year III

The antibody tests will be evaluated for the sensitivity and specificity. The suitability of various formats such as dot blot, coagglutination, ELISA for development of kit will be studied. The antibody based kits will be developed and field evaluated.

DNA based kits will be developed and field evaluated.

Work Sharing by Various Countries

Korea will provide metacercaria for India and antigen analysis and gene library for *C. sinensis* will be done in India. Thailand will do similar work with *O. viverrini*. The antibody screening for cross reaction with other species and choosing the specific antigen for *C. sinensis* will be done in Korea and China. Further development of antibody kits will be done in India and the bits will be used for field evaluation in Korea and China. A similar approach will be used for DNA-based test development.

Duration **3 Years**

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Budget (US$)

	I year	**II year**	**III year**	**Total**
INDIA				
Equipment	20,000	-	-	
Salaries	10,000	10,000	10,000	
Contingencies	15,000	15,000	20,000	
International Travel	5,000	5,000	10,000	
	60,000	40,000	40,000	$ 140,000
KOREA				
Equipment	20,000	-	-	
Salaries	20,000	20,000	20,000	
Contingencies	20,000	20,000	20,000	
International Travel	10,000	10,000	10,000	
	70,000	50,000	50,000	$ 170,000
CHINA				
Equipment	20,000	-	-	
Salaries	10,000	10,000	10,000	
Contingencies	15,000	15,000	15,000	
International Travel	5,000	5,000	5,000	
	50,000	30,000	30,000	$ 110,000

THAILAND				
Equipment	20,000	-	-	
Salaries	20,000	20,000	20,000	
Contingencies	15,000	15,000	15,000	
International Travel	5,000	5,000	5,000	
	60,000	40,000	40,000	$ 140,000

GRAND TOTAL **$ 560,000**

2. Proposal from P.R. China :

Title : Epidemiology of Clonorchis sinensis infection in cultured and captured fish and survival trial on metacercaria in fish in relation to cooking/eating habits in China.

Background

1. *Clonorchis sinensis* infection in human populations

Clonorchiasis sinensis is one of the most severe food-borne parasitic diseases in China. Human infection has been reported in 27 provinces/autonomous regions/municipalities in China except Inner Mongolia, Ningxia, Qinghai, Yunnan and Tibet. The national average infection rate is 0.37%, and it is relatively higher in Guangdong and Guangxi provinces. In Guangdong, among the 95 counties/cities sampled in an epidemiological survey for Clonorchiasis sinensis more than once in 1973-1991, 62 were found with prevalence of the disease. Fecal examination showed *C. sinensis* infection in 16.2% of 680 671 persons, with the highest infection rate in Shunde City, being 26.6% (63 184/237 281). In Guangxi, 48 of 82 counties/cities surveyed, were endemic for the disease. 115 581 persons were surveyed by fecal examination in 129 observation spots of 40 counties/cities in 1992-1995, the average infection rate was 20.73%, and the highest one 74.68% .

2. *C. sinensis* infection in the first and second intermediate hosts

It has been confirmed that 9 species of fresh water snails in 3 families can serve as the first intermediate host of *C. sinensis.* In Guangdong and Guangxi, they are mainly *Parafossarulus sinensis, Alocinma longicornis* and *Bithynia fuchsianus,* and their infection rate for cercaria are 2.1% (365/17 400), 2.7% (167/6 258) and 0.6% (2/335) in Guangdong, and 0.45% (62/13 695), 2.58% (204/7 916) and 1.38% (197/14 261) respectively.

C. sinensis has no strong preference for its second intermediate host. To date, at least 84 species of fresh water fishes in 46 genera of 11 families are known to serve as second intermediate hosts of *C. sinensis* in China. Among them, 71 species in 36 genera belong to Cyprinidae. In Guangdong and Guangxi, they are mainly: (1) *Ctenopharyngodon idellus*, (2) *Mylopharyngodon aethiops*, (3) *Hypophthalmichthys nobilis*, (4) *Hypophthalmichthys molitrix*, (5) *Cyprinus carpio*, (6) *Carassius auratus*, (7) *Labeo kontius*, (8) *Rhodeus sinensis*, (9) *Pseudorasbora paria*, (10) *Hemibarbus maculatus*.

