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Project Name: Development and Investigation of the Delivery Mode of a Multivalent Bacterial Fish Vaccine in Zambia

Fish Innovation Lab

Final Technical Report: 05/01/2021 – End Date: 07/31/2023

Cooperative Agreement 7200AA18CA003

USAID
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MISSISSIPPI STATE UNIVERSITY
GLOBAL CENTER FOR AQUATIC HEALTH AND FOOD SECURITY

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Partners/Institutions

University of Zambia

School of Veterinary Medicine

Zambia

Central Veterinary Research Institute

Ministry of Fisheries and Livestock

Zambia

Zambia - Aquaculture Enterprise Development Project

Siavonga Aquaculture Cooperatives

Zambia

Mississippi State University

USA

Abbreviations and Acronyms

API	Analytical Profile Index
BHI	Brain heart Infusion
BLAST	basic local alignment search tool
cfu	Colony Forming Units
°C	Degree Celsius
DOF	Department of Fisheries
FAO	Food Agriculture Organization
g	gram
h	hour
LD	lethal dose
mL	milliliter
PBS	Phosphate Buffered Saline
RPS	relative percentage survivals
rpm	revolution per minute
RNA	Ribonucleic acid
spp	Specie

Glossary

Autogenous vaccine: a vaccine made from a pathogen (commonly a specific bacteria) that has been isolated from diseased fish on a farm and the vaccine is meant for the disease caused by the same bacteria.

Bacteria: is a single celled microorganism capable of independent life and exists almost anywhere on the planet.

Bivalent vaccine: A vaccine consisting of two pathogenic agents in its composition.

Pathogen: Any microorganism capable of causing disease.

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Abstract

The emergence of production related bacterial diseases in aquaculture is on the increase. These diseases occur as a consequence of increased biomass in the environment the fish is living. The desire for increased profits by farmers also leads to increase stocking densities, In Zambia, the aquaculture industry is rapidly growing as the government has placed a lot of emphasis in fish production. This has led to increased production losses as a result of diseases.

The project under discussion, considered the development of an autogenous vaccine using bacterial isolated from the sick fish collected from small scale farmers. The fish exhibiting clinical signs was collected and bacteria isolated. The identified bacteria genera included *Acinetobacter, Pseudomonas, Aeromonas, Bacillus, Clostridium, Klebsiella, Lactococcus, Micrococcus, Staphylococcus, Streptococcus* and *Vibrio*. Of these bacteria identified *Acinetobacter, Aeromonas, Klebsiella* and *Lactococcus* were documented as pathogenic after conducting pathogenicity tests. *Lactococcus garviae* and *Aeromonas hydrophila* were picked for vaccine formulation following their pathogenic potential. These bacteria were inactivated accordingly for vaccine formulation, after which, the fish were vaccinated using the intraperitoneally and immersion methods. The *Aeromonas hydrophila* prepared vaccine did not induce a high protection on either route under consideration. The relative percentage survivals for the *Lactococcus garviae* and *Aeromanas hydrophila* vaccines was 94.1% and 70.6% respectively for the intraperitoneal routes. The immersion route did not confer appreciable protection. Following the efficacy studies, the *Lactococcus garviae* vaccine was used in the field at a farm on Lake Kariba. As observed so far mortalities are not being recorded in the vaccinated cage as compared to the unvaccinated cages. This study provides a platform for autogenous vaccine formulation and usage in Zambia.

1.0 Introduction

Aquaculture represents the fastest growing food production sector over recent decades (FAO, 2022). This sector encompasses intensification of growing fish in a defined area. This leads to an environmental imbalance where disease susceptibility and incidence to infectious diseases in aquaculture increases. These diseases have been singled out as a major obstacle to the success of the industry. Many of the most serious infectious disease agents affecting cultured species in aquaculture are bacteria. Bacteria rarely act as primary pathogens. They occur most commonly as opportunistic pathogens in already damaged or severely immunocompromised fish which is growing in a stress environment (Wang et al., 2023). Various strategies are being developed to minimize the impact of disease emergence such as use of medicinal plants (Wangkahart et al., 2022), probiotics, prebiotics, immunostimulants (Hai, 2015; Kocher, 2004; Ortuño et al., 2002) and vaccination (Ma et al., 2019). In some cases, antibiotics have been used as one of the easy alternatives. The use of antibiotics has attracted a lot of criticism as issues of antibiotic residues, bacterial drug resistance and toxicity arise (Smith, 2008). As a result, vaccination is seemingly the most probable disease management tool as fish disease outbreaks are now being reported in all areas where aquaculture is active (Barnes et al., 2022).

