Asian Fisheries Science **10**(1998):347-354
Asian Fisheries Society, Manila, Philippines
https://doi.org/10.33997/j.afs.1998.10.4.008

Isolation of *Aeromonas hydrophila* from *Oreochromis niloticus* during Fish Disease Outbreaks in the Philippines

A. V. YAMBOT

Freshwater Aquaculture Center
Central Luzon State University, Nueva Ecija, Philippines

Abstract

The study focused on the bacteriological examination of Nile tilapia, *Oreochromis niloticus*, during fish disease outbreaks in various aquaculture farms and projects in Luzon, Philippines. Farm sites and aquaculture research projects which had outbreaks were visited from January 1994 to February 1996. Fish samples were observed to have disease signs like skin lesions, ulcerations, fin rot, body discoloration, mouth sore, eye opacity, exophthalmia, dislodged eyeball, and sluggishness. High mortalities of Nile tilapia were monitored in cage culture projects notably during the rainy season and cold months.

Streaking into plates of nutrient agar, trypticase soy agar and Rimler-Shotts media was done for primary isolation of bacteria. Isolates were obtained from different tissues of diseased Nile tilapia samples such as ulcerated skin and muscle, liver, kidney, spleen, gall bladder and opaque eyes. All isolates had heavy growth of yellow colonies in Rimler-Shotts media. In primary characterization tests, they were gram negative, rod-shaped, motile, oxidase positive, fermentative, 0/129 resistant and novobiocin resistant, suggesting that the colonies were aeromonads. Detailed examination using differential tests and API 20E showed the isolates to be *Aeromonas hydrophila*.

All isolates were sensitive to chloramphenicol and oxolinic acid but resistant to ampicillin. Reaction to other antibiotics such as streptomycin, erythromycin and oxytetracycline varied from resistant, intermediate and sensitive. Experimental infection of the disease by immersion route was done and mortalities were monitored for 96 hrs. Results showed LC_{50} at 1.5 x 106 at 96 h, LC_{100} at 108, and no mortality at 103 and control. Moribund fish manifested the same clinical signs as the naturally infected Nile tilapia.

Consistent isolation of *A. hydrophila* from various tissues of diseased Nile tilapia during disease outbreaks shows a recurring septicemia. Occurrence of the disease has been observed in low-volume and high-density aquaculture of Nile tilapia during the rainy season and cold months when temperatures are low. Regular disease outbreaks pose a threat to the Nile tilapia industry, necessitating steps to control the disease and minimize economic losses.

Introduction

Nile tilapia is a popular freshwater aquaculture species in the Philippines. Tilapia hatcheries continue to sprout throughout the country as a result of technology dissemination generated by Nile tilapia genetics projects based at the Central Luzon State University (CLSU), Nueva Ecija, Philippines. Most farmers are going into semi-intensive and intensive farming of the fish in

tanks, ponds and cages. This could make the country one of the leading producers of cultured tilapia in the world (Bimbao *et al.* 1993).

Although most freshwater fish species in the country have been affected by the epizootic ulcerative syndrome (EUS), the Nile tilapia remains resistant to the disease. *Aeromonas hydrophila*, a bacterial pathogen, is considered a secondary invader of EUS-infected and already compromised host (Roberts *et al.* 1993). *A. hydrophila* is incriminated in a hemorrhagic septicemia involving the Nile tilapia with fish showing disease signs like external ulcerations and hyperaemia at the margins of the eye orbit (Roberts 1993; Roberts and Sommerville 1982). Lightner *et al.* (1988) likewise isolated *A. hydrophila* from kidney and peritoneal fluid of tilapias with external ulcerations and ascites.

Reports of diseases with significant economic losses in cultured Nile tilapia in the country were rare before 1990. However, in the early 1990s, disease outbreaks with high mortalities were noted in low-volume and high-density aquaculture, especially in cage culture projects, leading to severe economic losses for tilapia breeders. The disease outbreaks in cultured Nile tilapia were investigated primarily by bacteriological examination of affected fish. Results are herein presented.

Materials and Methodologies

Bacteriological examination was done on different sizes of Nile tilapia showing signs of disease such as lesions and other abnormalities and obtained from cage projects in reservoirs and different aquaculture farms in Luzon, Philippines, where disease outbreaks and mortalities reportedly occurred from January 1994 to February 1996. The number of fish sampled per farm was limited by the fishfarmer/owner from five to 10 fish since disturbing the diseased stocks during outbreaks would cause mortality of fish immediately after handling.