Chen Xiqi *et al.* (1993) reported that of 5947 fishes of different species examined in Guangdong, 1610 harbored metacercaria of *C. sinensis*, the average infection rate was 27.1%. Zhou Shihu *et al.* (1980) reported that of 14 species of fishes examined in Hengxian County, Guangxi, 4 were infected with metacercaria of *C. sinensis,* namely, *Pseudorasbora paria, Ctenopharyngodon idellus, Hypophthalmichthys molitrix* and *Hemiculter leucisculus.* Their infection rate was 95.7% (45/47), 30.0% (6/20), 33.3% (3/9) and 2.5% (2/80) respectively. Zhu Qunyou, *et al.* (1995) examined 307 fresh water fish of 10 species in Taiping Township of Wuming County, and found that 36 fish of 6 species were infected with metacercaria. Among 113 *Pseudorasbora paria,* 73 *Rhodeus sinensis* and 63 *Carassius auratus* examined, 25, 6 and 2 were found positive for metacercaria respectively; and among 5 *Ctenopharyngodon idellus*, 8 *Mylopharyngodon acthiops* and 10 *Cyprinus carpio* examined, one of each species had metacercaria parasitism.

Besides fresh water fish, metacercaria of *C. sinensis* was also found in shrimps (*Caridina nilotica Gracilipes, Macrobrachium superbum, Palemonetes sinensis* and *Leander miyadii*). However, their infection rate is not high. In Guangdong, among 251 *Macrobrachium superbum* examined, only 1 was positive, and the infection rate was 0.4%. In Guangxi, among 61 *Macrobrachium superbum* examined, none was positive.

3. Transmission factors and mode of infection

The basic conditions for the transmission of the disease are the presence of a source of infection (infected human population and animals), and the first and second intermediate hosts with a relatively high infection rate.

Water contamination by human and animal faeces is the key factor for the infection of snails and fish. In some endemic areas, people are used to set simple latrines and animal sheds by the ponds or ditches. Therefore, during the raining season, faeces will be very easily flushed into the water, such as in Wuming and Hengxian counties where over 90% (1456/1606) fish ponds by the villages were contaminated with human and animal excreta. In fish-raising areas in Guangdong and Guangxi, local people like to build latrines over the fish ponds to feed the fish with human faeces. Thus, the faeces excreted would fall directly down into the water; increasing the chance for snail and fish infection.

However, the most important key factor causing endemo-epidemic of clonorchiasis in many areas in China is the habit of eating raw or under-cooked fish. Generally speaking for the whole country, there are several ways to get infection as follows:

(1) Eating “raw fish” or “porridge with raw fish”

People in Guangdong, Guangxi, Hong kong and Taiwan have the habit of eating raw fish or porridge with raw fish. “Raw fish” is made of fish slices, fish with big body size, such as big carps, etc., mixed with seasonings (sesame oil, brown sugar, vinegar, sweet sauce made of fermented flour, sesame, green onion shreds and ginger shreds), and is without cooking. “Raw fish porridge” is made by filling boiling thin porridge into a bowl which holds raw fish slices and seasonings. When the boiling porridge is mixed with raw fish slices, the temperature would be lowered down to 70-80°C, which usually cannot kill all the metacercaria. Generally, fish with big body size except carps have low infection rate; however, repeated ingestion would also result in heavy infection. During the nation-wide sampling survey in 1988-1992, 2.50% of 100 568 people having raw fish-eating history were

infected with *Clonorchis sinensis*; however, only 0.150% of 1 369 281 people without raw fish-eating history were infected, showing very significant difference between the two groups ($p<0.001$).

An investigation showed that in Guangdong, 80.2% (3165/3946) of people infected with *C. sinensis* had a raw fish-eating history. In Baise Prefecture of Guangxi, 709 of 731 people (97%) infected with *C. sinensis* had eaten "raw fish".