Vaccination has become an important tool in managing infectious diseases in fish culture because of the vaccine's ability to stimulate protective immunity and produce a memory response in vaccinated fish (Wang et al., 2023). This has attracted the need to draw local microbial profiles and consolidate on the need to develop autogenous vaccines. Zambia’s aquaculture production potential has increased. This can be made from the 19% registered increase in aquaculture from 63,418 metric tonnes in 2021 to 75,647 metric tonnes in 2022. This production came from the cage culture and intensive land-based production systems (DoF Annual Report, 2022). The larger part comes from the cage systems on Lake Kariba. Small scale operations are also increasing in number as the Government of the Republic of

Zambia has come up with a deliberate policy of empowering Zambian aquaculture producers with revolving funds to procure cages, fingerlings and feed. This initiative has led to small scale farmers forming cooperatives where platforms made up of 10 to 30 farmers are erected with each farmer owning one of more cages. The cages have capacity of stocking up to 25000 fish.

In commercial cage production systems, significant bacteria diseases have been reported. These include *Streptococcus agalactiae*, *Streptococcus iniae* and *Lactococcus garvieae* in 2014/2015 mass mortality events (Bwalya *et al.* 2020a). Little attention has been drawn to small scale cage producers who are become a strong sector in fish production. It was therefore important that this project embarked on profiling bacteria pathogens associated with fish mortalities in small scale farmers and develop an autogenous vaccine from the significant pathogens identified.

2.0 Research Methods

The study design employed a mixed study approach (cross-sectional experimental) and involved the following activities:

2.1. Identification of small-scale farmers growing fish in cages: The farmers were identified by the Ministry of Fisheries and Livestock officers working with cooperatives made of small-scale farmers. The Ministry officials informed the farmers the objective of the study. From the objectives all the farmers expressed interest to be included.

2.2. Sampling of sick fish from the cages: A purpose approach was used according to Creswell (2012). Fish exhibiting signs of disease were scooped. The signs of disease included erratic swimming (such as spiraling or spinning); loss of buoyancy control; lethargy; darkening; uni- or bilateral exophthalmia (pop-eye in one or both eyes); corneal opacity (whitish eyes); hemorrhages in or around the eye, the gill plate, base of the fins, vent/anus, or elsewhere on the body; ascites (i.e., distended abdomen); ulcerations and abscesses.

2.3. Sample collection: The sick fish was subjected to clove oil after which it was dissected to collect the brain, liver, spleen and kidneys. The collected samples were placed into sterile containers and then stored in a cooler box with ice. The samples were then transported to the laboratory. The abdominal cavity was also swabbed with sterile swabs, which were then placed into the carry Blair transport media.

2.4. Bacteria isolation and identification: The collected samples were directly inoculated onto Blood and Nutrient agar, where bacteria colonies were isolated for further identification and characterization. The identification involved biochemical reactions and the analytical profile index (API) system.

2.5. Molecular Identification: Using the 16S rRNA primers, the isolated bacteria were identified through the polymerase chain reaction, sequencing and the basic local alignment search tool (BLAST).

2.6. Antibiotic Susceptibility of Identified Bacteria: The disk diffusion method was used to determine the susceptibility of identified bacteria to antibiotics. This was done for food safety purposes.

2.7. Pathogenicity test: The healthy individuals of *Oreochromis niloticus* fish weighing 50 g were used. The fish acclimated at 25°C for one week in flat bottomed circular 150L and 250L plastic tanks. The tanks were filled with borehole water from the tap. After filling the tank with water, the outlet of the tank was slightly opened to provide a continuous water out flow while approximately the same amount of water was allowed into the tank continuously from the tap. The water also was properly aerated with the help of aerators. The fish were fed a normal recommended commercial diet. Only the healthy fish which were showing normal activities were selected for further experimentation. In vivo pathogenicity test was carried as described by Bwalya et al., (2020b) and the minimum lethal dose was calculated as detailed by Saganuwan (2020).

