Contamination of *Mycobacterium* spp. in Live Feeds

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ABSTRACT

The importance of water flea, mosquito larvae, tubifex and oligochaete as live feeds for tropical fish, especially carnivorous fish, is well known. These live feeds are usually contaminated with pathogens that cause diseases in aquarium fish. Mycobacteriosis is a chronic bacterial disease infecting many fish species including aquarium fish. The detection by isolation and polymerase chain reaction (PCR) technique of *Mycobacterium* spp. contamination in live feeds in six provinces around Bangkok revealed four pathogenic bacteria, namely: *Mycobacterium* spp., *M. fortuitum, M. marinum* and *M. chelonae.* Contamination by *M. fortuitum* showed high prevalence. Feeding of contaminated live feeds should be avoided. The practice of producing live feeds free from mycobacteria is recommended.

INTRODUCTION

Mycobacteriosis, or fish tuberculosis, is a contagious bacterial infection that causes problems for breeders of aquarium fish such as Siamese fighting fish (*Betta splendens Regan*), discus (*Symphysodon aequifasciata*) and oscars (*Astronotus ocellatus*) (Pungkachonboon *et al*., 1992; Puttinaowarat, 1999). This disease is caused by various species of the genus *Mycobacterium*. Most infected fish normally do not exhibit any symptoms, but when they are stressed they will die in large numbers. Infection can be transovarian (Chinabut *et al*., 1994), through the water, and by oral transmission (Sodjit *et al*., 1993). The disease can also be spread to humans, particularly those who have direct contact with the fish (Kullavanijaya *et al*., 1993).

Water fleas (*Moina* spp.), mosquito larvae, tubifex (*Chironomus* spp.), and oligochaete (*Bothrioneurum iris*) are the most common live feeds used by aquarium fish breeders and exporters. Some breeders of food fish and moreover, some black tiger shrimp farms also use water fleas rather than artemia (*Artemia salina*) to feed fish fry and shrimp larvae. *Mycobacterium* spp. were recently reported as flora of water fleas, as well as mosquito larvae (Colorni *et al*., 1998; Puttinaowarat, 1999). It is possible that aquarium fish and other aquatic animals could be infected by *Mycobacterium* spp. through the use of live feeds. This could cause serious damage to the Thai aquarium fish market in the future because there is no known treatment for mycobacteriosis. It is essential that we work at finding ways to control the spread of this disease to reduce its effect on the aquarium fish

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industry. Measures to control the spread of mycobacteriosis in fish are one way of helping solve the problem. This research project studied the extent of contamination of *Mycobacterium* spp. in live feeds from wild sources and breeding farms that supply aquarium fish farms. This information can be used to prevent further spread of the disease. This is a pioneering study on *Mycobacterium* spp. contaminations in live feeds.

The objectives of the study were:

(a) to study the contamination of Mycobacterium spp. in water fleas, mosquito larvae, tubifex and oligochaetes in breeding areas and collection areas; and

(b) to establish ways of preventing the spread of mycobacteriosis obtained from live fish feed.

MATERIALS AND METHODS

Collection of samples

Breeding and wild collection areas that supply water fleas, mosquito larvae, tubifex, and oligochaetes as live feeds for fish were identified by asking aquarium fish breeders in central Thailand where they obtained their feed. Samples of water fleas, mosquito larvae, tubifex, and oligochaetes were collected during September 2001 to January 2002 from both breeding and wild collection areas. Three weekly samples were taken from each site. Each sample of water and live feed was transported to the laboratory within three hours. The sample was then divided into two portions: one was for conventional detection and another was for polymerase chain reaction (PCR) detection.