In the three provinces in northeast China, residents of the Korean ethnic group and a few Chinese people also have the habit to eat raw fish. They capture the wild small fish from rivers, ditches, pits and ponds, then immediately remove their heads and viscera, and chop them into small pieces, add seasonings (sugar, vinegar, soy sauce, monosodium glutamate and chili) for eating. Usually, the infection rate of such wild small fish is high, and their intensity of infection is high too. People will easily get infected after eating.

(2) Eating under-cooked small fish and shrimps

In Henan, Anhui, Shandong, Sichuan and the mountainous regions in the upper reaches of the Pearl River and Hanjiang River, children and teenagers frequently go to catch small fish and shrimps in rivers, ditches and ponds, then eat them after incomplete barbecuing, baking and roasting. In some areas, people have the habit to eat freshly sun-dried or dry salted fish.

(3) Infection due to using metacercaria-contaminated utensils or hands

The knives and boards for cutting raw fish or the bowls, trays, saucers for holding raw fishes are all easily contaminated with metacercaria of *C. sinensis*, and are often used for cutting and holding cooked food without washing. People who always catch fish and shrimps, housewives and cooks are used to taking food with hands unwashed after treating the fish and shrimps. Fish-catching people are used to holding the fish in their mouths. All these habits could result in the infection of people with *C. sinensis.*

4. New epidemiological characteristics of clonorchiasis

(1) Along with the improvement of living standard, the infection rate of the disease tends to increase gradually as well, showing a remarkable family clustering.

Along with the social economic development of the country, people's living conditions improve gradually. People in town and country have changed their diet from mainly grain and vegetable to fish and meats, vegetables and fruits; and some typical local dishes become more popular. At present, raw fish has entered the menu of restaurants and families in rural and urban endemic areas. In Guangxi, some newspapers published articles in praise of raw fish as the first dish of Zhuang Ethnic Group. On New Year's Day and any festivals or dinners with relatives and intimate friends, it will be regarded as inhospitable and shaming if there is no raw fish dish on the table.

In 1996, two adjacent villages in Wuming County, Guangxi, i.e., Jumao village and Tangjia village, were investigated. The living standard was relatively higher in Tangjia village than in Jumao village. People of Tangjia village ate raw fish every day, and in some families even had raw fish for all the three meals daily. However, ordinary people in Jumao village

could only eat raw fish occasionally. Therefore, the infection rate of the two villages varied significantly, being 25.88% (118/495) for Jumao village and 40.22%(72/179) for Tangjia village. Based on a nation-wide sampling survey in 1988-1992, binomial distribution goodness of fit test was used for analyzing the data from 1,297,409 persons in 382,700 households which had complete data of survey. The results indicated a very remarkable family clustering for *Clonorchis sinensis* infection (X^2=2195300344.003, P<0.001).

(2) The difference of infection rate between urban and rural areas is reducing. In towns and cities, infection rate of officials is higher than that of workers, especially for those officials often going down to the countryside or moving about frequently.

The results of a nation-wide sampling survey in 1988-1992 show that people of all kinds of occupations can get infection. The average infection rate for different kinds of occupations were: farmers 0.3999%, workers 0.347%, officials 0.500%, teachers 0.593%, businessmen 0.787%, part agriculture and part business 2.407%.

(3) Endemic area tends to expand gradually. As live food fish and fish fry are transported to different parts of the country, men and animals in different areas will be likely infected. For instance, although there is no first intermediate host of *C. sinensis* in Bopu Town, Wuchuan county, Leizhou Peninsula, carps are infected with metacercaria, and 2 local cats were infected too. The reason is that the fish fries come from the endemic areas of Guangdong, namely, Pearl River Delta. However, since local people like to eat well-cooked fish, among the 678 people examined, no one was infected with *C. sinensis*.

In recent years, the fish culture industry is developing rapidly in China. For instance, since implementing the reform and opening policy in Guangxi, fish product has increased by 5-6 times. In 1997, water surface area for pisciculture was 2,590,000 mu (15 mu = 1 hectare), fish output was 636,000 tons, and it is estimated will be 700,000 tons. Fish will be transported to different parts of the country. Nowadays, eating raw fish becomes fashionable across the country. If men and animals in non-endemic areas eat raw or under-cooked infected fish, they will get infected, even resulting in new endemic areas.