2.8. Vaccine development: Isolates exhibiting pathogenicity were used for vaccine formulation. In this case *Lactococcus garvieae* and *Aeromonas hydrophila* were used. The bacteria (*L. garvieae* and *A. hydrophila*) were separately propagated in Brain heart Infusion broth and incubated at 30°C on a shaker at 150 rpm for 72 hours. Thereafter, the cells were pelleted by centrifugation at 1000 *xg* for 20 min at room temperature. The pelleted cells were washed three times and then suspended into PBS with 10^9 cfu/mL. This suspension was inactivated with 4% formalin for 72h. The formalin was removed by washing the cells in PBS. This was done by suspending the cells in PBS buffer and

pelleting the cells at 1000 *xg* for 20 min. This procedure was repeated 4 times. The inactivation process was confirmed by inoculation of the antigen on blood agar followed by incubation at 37°C for 72h to demonstrate the absence of bacterial growth.

a. Intraperitoneal administration: The vaccine was formulated using 10^9 cfu/mL in a water-in-oil emulsion using the ISA 763 VG adjuvant (Seppic, France) according to the manufacturer's guidelines. The adjuvant only group was prepared in the same way but without bacteria. The preparations were then stored at 4°C until used. The vaccine and adjuvant only were determined to be sterile by streaking them on blood agar and observing for bacterial growth after 72h incubation at 37°C.

b. Immersion administration: The cells to be used as antigen under the immersion method were re-pelleted to the original volume of 10^9 cfu/mL after and then stored at 4°C until use.

2.9. Bivalent Vaccine formulation: The inactivated cells of 10^9 cfu/mL *L. garvieae* and *A. hydrophila* were mixed together and then suspended in a water-in-oil emulsion. The preparation was then stored at 4°C until use.

The mixing of two antigens was also done under the immersion preparation. Following the removal of formalin by washing cells with PBS, the two antigens were mixed and stored as previously indicated.

2.10. Vaccine efficacy:

a. Intraperitoneal administration: The fish were divided into 3 groups consisting of 60 fish per group as control, adjuvant and vaccine groups. The control fish were injected with PBS only, while the adjuvant group were injected with adjuvant only and the vaccine group with *L. garvieae* and *Aeromonas hydrophila* vaccines separately. Each group was further split into two replicates, one for

observation (surveillance) and the other for the challenge experiment. For the fish meant for challenge observations, each group was placed in a separate tank. Tank A (PBS only), tank B (adjuvant only) and tank C (vaccinated) containing 30 fish each. The rest of the fish were pooled together in tank D (surveillance), containing 75 fish as control, adjuvant-only and vaccinated groups (all mixed together). The fish in tank D were marked by clipping of the dorsal fin, caudal fin or left unclipped to differentiate between groups. All fish were injected intraperitoneally with 0.1ml of vaccine, adjuvant-only or PBS according to the group.

An attempt was made with a bivalent vaccine prepared. This was administered as described above, only that the vaccine consisted of two antigens as opposed to one antigen.

b. Immersion administration: Under this route of administration, a similar approach as for the intraperitoneal route was taken. Only that, instead of administering the vaccine through an injection, in this case the pelleted antigens were suspended into 5L of water to give a 1.5×10^8 cfu/mL. The fish was suspended into the water for 30 minutes. This process was repeated using the combined antigens accordingly.

2.11. Challenge experiment: Following vaccination, all fish in the four tanks (A, B, C and D) were allowed a period of 6 weeks for immune induction. On day 36 days post vaccination, the fish were challenged by intraperitoneal injection of 0.1 ml of 10^6cfu bacteria/fish. Immediately following challenge, the fish were observed for clinical signs and sampling was done for bacterial re-isolation and pathology observation. The fish that exhibited distress signs of sickness was anaesthetized using Benzocaine and then subjected to pathology observation.

2.12. Field vaccination of fish: Using the results obtained, the only antigen delivered for trials was the Lactococcus vaccine. This was injected in 5000 fish weighing 50g. As of now the fish is growing steadily without mortalities. Further observations are still being made.