Inoculation of nutrient agar media, tryptone soya agar and Rimler-Shotts media prepared in the laboratory was done in farm sites using an improvised isolation carton box to collect possible bacterial pathogens. Isolates were plated for primary culture and these were obtained from different tissues such as ulcerated skin and muscle, liver, kidney, spleen, gall bladder and opaque eyes.

Bacterial colonies growing in the different petri plates were subcultured the following day. Primary characterization tests were done on the bacterial culture at the laboratory. Isolates were also sent to a collaborating laboratory, the Institute of Aquaculture, University of Stirling, for detailed examination. The isolates were further characterized using detailed differential biochemical tests and API 20E. Sensitivity to selected antibiotics such as erythromycin (15 mcg), tetracycline (30 mcg), streptomycin (10 mcg), ampicillin (10 mcg), oxolinic acid (2 mcg), and chloramphenicol (30 mcg) was determined by adopting the Kirby-Bauer disc diffusion technique in Mueller Hinton Agar.

Experimental induction of the disease was done through immersion of the Nile tilapia fingerlings, with an average weight of 4 g each, in different concentrations of the identified isolate cultured in tryptic soy broth. The concentrations used were at 1.5 x 103, 104, 105. 106, 107, and 108 bacterial

cells per ml, with three replicates in each concentration using serial dilution. One treatment, without the isolate, served as a control. Before the induction, the isolate was passaged for five times into healthy Nile tilapia samples to enhance the virulence of the bacteria. The experiment used plastic bags containing 10 liters of aerated water with 20 Nile tilapia in each bag. Total fish mortality was noted after 96 h of monitoring. Reisolation of the bacteria was conducted by plating from kidney, liver and spleen. Isolated bacteria from various tissues were also identified.

Results

Gross Clinical Signs of Naturally Infected Nile Tilapia

The disease was observed affecting all sizes of fish from fingerling to adult stage regardless of sex. Disease signs were frequently accompanied by hemorrhagic skin, ulcerations, loss of scales, mouth sores, eye abnormalities, fungal growth and/or tail and fin rot. Those with severe mouth sores displayed deformation of the upper and lower lips. Eye abnormalities, unilateral or bilateral, included hemorrhage in the orbit which degenerated to opacity, exophthalmia and bursting that dislodged the eyeball out of the socket. When both eyes were affected, darkening of the body was noted. Internal gross signs included pale liver occasionally with hemorrhagic spots and necrotic kidneys showing liquefaction.

Some samples of Nile tilapia exhibited pupil constriction and total pupil closure. However, no other abnormality previously described in diseased fish was noticed. No bacterial growth was observed on the media inoculated with tissues from tilapia samples.

Bacterial Isolates from Diseased Nile Tilapia Samples

Streaking of inoculum from affected tissues into the agar (NA and TSA) plates led to heavy growth of bacterial colonies with similar morphological characteristics. Those which were inoculated into the Rimler-Shotts media grew into yellow colonies. All the isolates were found to be gram negative, motile, fermentative, oxidase positive, 0/129 resistant and novobiocin resistant. The isolates appeared to behave as aeromonads in the primary characterization tests. A total of 82 isolates of aeromonads were collected (Table 1).

Six bacterial isolates sent to the collaborating laboratory at the Institute of Aquaculture, University of Stirling, were examined in more detail using the scheme of Cowan and Steel (Barrow and Feltham 1993) based on the criteria given in Kreig and Holt (1984). The isolates were all presumptively identified as Aeromonas hydrophila. Additional tests were done on all isolates at the laboratory of the Freshwater Aquaculture Center at the CLSU to further characterize the bacteria. Results of the tests are presented in Table 2. Six isolates were selected at random for confirmatory identification of the species using the API 20E (since only six units were available, the number of samples was also limited to six isolates). Responses to the tests also revealed characteristics typical of *A. hydrophila.*

Table 1. Aeromonads isolates which were collected from various aquaculture projects in Luzon, Philippines from January 1994 to February 1996.

No. of Isolates	Culture Confinement	Month of Collection
2	aquaria	Jan 1994
1	tank	Jul 1994
8	cage	Aug 1994
1	tank	Aug 1994
4	aquaria	Aug 1994
12	aquaria	Sep 1994
4	tank	Sep 1994
2	cage	Sep 1994
4	aquaria	Oct 1994
19	tank	Feb 1995
4	cage	Dec 1995
6	tank	Jan 1996
15	hapa	Feb 1996
Total - 82		

Table 2. Characteristics of bacterial isolates from diseased Nile tilapia identified as *Aeromonas hydrophila*.