A great amount of fresh water fish is shipped from inland of China to Hong Kong, Macao, and exported to Japan, South Korea, USA, Canada, Netherlands, Australia, and other countries. A rough estimation indicated that, in Guangdong the amount of fish exported from November 25, 1996 to November 25, 1997 is as follows:

(a) Live fresh water fishes to Netherlands: 0.15 ton,
(b) Frozen fresh water fishes to Hong Kong, Japan, Canada: over 72 tons,
(c) Fish slices to South Korea: 50 tons,
(d) Other kinds of fish flesh to Hong Kong, Macao and Australia: 277 tons,

and from November 26, 1997 to May 25, 1998:
(a) Frozen fresh water fishes to USA: 11 tons,
(b) Fish slices to Hong Kong: 1007 tons,
(c) Fin to Hong Kong, U.K. and France: 64 tons,
(d) General fish products to Hong Kong, Japan and USA: 1367 tons.

It is understood that all the fish products are exported after the examination by provincial animal quarantine. How to strengthen the quarantine in the future to ensure the safety of the

fresh water fish shipped to Hong Kong, Macao and foreign countries will be of utmost importance.

Objectives

This project aims at working out a guideline which can not only ensure safe eating of raw fish, but also keep the original good taste and nutrition of fishes for the health of consumers at home and abroad.

(1) To investigate metacercaria-infection rate in major fish host cultured and captured in endemic area.
(2) To investigate factors relevant to fish infection and human infection.
(3) To test thermal resistance of metacercaria in fish flesh by freezing, heating or other treatments in relation to eating habits.
(4) To discuss with local sectors the policies of agriculture practices and public health.

Method and Design

1. Field investigation in Guangdong and Guangxi simutaneously.

(1) Kato-Katz test for investigating human infection.

(a) Select 1-3 villages in one endemic county to investigate *C. sinensis* infection of residents, and interview the examinees for their raw fish-eating history. A total of 600-700 persons will be examined.

(b) Select 100-200 persons respectively from people eating raw fish and people not eating raw fish in the same endemic county for investigating *C. sinensis* of residents.

(2) Digestion technique for investigating *C. sinensis* metacercaria infection in cultured fresh water fish and wild fresh water fish.

(a) Select 4 fish ponds with hanging latrines over them (or fed with human and animal faeces) and 4 fish ponds fed with synthetic feed in the vicinity of the above villages. Catch cultured fish randomly from each pond, 10-20 fish for each species, then take the flesh from fish back y 5% of its body weigh for examination.

(b) Catch wild fish near the above village, about 20 fish for each species, then take the flesh from the back part by 5% of its body weight for examination.

(c) Select 2-3 restaurants serving raw fish, take 20 fish for each species from each restaurant, then get the flesh from the back by 5% of its body weight for examination.

2. Laboratory study on the resistance of *C. sinensis* metacercaria

(1) Materials

(a) Fresh water fish: Fresh water fish fries and wild small fish (whole body) which are confirmed to be infected with metacercaria of *C. sinensis* by squash method, average body

weight being 10g ±, and big size cultured fresh water fish (cut into slices of 1-2 mm, 5 mm thickness).

(b) Guinea pigs: select healthy and free from parasite eggs confirmed by stool examination (Average body weight 0.6 kg ±).

(2) Method

(a) Effect of cold storage and freezing on the infectivity of *C. sinensis* metacercaria to fish. They will be divided into two groups. The first group will be subdivided into 14 sub-groups for experiment and 1 for control. Five fries (or wild small fish) will be taken from each group. The second group will be subdivided into 24 sub-groups for experiment and 2 for control. Five fish slices 1-2 mm thick will be taken respectively from each of 12 sub-groups, and 5 fish slices 5 mm thick respectively from each of the other 12 sub-groups.

Materials from 42 experimental sub-groups will be stored in refrigerator of -4°C ~ -8°C or frozen at -18°C ~ 20°C, then be taken out on d1, d2, d3, d7, d14, d21 and d28 for microscopic examination of the structure of metacercaria and the viability of the larva in the cyst by using squash method, then the metacercaria will be separated by using digestive method for infecting animals.