2.13. Ethical Considerations: All aspects of project activities were subjected to review by the ERES Converge Institutional Review Board which is accredited to the Office for Protection of Human Research Participants in the United States of America and has IRB and Federal Wide Assurance numbers, 00005948 and 00011697 respectively.

3.0 Research Results

a. Identified Bacteria associated with sick fish: The sampled fish samples subjected to bacteria culture and isolation yielded the following bacteria genera as associated with sick fish; *Acinetobacter, Pseudomonas, Aeromonas, Bacillus, Clostridium, Klebsiella, Lactococcus, Micrococcus, Staphylococcus, Streptococcus* and *Vibrio*. Of the isolated bacteria, *Aeromonas spp, Pseudomonas spp, Micrococcus spp, Klebsiella spp, Lactococcus spp, Streptococcus spp*, and *Acinetobacter spp* are well known fish pathogens. The results are shown in Appendix 1.

b. Antibiotic susceptibility testing: The identified bacteria were subjected to antibiotic susceptibility tests for food safety consideration. The overall susceptibility results indicated that sensitivity to broad spectrum antibiotics tested was; doxycycline (52.9%), oxytetracycline (51.7%) and ciprofloxacin (50.3%). Complete resistance to ciprofloxacin, co-trimoxazole, and cephalothin was recorded in the *Bacillus spp*. Doxycycline was the only antibiotic that showed effectiveness against all the bacterial isolates, while other antibiotics used were ineffective on one or more bacterial isolates.

c. Pathogenicity tests: In order to determine bacteria of relevance in disease causation the isolated identified bacteria were exposed to *Oreochromis niloticus* weighing 50g. Of the bacteria tested, *Acinetobacter, Aeromonas, Klebsiella* and *Lactococcus* were documented as pathogenic with lethal doses (LD50) calculated as 10^6 cfu/ml, 10^5 cfu/ml, 10^6 cfu/ml and 10^5 cfu/ml respectively.

d. Laboratory Vaccine efficacy: Of the identified bacteria, *Lactococcus garvieae* and *Aeromonas hydrophila* were picked for vaccine formulation following their pathogenic potential as exhibited by the LD50. The *Lactococcus garvieae* vaccine formulated and administered through the intraperitoneal route provided protection as no clinical signs were observed in the vaccinated fish as opposed to the control and adjuvant only groups. The relative percentage survivals (RPS) for *Lactococcus garvieae*

vaccine was 94.1% and 41.7% for the intraperitoneal and immersion routes respectively. As for the *Aeromonas hydrophila* vaccine the RPS was calculated at 70.6% and 21.9% for the intraperitoneal and immersion routes respectively. As for the bivalent vaccine, the RPS was 39.1% and 22.4% for the intraperitoneal and immersion routes respectively.

e. Field vaccination of fish: Following the efficacy studies, the *Lactococcus garvieae* vaccine was used in the field trails at a farm. As observed so far mortalities are not being recorded in the vaccinated cage as compared to the unvaccinated cages. The study team is still monitoring the results.

f. Lessons learnt from the interaction with small scale farmers: As the study was on going, the team interacted with farmers and a questionnaire (Appendix 2) was administered to determine the level of knowledge and understanding on various aspects of fish health by the farmers. From the analyzed questionnaires, it was clear that most farmers ventured into aquaculture without clear understanding on how to manage the enterprises. The farmers did not have any knowledge on fish health and biosecurity. There were no measures aimed at disease prevention or control in any case of a disease outbreak.

4.0 Outputs

1. Profiling of Bacteria associated with sick fish in small scale establishments. The study identified key pathogens (production related bacteria pathogens) that could affect the productivity of the aquaculture enterprises.

2. Aquatic food safety indictment: The antibiotic susceptibility tests highlighted the possibility of fish being a driver of antibiotic resistance bacteria.

3. Vaccine development/formulation: A Lactococcosis vaccine was developed and applied on a selected fish farm.

4. Challenges encountered by small scale farmers: Farmer associated challenges were identified and highlighted through this work.

6. Human resource capacity building on local autogenous vaccine formulation and administration. The project has supported two masters and doctorate students who are on course to completion.

