Original Research Article

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Comparison between conventional and recent culture methods for detection of fish mycobacteria

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Abstract

The present study aimed to isolate and identify mycobacteria from Nile tilapia and fresh water as well as ornamental fish and aquarium water in Sohag Assuit and Qena Governorates. Conventional and recent methods (MB-Redox system) were used for isolation and detection of mycobacteria with comparison between them regarding to the rate of isolation time for growth and growth character of colonies. Three isolates were isolated from fresh water and identified as unclassified atypical mycobacteria and four isolates were recovered from aquarium water and identified as *M. fortuitum* and *M. marinum*. Eleven isolates were isolated from goldfish and identified as *M. marinum*, *M. fortuitum* and unclassified atypical mycobacteria. The recovery rate of MB-Redox system for mycobacteria was 12%, while in case of L-J medium was 10.66%. The mean time for detection of *M. marinum* was 3-7 days and 7-10 days for MB-Redox system and Lowenstein – Jensen medium, respectively. The mean time for detection of *M. fortuitum* was 1-3 days for MB-Redox system, while in case of L-J medium was 3-6 days. The mean time for detection of other unidentified atypical mycobacteria was 3-8 days for MB-Redox system, while in case of L-J medium was 3-14 days. The detection of mycobacterial growth on MB-Redox system at different times revealed that the color of MB- Redox tubes appear yellow at time of inoculation changed to pink color after 3 days. The colonies appear as red particles on the surface. After 15 days, the color of MB-Redox medium changed to yellow with red particles on the surface.

Keywords: MB-Redox, mycobacteriosis, ornamental fish, tilapia fish

Introduction

Fish mycobacteriosis once known as piscine tuberculosis, is a chronic progressive disease caused by several species of the genus *Mycobacterium*. Mycobacterial species are capable of causing serious and costly disease in most vertebrates including humans, livestock and fish. While commonly reported in aquaculture and the fish aquaria trade, reports in wild fish have been infrequent (Jacobs *et al.*, 2009). The high susceptibility of some ichthyic species toward mycobacteria makes them perfect carriers, able to transmit pathogens to other fish and human. In recent years, several studies have reported the presence of mycobacteria in ornamental as well s farmed species (Macri *et al.*, 2008). A variety of different media for the cultivation of mycobacteria have been described but a few of them are in use today (Satio, 1998). Those currently used can be characterized by three basic types. The first is egg-based media represented by Ogawa and Lowenstein-Jensen medium. The second type is agar-based media, the most common one is Middlebrook 7H10 and 7H11. The third type is liquid media such as Middle

brook 7H9. Several weeks of incubation may be required for the isolation of mycobacteria on solid media. Substantial improvement in the time to detection and recovery rate was realized by using broth-based culture system such as the BACTEC 460 TB, Septi-Chek AFB, BACTEC 9000 and MGIT. A novel system is MB-Redox which is a modified serum-supplemented Kirchner medium containing iodonitro-tetrazolium salt as indicator of microbial growth (Satio, 1998).

Materials and Methods

1. Samples:

A. A total number of 240 Tilapia fish were collected from Nile River (at Sohag 85 fish, at Assuit 80 fish and at Qena 75 fish). Sampled fish were chosen either with necrotic skin lesions or with ascites. Tissue samples from skin, skeletal muscles, gills, kidney, spleen and liver were taken for bacteriological examination.

B. Sixty fantail gold fishes (Carassius auratus) were collected from pet shops. Sampled fish were showed ulcers on the head or body, exophthalmia and ascites in some of them. Samples were aseptically collected from lesions using the needle of a platinum loop. Tissue samples were taken from ulcers, kidney, spleen, liver, eye, gills and ascitic fluid for bacteriological examination. A direct film was prepared as an impression smear from each lesion as well as from ascetic fluid.

C. Moreover, 50 water samples were collected in sterile glass bottles from different locations of Nile River in Sohag, Assuit and Qena Governorates.

D Forty water samples from aquaria were collected in sterile glass bottles.

2. Isolation of mycobacteria:

I- Conventional culture methods [Lowenstein – Jensen medium (Difco)] (Marks, 1972):

-Organs and tissue samples showing gross lesions or congestion were cut and divided into small parts in a small sterile mortar containing washed sterilized sand.

-The tissue was homogenized in sterilized distilled water, then the homogenate was transferred into a sterilized centrifuge tube of 30-50 ml capacity.

-NaOH (4%) was added to the homogenate in a volume equal to that of specimens.

-The tubes were sealed and shaken on a shaker for 25 min.

-Then sterile distilled water was added to the top ring in the centrifuge tube and centrifuged at 3000 rpm for 20 min.

-Then the supernatant was discarded and the sediment was re-suspended in 1ml of sterile normal saline.