Sample extraction

For conventional detection, *Mycobacterium* spp. were isolated from the samples of live feeds and water. 1 g of live feed was washed with 30 ml of sterile distilled water and centrifuged at 3,000 rpm for 10 min. The pellet was crushed in a mortar, mixed with 1 ml 4% NaOH solution, left for 5 min, then neutralized with 1 ml 0.1 N HCl. 100 ml of the water samples were centrifuged at 6,000 rpm for 15 min, 10 ml was pipetted from the bottom of the centrifuge tube and 1 ml 4% NaOH solution was added. After 5 min, the solution was neutralized with 1 ml 0.1 N HCl. The treated cell suspensions were inoculated on an Ogawa egg medium (Tsukamura, 1984) and incubated at 28°C for 3 - 7 days. A Ziehl-Neelsen stain was used to test the acidity of the colonies, which grew on Ogawa egg medium. Colonies were tested for acid-fastness by the method of Ziehl-Neelsen (Cheesbrough, 1984). A presumptive identification as a *Mycobacterium* species was made if the cells were acid-fast and unbranched.

Polymerase chain reaction

For PCR detection, the samples (colonies, water and live feeds) were analysed as previously described by Puttinaowarat *et al*. (2002).

Statistical analysis

The data on *Mycobacterium* infection in the live feed was analysed using a chi- square test at a confidence level of 95%.

RESULTS

The survey of sources of live feeds for aquarium fish found 24 breeding or collecting areas in six provinces (Pathumthani, Nakornpathom, Ratchaburi, Singhaburi, Angthong and Ayutthaya) in the central region of Thailand. Most of the sources were close to natural water sources and irrigation canals. The water temperature was 26-30°C. Two types of water flea farms were found; those that use tank and those that use earthen ponds. Water flea cultured in 50 m^2 cement tanks was used by 15 breeders. Chemical fertilizer, calcium carbonate and 'ami ami' (a by-product of monosodium glutamate production) were used to create *Chlorella* spp. blooms as food for the water fleas. Water flea cultured in 800-1,600 m^2 earthen ponds were used by 5 breeders. Solid or liquid manure, pig or chicken, were used as a nutrient source.

The survey found two distributors who collected and sold wild water fleas. One collected the water fleas from wastewater of pig farms in Ratchaburi. The other collector harvested fleas from public water sources, wastewater holding areas and canals. Mosquito larvae were grown by two breeders using cement tanks under chicken coops (i.e., cage or hen house, usually 10-20 cages in a row housing an individual chicken in a cage).

Tubifex and oligochaetes sold as live feeds for fish were all collected from the wild, mainly from streams, swamps, and ponds that contained wastewater from residential communities, aquarium fish farms, food fish farms, and sugar factories.

Mycobacterium spp. were detected by PCR and in culture in most samples both from wild collections and breeders (Tables 1, 2 and 3). Statistical analysis (chi-square test) of the *Mycobacterium* spp. in the samples showed that there was no difference among samples from different provinces tested by the conventional method. However, the samples were significantly different among provinces analysed by the PCR method. There was no difference between the bacterial concentrations in the live feed samples and the water samples, no matter which testing method was used. There was a significant difference in bacteria detection of samples when comparing the bacteriological testing method and the PCR method. A comparison of the prevalence of *M. marimum, M. fortuitum* and *M. chelonae* species found that the prevalence of *M. fortuitum* varied among provinces, whereas the prevalence of *M. fortuitum* and *M. chelonae* were approximately the same in each province. The prevalence of *Mycobacterium* spp. in samples of water fleas cultured using chemical fertilizer and using manure were not different.

Our survey found contamination by *M. fortuitum* at levels of 82.61%, 75.00% and 63.33% in the samples of live feeds from Pathum Thani, Singhaburi, and Ayutthaya provinces, respectively. Contamination by *M. chelonae* was only found in Nakorn Pathom. The highest prevalence was 80.95% for *Mycobacterium* spp., followed by *M. marinum* at 11.90% and *M. fortuitum* at 4.76%. In Ratchaburi Province, *Mycobacterium* spp. had the greatest prevalence at 63.16%, followed by *M. fortuitum* at 26.32% prevalence and *M. marinum* at 10.53% prevalence. *M. marinum* and *M. chelonae* were not found in Ayutthaya and Ang Thong provinces. In Ang Thong Province, the prevalence of *Mycobacterium* spp. was highest (60%), followed by *M. fortuitum* (40%).