5.0 Conclusions

The project has impacted positively on aquatic health as pathogens associated with fish losses have been identified and potential strategies to control these pathogens through an autogenous vaccine that can be formulated locally. The trials on locally made vaccines should be sustained to perfect the products.

6.0 Technologies/Innovations developed, and what phase was achieved

The innovation being developed is on the biological control of fish pathogens by using autogenous vaccines which are custom vaccines produced from pathogens isolated directly from affected farm(s) on which the vaccines are subsequently deployed under a minor use or restricted permit.

The clinical trial phase of the vaccine has been achieved as currently the vaccine has been administered on the farm on Lake Kariba. Further observations are being recorded and will be determined at the point of harvesting the fish. The targeted disease is a production related disease that occurs as the fish biomass increases.

7.0 Key Beneficiaries

1. Fish farmers cooperatives: The farmers benefited from the interaction with project team and the results that have been generated from the activities undertaken will assist in programs meant for improved productivity and implementation of biosecurity measures.

2. Government Ministry of Fisheries and Livestock extension staff: The Government extension workers attached to the project, learnt fish sampling, sample preservation and packaging. Strategies for fish health and biosecurity was explained to the staff.

3. Central Veterinary Institute: This a vaccine manufacturing institute for the Ministry of Fisheries and Livestock. The doctorate student is a member of staff, who has been trained and learnt on how to proceed with autogenous vaccine formulation, challenge and molecular analysis of bacteria.

4. Copperbelt University, faculty of Natural Resources: This is a university that offers courses in aquaculture and their staff is one of the Master's students pursuing aquatic health.

8.0 How the scientific results were disseminated

1. The results have been disseminated in the country through a workshop hosted by the Food Agriculture Organization and the Ministry of Fisheries and Livestock. The workshop was on Aquatic health and Biosecurity. The results were disseminated to inform the workshop participants on the emerging diseases documented in aquaculture in Zambia.

2. Scientific publications are being prepared for submission to international journals. The manuscript under preparation and ready for submission is:

An Investigation of Bacterial Pathogens Associated with Diseased Nile Tilapia in Small Scale Cage Culture Farms on Lake Kariba, Siavonga, Zambia

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10.0 Appendices

Appendix 1: Occurrence of the different bacteria genera on the 11 farms in the study area

Bacterial isolates	Number (%) of Isolated Bacterial Species by Source											
	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5	Farm 6	Farm 7	Farm 8	Farm 9	Farm 10	Farm 11	Total
Acinetobacter spp	0 (0.0)	1 (0.3)	1 (0.3)	4 (1.3)	3 (1.0)	0 (0.0)	2 (0.7)	3 (1.0)	2 (0.7)	3 (1.0)	2 (0.7)	21 (7.0)
Aeromonas spp	2 (0.7)	2 (0.7)	6 (2.0)	3 (1.0)	7 (2.3)	1 (0.3)	9 (3.0)	3 (1.0)	1 (0.3)	1 (0.3)	4 (1.3)	39 (13.0)
Bacillus spp	2 (0.7)	2 (0.7)	3 (1.0)	1 (0.3)	1 (0.3)	1 (0.3)	2 (0.7)	1 (0.3)	1 (0.3)	0 (0.0)	0 (0.0)	14 (4.7)
Citrobactor spp	0 (0.0)	0 (0.0)	1 (0.3)	1 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.7)
Klebsiella spp	4 (1.3)	1 (0.3)	2 (0.7)	1 (0.3)	2 (0.7)	4 (1.3)	6 (2.0)	3 (1.0)	2 (0.7)	1 (0.3)	0 (0.0)	26 (8.7)
Lactococcus spp	6 (2.0)	1 (0.3)	3 (1.0)	0 (0.0)	2 (0.7)	2 (0.7)	4 (1.3)	2 (0.7)	1 (0.3)	1 (0.3)	0 (0.0)	22 (7.3)
Micrococcus spp	4 (1.3)	2 (0.7)	6 (2.0)	1 (0.3)	4 (1.3)	1 (0.3)	6 (2.0)	2 (0.7)	2 (0.7)	0 (0.0)	1 (0.3)	29 (9.7)
Pseudomonas spp	2 (0.7)	1 (0.3)	4 (1.3)	1 (0.3)	5 (1.7)	2 (0.7)	9 (3.0)	2 (0.7)	1 (0.3)	1 (0.3)	3 (1.0)	31 (10.3)
Staphylococcus spp	0 (0.0)	2 (0.7)	1 (0.3)	0 (0.0)	2 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (1.7)
Streptococcus spp	3 (1.0)	2 (0.7)	2 (0.7)	1 (0.3)	1 (0.3)	4 (1.3)	4 (1.3)	2 (0.7)	2 (0.7)	0 (0.0)	0 (0.0)	21 (7.0)
Unidentified	2 (0.7)	3 (1.0)	3 (1.0)	2 (0.7)	5 (1.7)	2 (0.7)	7 (2.3)	2 (0.7)	1 (0.3)	1 (0.3)	2 (0.7)	30 (10.0)
No Growth	5 (1.7)	3 (1.0)	8 (2.7)	5 (1.7)	13 (4.3)	3 (1.0)	11 (3.7)	5 (1.7)	2 (0.7)	2 (0.7)	3 (1.0)	60 (20.0)
Total	30 (10.0)	20 (6.7)	40 (13.3)	20 (6.7)	45 (15.0)	20 (6.7)	60 (20.0)	25 (8.3)	15 (5.0)	10 (3.3)	15 (5.0)	300 (100.0)