Test	Characteristics
Gram stain	--
Shape	rods
Motility	+
Oxidase	+
Glucose, APW	+
Glucose, O/F	Fermentative
Simmon's Citrate	+
ONPG for B-galactosidase	+
Arginine dihydrolase	+
Lysine decarboxylase	+
Ornithine decarboxylase	--
H2S production	+
Salicin APW	+
Arabinose	--
Indole	+
Voges Proskauer	+
Gelatin hydrolysis	+
Catalase	+
Aesculin hydrolysis	+
Antibiotics sensitivity:	
Chloramphenicol (30mcg)	Sensitive
Oxolinic acid (2 mcg)	Sensitive
Ampicillin (10 mcg)	Resistant

Antibiotic Sensitivity Test

All of the 25 isolates randomly selected were sensitive to chloramphenicol and oxolinic acid but resistant to ampicillin (Table 2). Responses to the other three antibiotics, namely, streptomycin, oxytetracycline and erythromycin varied from

resistant, intermediate, and sensitive (Table 3). About 68% (17 out of 25) of the isolates showed sensitivity to streptomycin while the rest had intermediate reactions. Against oxytetracycline, 76% (19 out of 25) of the isolates were sensitive while 24% showed resistance. A certain degree of resistance to erythromycin was also manifested by 64% (16 out of 25) of the isolates while the rest had intermediate sensitivity.

Experimental Infection of the Disease

Results of mortality of tilapia fingerlings at different concentrations of A. hydrophila are presented in Table 4. LD_{50} fell exactly at the concentration of 1.5 x 106 bacterial cells$\cdot ml^{-1}$ at 96 h even when plotted arithmetically. Mortality of 100% was observed at the concentration of 108 while no mortality was monitored in the control treatment and at the concentration of 103 at 96 h. Percent mortalities at 104, 105 and 107 were 10%, 30% and 70%, respectively. Isolated bacteria from moribund fish were also characterized as *A. hydrophila.*

Discussion

The outbreaks of the disease were first reported by the farmers during the later months of 1992 (Yambot and Inglis 1994). The colony appearance and primary test results of the bacteria isolated from the 1992-93 outbreaks of the disease were the same as the isolates in the 1994-96 outbreaks. Gross clinical signs were similar in all outbreaks with occurrences during the rainy season and cold months. Mortality of cultured fish during these outbreaks was alarmingly high, with some cages having 100% death rates. It was during these same months that epizootic ulcerative syndrome (EUS) was likely to occur, although other freshwater species were also affected.

The presence of aeromonads, particularly *A. hydrophila*, in diseased samples of the Nile tilapia *O. niloticus* obtained from different localities suggests an epizootic. The isolation of aeromonads from different tissues corroborates the findings of Yambot and Inglis (1994) regarding the existence of a septicaemic disease in the Nile tilapia in a magnitude previously unreported in the country. Roberts and Sommerville (1982) reported a case of hemorrhagic septicemia with *A. hydrophila* in tilapias in other countries. Aeromonads were also isolated from diseased fish samples in the United States and various methods or systems are used to identify the pathogen (Taylor *et al.* 1995).

In one study, Liu *et al.* (1990) described a disease outbreak in Taiwan involving cultured tilapia manifesting exophthalmia, corneal opacity and external lesions. This was attributed to *Streptococcus* sp. and/or *A. hydrophila.* Experimental infection of fish with *A. hydrophila* caused petechia, corneal opacity and exophthalmia. Hargis (1991) cited eye disorders, particularly corneal opacity, in teleosts which may be due to infection. Severe eye infection with opacity and orbital bursting in the Nile tilapia was not given emphasis in many reports, although Roberts and Sommerville (1982) and Lightner *et al.* (1988) mentioned the isolation of *A. hydrophila* from diseased tilapias. Yambot and Inglis (1994) focused their investigation on eye disorders in

Table 3. Sensitivities of the isolates of *Aeromonas hydrophila* to antibiotics such as streptomycin (Strept, 10 mcg), oxytetracycline (Oxytet, 30 mcg), and erythromycin (Erythro, 15 mcg).