Table 1. *Mycobacterium* spp. in water flea farms.

Farm*	Sampling time	Sample	Bacterial isolation	PCR	Mycobacterium spp.
1-4	1-3	Moina & Water	-	+	*M. fortuitum*
5	1	Moina & Water	-	+	*M. fortuitum*
	2	Moina	+	+	*Mycobacterium* spp.
		Water	-	+	*M. fortuitum*
	3	Moina & Water	-	-	-
6	1-2	Moina & Water	-	+	*M. fortuitum*
	3	Moina	-	+	*M. fortuitum*
		Water	+	+	*Mycobacterium* spp.
7	1	Moina	-	+	*M. fortuitum*
		Water	-	-	-
	2	Moina	-	+	*M. fortuitum*
		Water	+	+	*Mycobacterium* spp.
	3	Moina & Water	-	+	*M. fortuitum*
8	1-2	Moina	-	+	*M. fortuitum*
		Water	-	-	-
	3	Sampling not possible as Moina culture was stopped temporarily			
9,10	1-3	Moina & Water	-	-	-
11	1	Moina	+	+	*Mycobacterium* spp.
		Water	-	-	-
	2	Moina & Water	-	-	-
	3	Moina	-	-	-
		Water	+	+	*Mycobacterium* spp.
12	1-2	Moina	-	-	-
		Water	+	+	*Mycobacterium* spp.
	3	Moina & Water	-	-	-
13	1	Moina	-	+	*Mycobacterium* spp.
		Water	+	+	*M. fortuitum*
	2	Moina	-	-	-
		Water	+	+	*M. fortuitum*
	3	Moina	-	-	-
		Water	-	+	*M. marinum*
14	1-2	Moina & Water	-	-	-
	3	Moina	-	-	-
		Water	-	+	*Mycobacterium* spp.
15	1-2	Moina & Water	-	+	*M. marinum*
	3	Moina	-	+	*M. fortuitum*
		Water	-	-	-
16	1	Moina	-	+	*Mycobacterium* spp.
		Water	-	+	*M. fortuitum*
	2	Moina	-	+	*M. fortuitum*
		Water	-	+	*Mycobacterium* spp.
	3	Moina & Water	-	+	*M. fortuitum*

17	1	Moina Water	- -	+ +	*Mycobacterium* spp. *M. fortuitum,* *M. marinum*
	2	Moina Water	- -	- +	- *Mycobacterium* spp.
	3	Moina Water	- -	+ +	*M. fortuitum* *M. fortuitum,* *M. marinum*
18	1-2	Moina Water	- -	- +	- *M. fortuitum*
	3	Moina & Water	-	+	*M. fortuitum*
19	1	Moina & Water	-	-	-
	2	Moina Water	- -	+ +	*Mycobacterium* spp. *M. fortuitum*
	3	Moina Water	- -	+ -	*M. fortuitum* -
20	1	Moina Water	- -	+ +	*Mycobacterium* spp. *M. fortuitum*
	2	Moina Water	- -	- +	- *Mycobacterium* spp.
	3	Moina & Water	-	+	*M. fortuitum*

* Farm nos. 1-8 are located in Pathumthani, farm nos. 9-14 are located in Nakornpathom, farm nos. 15-18 are located in Singhaburi, farm no. 19 is located in Angthong and farm no. 20 is located in Ayutthaya). Farm nos. 13 and 14 cultured *Moina* in earthern ponds, while the rest were cultured in cement tank.