Appendix 2

Section A. Your knowledge about Fish Diseases Incidences

This section aims at obtain the KNOWLEDGE and AWARENESS that you and your farm/ Institution have about fish diseases outbreaks in farmed fish.

Question 1. Please indicate the level of your knowledge in fish diseases incidences in fish farms/ District.

None	Little	Some	Fair	Good	Excellent	Do not know

Question 2. Please indicate the level of fish diseases incidences at your farm/ District

- None: No occurrences
- Little: Once at stocking
- Some: Monthly
- Fair: Weekly
- Do not know: Never paid attention.

N.	Fish Diseases incidences	None	Little	Some	Fair	Good	Do not know
1.	In the past 10 years						
2.	In the past 5 years						
3.	In the past 2 years						
4.	In a year						
5.	Mortalities in fingerlings / month 1						
6.	Mortalities in juveniles /month 2-3						
7.	Mortalities in adult fish month 4-6						
8.	Level of mortalities at your farm/district						

Question 3. Please indicate HOW OFTEN your farm/Institution has taken measures to prevent fish diseases outbreaks.

- Never: No occurrences
- Rarely: Once at stocking
- Occasionally : Monthly
- Sometimes: Weekly
- Often : Every other day
- Always: Daily.
- Do not Know: Never paid attention

N.	Preventive measures taken	Never	Rarely	Occasionally	Some-times	Often	Always	Do not know
A.	Foot baths							

B.	Attending workshops on fish diseases							
C.	Cleaning of aquaculture equipment's							
D.	Water quality measurements							
E.	Taking of fish mortalities samples for diagnostics							
F.	Sharing of equipment's with other farmers							
G.	Sampling and taking adjustments of fish feed							
H.	Cleaning of culture nets							
I	Do you find out about the health practices of hatcheries were we obtain the fish seed from.							
J	Storage of fish feed/ any chances of contamination							
L	Workers' Knowledge on proper handling of fish, feed and equipment's.							

Question 4. Please indicate which areas you consider to be a priority in preventing fish diseases incidences according to your priority ranking.

N.	Areas of work	First priority	Second priority	Third priority
1.	Training in fish diseases outbreaks			
2.	Culture facilities management			
3.	Biosecurity measures			
4.	Quality of fish seed from hatcheries			
5.	Supply of fish vaccines			

Question 6. What else do you think are areas of concern that would be contributing to fish diseases incidences in culture facilities?

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THE INFORMATION THAT WILL BE OBTAINED FROM THIS RESEARCH WILL BE TREATED WITH HIGH CONFIDENTIALITY. NO FARMER WILL INDIVIDUALLY BE IDENTIFIED.

MANY THANKS AGAIN FOR YOUR CONTRIBUTION: IT IS HIGHLY APPRECIATED AND THE FINDINGS WILL CONTRIBUTE TO THE UNDERSTANDING OF RISK FACTORS CONTRIBUTING TO FISH DISEASES INCIDENCES IN CULTURE FACILITIES.