Isolate	Strept	Oxytet	Erythro
1	I	S	I
2	I	S	R
3	S	S	I
4	I	R	R
5	S	R	R
6	I	R	R
7	I	R	R
8	S	R	R
9	S	S	I
10	S	S	R
11	S	S	R
12	S	S	R
13	S	S	R
14	I	S	R
15	S	S	R
16	I	R	R
17	S	S	R
18	S	S	R
19	S	S	I
20	I	S	I
21	S	S	I
22	S	S	R
23	S	S	I
24	S	S	I
25	S	S	I

Legend: S - Susceptible
I - Intermediate
R - Resistant

Table 4. Mortality of Nile tilapia immersed at different concentrations of *Aeromonas hydrophila* after 96-hrs observation (n=20 fish).

Concentration (x 1.5)	Fish Mortality					
	Rep.1	Rep.2	Rep.3	Total	Mean	%
108	20	20	20	60	20	100
107	15	15	12	42	14	70
106	9	11	10	30	10	50
105	6	7	5	18	6	30
104	2	1	3	6	2	10
103	0	0	0	0	0	0
control	0	0	0	0	0	0

the Nile tilapia yet the disease appeared systemic because of the isolation of *A. hydrophila* from various tissues of the affected fish. This investigation, however, revealed various gross clinical signs of disease at varying degrees present in affected Nile tilapia with or without eye disorders. Torres *et al.* (1990) were even able to isolate *Aeromonas* spp. from "healthy" tilapia tissues such as kidney,

spleen and liver. Different types (or degree) of infection caused by *A. hydrophila* in the channel catfish were mentioned by Grizzle and Kiryu (1993)

The problem of resistance of microorganisms to antibiotics is a global concern (Inglis *et al.* 1993; Austin 1993). Drug resistance has been reported in microbial organisms, including *A. hydrophila* (Aoki *et al.* 1990). Misuse and unregulated use of antibiotics are common, leading to the development of resistance to these drugs.

Percent mortality of Nile tilapia in this study was noted from 10% to 50% in treatments 104 to 106 bacterial cells$\cdot ml^{-1}$. It is to be noted that the route of experimental infection was through immersion. It is presumed that immersion requires more concentration of bacterial cells to infect a fish than injection route, either intraperitoneal or intramuscular. The said concentrations, except at 103 bacterial cells$\cdot ml^{-1}$, appeared to corroborate the findings of Saitanu (1986) that high density of *A. hydrophila* in infected areas at 103 to 105 cells$\cdot ml^{-1}$ indicates a relationship between high numbers of pathogen and outbreaks of disease.

Conclusion

The consistent isolation of *A. hydrophila* from diseased Nile tilapia in several farms and different places during the rainy season and cold months suggests an epizootic type of disease. The bacteria is systemic since it has been isolated from various tissues of diseased fish. It is also revealing that all diseased fish sampled were collected from low-volume and high-density fish farms classified under intensive aquaculture and none from the extensive or semi-intensive system. *Aeromonas* spp. are described as opportunistic and secondary bacterial pathogens of fishes whose health has been compromised. Hence, two possible predisposing factors which compromised the health of the fish and were common in all the disease outbreaks could be identified: a) rainy seasons and cold months with low temperatures; and b) low-volume and high-density culture.

The emergence of this septicemic disease in the Nile tilapia necessitates the re-evaluation of standard practices in Nile tilapia culture, especially in cages where economic losses have been severe. Cages with high stocking density as well as reservoirs that prevent circulation of water and cause nutrification and pollution should be evaluated. Setting up of cages was observed to have followed the chessboard type of cage positioning and not the ideal single line. Likewise, stocking density of Nile tilapia during the rainy season and cold months needs to be assessed. Based on the magnitude of fish mortality during outbreaks, this type of infection poses a significant threat to the culture of Nile tilapia in the country and needs to be checked.

Acknowledgment

The author would like to thank the Aquatic Animal Health Research Institute, Department of Fisheries, Bangkok, Thailand, and Dr. Valerie Inglis of the Institute of Aquaculture, University of Stirling, U.K., for their assistance and support.

References

Aoki, T., T. Umeda, K. Takami, T. Kitao, K. Saitanu, A. Chongthaleong and P. Punyaratabandhu. 1990. Drug-resistant *Aeromonas hydrophila* in Thailand. In: Proceedings of the Second Asian Fisheries Forum (eds. R. Hirano and Hanyu), pp. 693-696. Asian Fisheries Society, Manila.