Guinea pigs will be divided into 42 sub-groups for experiment and 3 for control, 5 guinea pigs for each group. 100 Metacercaria from each experimental sub-group and from fresh fish slices of control group or live fries will be fed into the stomach of guinea pigs per os. The guinea pigs will be raised for 25 days, then examined by using aldehyde amidine technique to confirm whether there is *C. sinensis* egg in the faeces. Stool examination will be done every other day. The guinea pigs will be dissected on d60 after infection, and examined. Its *C. sinensis* infection and worm count will be recorded. If the result is negative, microscopic examination of its bile sediment for *C. sinensis* egg will be followed.

(b) Experiment on resistance of *C. sinensis* metacercaria against seasoning

Whole body of the above mentioned small fish and fish slices of various thickness will be immersed respectively in 5%, 10%, 15% table salt solution, soy sauce (containing 12.5% sodium chloride), vinegar (containing 6.9% acetic acid), pure garlic sauce and 1:6 chili sauce. Each of the above 7 kinds of solutions will be divided into 6 groups by immersion time, namely, 0.5h, 4h, 8h, 16h, 24h, 48h. Each group has 5 small fish and 5 pieces of fish slices of different thickness. In addition, 3 control groups will be set in parallel. After immersion, fish bodies and fish slices will be washed clean, then metacercaria are isolated with digestive technique. The isolated metacercaria will be put into artificial pancreatic juice, and the morphology and activity of the larva in the cyst are observed under microscopy. They will be put into an incubator of 37°C for 1-2 hours, and then reexamined for excystation to determine whether it is live or not. Those already excysted will be regarded as live metacercaria. For dead metacercaria, the larva in the cyst will have no reaction under heating stimulation in the artificial pancreatic juice, and will curl up in the cyst, no molting occurs although the cyst wall is already very thin, the granules in the excretory bladder will become light yellow or yellowish-brown from black, and lose their lustre.

(c) Experiment on the resistance of *C. sinensis* metacercaria in boiling water, “raw fish porridge” and cooking on fire

(i) Boiling in water

Put the above mentioned small fish and fish slices of various thickness into boiling water. Five small fishes and 5 pieces of fish slices will be put into each group treated at different temperature and time. The temperatures tested will be 50°C, 60°C, 70°C, 80°C, 90°C, 100°C respectively, and the duration of boiling will be 15 sec., 30 sec., 1 min., 3 min., 5 min., 10 min. respectively. After boiling, they will be taken out for isolating metacercaria with digestion technique.

(ii) Raw fish porridge

First cook the porridge to boiling point, then put the above mentioned small fish and 5 pieces of fish slices of different thickness into the porridge, and maintain the temperature of porridge respectively at 50°C, 60°C, 70°C, 80°C, 90°C. The duration of immersion will be 30 sec., 1 min., 2 min., 5 min., and 10 min. respectively. In each group of different temperature and duration, five small fish will be tested. Then, they will be taken out and the metacercaria will be isolated with digestion technique.

(iii) Baking on fire

Wrap the above-mentioned whole fish and fish slices in cabbage leaves or mud, then bury it into live charcoal for baking (the temperature of live charcoal is about 200°C), for 1, 3, 5, 10 and 15 min respectively, then take it out for isolation of metacercaria with digestive technique.

One control group will be set for each of the above methods.

Among the metacercaria isolated from various groups with the above 3 methods, those with normal morphology and structure will be selected to infect guinea pigs, 4 guinea pigs for each group, and 100 metacercaria for each guinea pig. Sixty days after infection, they will be dissected for observation of the adult and immature worms of *C. sinensis* in the guinea pigs. If it is negative, its bile sediment will be microscopically examined for eggs.

(d) Influence of ionizing radiation on the infectivity of *C. sinensis* metacercaria in fish body.

(i) The radiation source is ^{60}Co γ ray. The radiation dosage on fish body is 0.15, 0.5, 1.00, 2.00, 2.50, 3.00 and 4.00 KGy respectively.