DISCUSSION

Most live feed breeding and collection areas are located close to places where there are aquarium fish breeders. The use of live feed organisms as food for aquarium fish results in a high survival rate and good growth, as well as beautiful colours. This is especially true for carnivorous fish such as oscars (*Astronotus ocellatus*), discus (*Symphysodon aequifasciata*), and Siamese fighting fish (*Betta splendens* Regan). Shim and Bajrail (1982) reported that water fleas were the best source of protein for fish fry. Water fleas contained 70% protein while tubifex contained 62.5% protein. This may explain why live feeds are popular among aquarium fish breeders.

Most live feed breeders in Thailand use pig or chicken manure as the main source of nutrients for the feed organisms and for growing phytoplankton. This can lead to various problems such as traces of antibiotics in the manure being fed to the pigs or chickens, as well as various bacterial and parasitic diseases. This survey found that health standards were higher at the water flea breeding areas that used chemical fertilizer rather than those which used manure to promote phytoplankton growth. However, the costs for chemical fertilizer are higher. Also, yield per unit area was lower at the water flea breeding areas than those which used manure. Studies to determine ways to improve yield and reduce the costs of raising water fleas using chemical fertilizer are desirable.

Our survey found that chicken manure was the only input used for raising mosquito larvae. As for tubifex and oligochaetes, these are not yet used by any commercial breeders in Thailand. Most of the tubifex and oligochaetes used as live feeds for fish are collected from wastewater resting areas which drain wastewater from residential communities, sugar factories, or whisky distilleries. In a trial experiment on raising tubifex using chicken manure, Shaw and Mark (1980) were able to produce 28 g/m^2/week, compared to the results of Yashouv (1970), who also cultured tubifex using chicken manure but obtained yields of 250-375 g/m^2/week. It is interesting to note that if tubifex were cultured with chemical fertilizer, as has been done with water fleas, it should be possible to produce tubifex that are free from *Mycobacterium* spp. contamination.

Our survey of live feeds and water samples from breeding and collection areas found *M. marinum, M. fortuitum, M. chelonae* and *Mycobacterium* spp. in every area. *Mycobacterium fortuitum* were most common, followed by *Mycobacterium* spp. and *M. marinum*, in descending order. *Mycobacterium marinum* was very common in samples of mosquitoes and mosquito larvae, but was found in only one sample of water fleas. *M. chelonae* was found only in Nakorn Pathom Province. The species of *Mycobacterium* that were found in live feed depended on the species present in the environment where the samples were taken. This study did not find any *Mycobacterium* in tubifex. This may be due to the small number of samples compared to the number of samples for other types of live feeds studied. Further studies using larger number of samples are required.

Mycibacterium marinum is a bacterium that causes disease in fish. Giavenni *et al*. (1980) reported finding *M. marinum* in 97 samples of 17 different species of fish in tropical seas. Rhodes et al. (2001) were able to isolate *M. marinum* and *M. ulcerans* from wild striped bass (*Morone saxatilis*) in the Chesapeake Bay in the USA at a time when there was an outbreak of mycobacteriosis. These bacteria can also cause a skin disease called "fish tank granuloma" in humans, as first reported in 1954 (Linell and Norden, 1954). The victims had bumps, rashes, or sores and irritations on the hands, fingers, arms and legs (Lim *et al*. 2000). Kiesch (2000) reported that fish breeders contracted this disease from their aquarium fish. Kullavanijaya *et al*. (1993) reported 18 cases of *M. marinum* infections in humans in Thailand during a 20-year period (1980-1990). All were people whose jobs or hobbies put them in contact with either freshwater or seawater fish.

M. fortuitum has been isolated in three types of freshwater fish (i.e., oscars, guppies and discus) in South Africa (Bragg *et al*., 1990). The infected fish had a high mortality rate and the bacteria that were isolated were resistant to many types of antibiotics. *M. fortuitum* is a fast growing species. It can be found in soil, dust and water. It can cause diseases of the skin and soft tissues in humans who have trauma injuries (Midani and Rathore, 1999).