Austin, B. 1993. Environmental issues in the control of bacterial diseases of farmed fish. ICLARM Conference Proceedings 31:237-251. International Center for Living Aquatic Resources Management, Manila.

Barrow, C.I. and R.K. Feltham. 1993. Cowan and Steel's manual for the identification of medical bacteria. Cambridge University Press, Cambridge.

Bimbao, M.P., M. Ahmed, R.S. Pomeroy and R.S.V. Pullin. 1994. The economic potential of tilapia farming in Asia: The Philippine experience. In: Socioeconomics of aquaculture (eds. Y.C. Shang, P.S. Leung, C.S. Lee, MS. Lu and I.C. Liao), pp. 157-172. TML Conference Proceedings 4. Tungkang Marine Laboratory, Tungkang, Taiwan.

Grizzle, J.M. and Y. Kiryu. 1993. Histopathology of gill, liver, and pancreas, and serum enzyme levels of channel catfish infected with *Aeromonas hydrophila* complex. Journal of Aquatic Animal Health 5:36-50.

Hargis Jr., W.J. 1991. Disorders of the eye in finfish. Annual Review of Fish Diseases 1:95-117.

Inglis, V., R.H. Richards, and K.N. Woodward. 1993. Public health aspects of bacterial infections of fish. In: Bacterial diseases of fish (eds. V. Inglis, R.J. Roberts and N.R. Bromage), pp. 284-303. Blackwell Scientific Publications, London.

Kreig, N.R. and J.G. Holt. 1984. Bergey's manual of systematic bacteriology, vol. 1. Williams and Wilkins, Baltimore.

Lightner, D., R. Redman, L. Mohney, G. Dickenson and K. Fitzsimmons. 1988. Major diseases encountered in controlled environment culture of tilapias in fresh- and brackishwater over a three-year period in Arizona. In: The Second International Symposium on Tilapia in Aquaculture (eds. R.S.V. Pullin, T. Bhukaswan, K. Tonguthai and J.L. Maclean), pp. 111-116. Bangkok.

Lilley, J.H., M.J. Philipps and K. Tonguthai. 1992. A review of epeizootic ulcerative syndrome (EUS) in Asia. Aquatic Animal Health Research Institute and Network of Aquaculture Centres in Asia-Pacific, Bangkok. 73 pp.

Liu, C.I., J.L. Huang, H.H. Hung and C.F. Pen. 1990. The experimental infection of *Streptococcus* and/or *Aeromonas* to induce bacterial septicemia in cultured tilapia. COA Fisheries Series No. 24, Fish Disease Research (X), 40-45, 6.

Roberts, R.J. 1993. Motile aeromonad septicemia. In: Bacterial diseases of fish (eds. V. Inglis, R.J. Roberts and N.R. Bromage), pp. 143-155. Blackwell Scientific Publications, London.

Roberts, R.J. and C. Sommerville. 1982. Diseases of tilapias. ICLARM Conference Proceedings 7:247-263. International Center for Living Aquatic Resources Management, Manila.

Roberts, R.J., L.G. Willoughby and S. Chinabut. 1993. Mycotic aspects of epizootic ulcerative syndrome (EUS) of Asian fishes. Journal of Fish Diseases 16(3):169-184.

Saitanu, K. 1986. *Aeromonas hydrophila* infections in Thailand. In: The first Asian Fisheries Forum (eds. J.L. Maclean, L.B. Dizon, and L.V. Hosillos), pp. 231-234. Asian Fisheries Society, Manila.

Taylor, P.W., J.E. Crawford and E.B. Shotts Jr. 1995. Comparison of two biochemical test systems with conventional methods for the identification of bacteria pathogenic to warmwater fish. Journal of Aquatic Animal Health 7:312-317.

Torres, J.L., M. Shariff and A.T. Law. 1990. Identification and virulence screening of Aeromonas spp. isolated from healthy and epizootic ulcerative syndrome (EUS)-infected fish. In: The second Asian Fisheries Forum (eds. R. Hirano and Hanyu), pp. 663-666. Asian Fisheries Society, Manila.

Yambot, A.V. and V. Inglis. 1994. *Aeromonas hydrophila* isolated from Nile tilapia (*Oreochromis niloticus* L.) with "eye disease". In: International congress on quality veterinary services for 21st century (eds. M.K. Vidyadaran, M.T. Aziz and H. Sharif), pp. 87-88. Kuala Lumpur.

Manuscript received 30 October 1995; accepted 10 January 1998.