(ii) Radiation on fish body. The radiation source is electron linear accelerator, producing 10 MeV electron ray. The dosage on fish body is 0.5, 1.00, 2.00, 3.00, 4.00 and 5.00 KGy.

Divide the experimental objects into groups by different radiation dosages of the above mentioned 2 methods, five small fish and 5 pieces of fish slices of different thickness for each group, and 1 group for control. Fish will be treated with different radiation dosages, and be dissected immediately after radiation for isolating metacercaria. The same procedure will be performed on control group. The metacercaria isolated will be used to infect guinea pigs per os at the dosage of 100 metacercaria per guinea pig, 4 guinea pigs for each experimental group. From d25 after infection, faeces of guinea pigs will be examined with aldehyde amidine technique to see whether there is *C. sinensis* eggs. Stool examination will be done

every other day. Dissection will be done on d60 after infection to examine *C. sinensis* infection and make egg counting. If the result is negative, the bile will be taken for microscopic examination for *C. sinensis* egg.

(e) Influence of electrolytic oxidation water on the infectivity of *C. sinensis* metacercaria in fish body

Adopt electrolytic oxidation water technique to treat wild small fish and fish chops of two different thickness, then separate the metacercaria from the treated fish body to infect guinea pigs. On day 25 after infection, the faeces of guinea pigs will be examined by formalin-ether technic once every other day to see whether there is *C. sinensis* egg. On day 60, the guinea pigs will be dissected for examination and counting of *C. sinensis* worm.

3. Coordinated by local administration, we will meet with personnel engaged in sanitation and epidemic prevention, business and trade, owners of aquatic product farms and restaurants to ask for their suggestions for aquatic products, then suggest our strategy and method in a guideline to local governments and departments concerned about how to produce and transport “safe fish”, and serve people “trustful fish”.

Timetable: From approval of proposal to the date receiving grant

1. First year

(1) Site selection
(2) Field survey
(3) Experimental study on the effect of cold storage and freezing on the infectivity of *C. sinensis* metacercaria in fish body.

2. Second year

(1) Experiment on the resistance of *C. sinensis* metacercaria in seasoning, boiling water, “raw fish porridge” and baking on fire.
(2) Effect of ionizing radiation and electrolytic water on the infectivity of *C. sinensis* metacercaria in fish body.

3. Third year

(1) Data analysis and collation
(2) Discuss with personnel concerned to ask for their requirement of aquatic products.
(3) Conclusion, suggest our strategy and method to local governments and departments concerned about how to produce and transport “safe fish”, and serve people “trustful fish”.

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Guangdong Provincial Institute of Parasitic Disease Control

Proposed Budget for two years: (in US$)

1. Field activities:

(1) Provincial personnel, administrative cost for
2 provincial institutes 3500/year x 2 x3 — 21,000

(2) Travel:
Airfare (Shanghai - Nanning) round trip, 3 times/year
Airfare (Shanghai - Guangzhou) round trip, 3 times/year
120 x 5 persons x 18 times: — 10,800

(3) Hotel and per diem for PIs and local workers in field investigation
7 person for each province plus 5 person from Shanghai,
total 24 persons
30 x 60 days x 24 persons: — 43,200

(4) Car rental and gasoline
500 x 3: — 1,500

Subtotal: 76,500

2. Consultants (WHO/FAO officers or international experts)

Two times, one at the beginning of project and the other at the end of the project,
Two persons for each time.

(1) Airfare: 3000 x 2 persons x 2 times: — 12,000
(2) Per diem: 100 x 2 persons x 10 days x 2: — 4,000

Subtotal: 16,000

3. Laboratory expenses		
(1) Animals: 6000 x 3		18,000
(2) Collaborative expense for radiation experiments:		16,000
(3) Fish sample collection at fish farm, restaurants and fish markets:		17,000
(4) Other supplies: 1000 x 3		3,000
	Subtotal:	54,000
4. Data processing and analysis, communication		6,000
5. Conference expenses (two times) one at the beginning, the other at the end		12,000
	Grand total:	**164,500**

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