Our tests for *Mycobacterium* spp. in this study showed that the PCR method is more sensitive than the conventional bacterial isolation method. The primary isolation of mycobacteria took 3-7 days, and the bacteria could only be isolated if there was a large volume of bacteria in the sample. PCR analysis took shorter time to get results and had a detection limit of 20 mycobacteria (Puttinaowarat *et al*., 2002). Knibb *et al*. (1993) carried out a PCR test using the direct sequencing of the gene of 16S rRNA at 600 bp to test for *Mycobacterium* spp. in the European sea bass (*Dicentrachus labrax*) and found the test to be very specific and highly accurate. Talaat *et al*. (1997) used PCR technology to identify *M. marinum,*

M. fortuitum, and *M. chelonae* using primers for 16S rRNA amplification yielding a 924 bp product.

The prevalence rates of *Mycobacterium* spp. found in samples of water fleas cultured using chemical fertilizer and using manure were not statistically different in this study. This might be because the farms that bred water fleas using chemical fertilizer might have obtained their breeding water fleas or their algae (*Chlorella* spp.) from sources that were contaminated with *Mycobacterium* spp. These farms should revise their management methods, clean and disinfect every part of their equipment, and get fresh, uninfected supplies stock of water fleas and algae so that they can produce high quality, water fleas free of *Mycobacterium* spp.

This survey showed that *Mycobacterium* spp. infections are widespread among aquarium fish farms via live feeds. This is a problem that warrants urgent attention because when the fish eat infected live feed, they are at risk of developing mycobacteriosis. This disease can spread through food and can be transmitted vertically (from parent fish to their fry). It is a chronic disease. Most of the infected fish do not display any clinical signs, but they may develop them after a long time or when they are stressed.

It is not possible to solve the problem by treating the live feeds with an antibacterial agent before feeding it to the fish because a very high concentration of antibacterial agent is required to kill this type of bacteria, and it would kill the live feeds as well. There is no known effective antibiotic for treating fish that have contracted mycobacteriosis. Extremely long treatments would be required, just as for treating tuberculosis in humans.

Since *Mycobacterium* spp. infections in fish can be passed to humans, people who culture or collect live feeds for fish and those who take care of aquarium fish should all be careful of this hazard. If they have any kind of scratch or open wound they should not let the affected part of their bodies come in contact with the water in the breeding tanks or ponds, or the live feed or fish.

CONCLUSIONS AND RECOMMENDATIONS

Live feed for fish, comprising water fleas, mosquito larvae, tubifex, and oligochaetes, - collected both from the wild and those bred in captivity in the central region of Thailand, were found to be contaminated with *M. marinum, M. fortuitum, M. chelonae* and *Mycobacterium* spp.

Testing using PCR for *Mycobacterium* spp. in live feeds for fish, was found to be faster and more accurate than the bacterial isolation method. It is recommended that people use the PCR method for testing for *Mycobacterium* spp. in live feed for fish and in the raw materials used to produce live feed in order to prevent the spread of this bacterium and to produce uncontaminated aquarium fish.

In order to prevent contamination of *Mycobacterium* spp. contamination in aquarium fish, the following are recommended: (a) avoid feeding water fleas, mosquito larvae, tubifex or oligochaetes to the fish; (b) avoid feeding fish with live feed that was collected from the wild; and (c) use only live feed that comes from a breeder using a production process that is free of *Mycobacterium* spp.

Production of water fleas and mosquito larvae-free of Mycobacterium spp. can be achieved by: (a) avoiding the use of manure as a substrate for algae growth; (b) using chlorine or another disinfectant to disinfect the water; and (c) testing the stock of *Chlorella* spp. to be used as food to make sure it is free from *Mycobacterium* spp. Since it is necessary to use water fleas, mosquito larvae, tubifex and oligochaetes as food for aquarium fish, development of vaccine against mycobacteriosis may be required.

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