-The prepared sediment was inoculated into two slants of Lowenstein-Jensen medium. Inoculated medium was incubated at 30°C for 8 weeks.

II. Recent method [MB-Redox System Heipha Diagnostica, Germany (Din, 1996)]:

-Before inoculation, the MB-Redox tubes were wormed up at least to room temperature (25-30°C).

- 0.25 ml of previously processed specimens was inoculated into each of the MB-Redox tubes under aseptic condition using an insulin syringe and the tubes were closed tightly before incubation in an incubator at 30°C.

-The MB-Redox tubes were examined after 24 hours incubation, the first time, thereafter twice weekly at intervals of 3 to 4 days. After 4 weeks incubation, the culture was examined once weekly.

-Each examination generally was performed in two steps:

a. Examination of the culture medium and the sediment without tilting the tube.

b. Examination of the sediment particles after slight tilting of the tube.

-The appearance of pink-red- or violet-coloured particles was a strong indication of the presence of mycobacteria also a complete colorization of the culture medium indicated the presence of mycobacteria.

-The presence of mycobacteria was confirmed by examination of a Kinyoun's Z.N. stained smear microscopically.

3. Identification of the recovered mycobacteria:

Identification of suspected colonies was carried out depending upon: morphological characters (Cruickshank *et al.*, 1973), rate of growth (Runyoun, 1959), pigmentation (Songer and Trautman, 1980), growth at different temperatures and biochemical tests (Kubica, 1973) for full identification of the isolated mycobacterium species namely Niacin test, nitrate reduction test, hydrolysis of Tween 80, arylsulfatase test, iron uptake, tolerance to 5% sodium chloride,

growth on MacConkey's agar, urease test and thiophen-2 carboxylic acid hydrazide growth inhibitor.

Results

1. Results of mycobacterial isolation:

All of the 240 Nile tilapia from Nile River in Sohage, Assuit and Qena Governorates were free from mycobacteria and no mycobacteria were detected by microscopic smear or by culture. Three isolates of atypical mycobacteria were recovered from water samples from the Nile. Four other isolates were isolated from aquarium water from different aquaria in shops present in these three Governorates. Also 11 isolates of atypical mycobacteria were isolated from fantail goldfish raised in aquaria (Table 1).

Table (1): Results of mycobacterial isolation from fish and water

Samples		Direct smear*		Growth on culture media		Isolates
Type	No.	No.	%	No.	%	
Tilapia fish	240	0	0	0	0	Negative culture
Water samples from Nile	50	3	6	3	6	Isolates No. 16, 17 and 18
Diseased gold fish (Ascitis and tissue from ulcer)	60	6 3	10 5	7 4	11.66 6.66	Isolates No. 1, 2, 3, 4, 5, 6 and 10 Isolates No. 7, 8, 9 and 15
Aquarium water	40	4	10	4	10	Isolates No., 11, 12, 13 and 14

** Acid fast smear*

2. Results of identification of the isolated acid-fast bacteria:

From morphological, growth characteristics and biochemical characteristics of the isolated mycobacteria, the isolates No. 1, 2, 3, 9, 11 and 15 were identified as *M. marinum*, while isolates No. 10, 12, 13 and 14 were identified as *M. fortuitum* (Photo 1, 2 and Table 2).

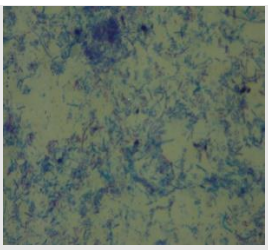

Photo. 1

Photo. 2

Photo (1): A film from Nile water sediment showing acid fast bacilli. **Photo (2):** Isolated atypical mycobacteria (*M. fortuitum*) on Lowenstein-Jensen medium (4 days post inoculation).

3. Results of comparison between using MB-redox system and Lowenstein-Jensen medium for recovery of mycobacteria:

The recovery rate of MB-Redox system for mycobacteria was 12 %, while in case of conventional method (L-J medium) was 10.66 % (Table 3).

4. Results of comparison of time for detection of different mycobacteria grew on MB-redox system and Lowenstein-Jensen medium:

The mean time for detection of *M. marinum* was 3-7 days and 7-10 days for MB-Redox system and Lowenstein-Jensen medium, respectively, with the average of days for detection was 5 days for MB-Redox and 8.5 days for L-J medium. The mean time for detection of *M. fortuitum* was 1-3 days and 3-6 days for MB-Redox system and L-J medium, respectively with an average of 2 days for MB-Redox and 4.5 days for L-J medium. While the mean time for detection of other unidentified atypical mycobacteria was 3-8 days and 3-14 days for MB-Redox system and L-J medium, respectively with an average of 5.5 days for MB-Redox system and 8.5 days for L-J medium (Table 4).

5. Results of detection of mycobacterial growth on MB-redox system at different times:

As illustrated in Table (5), the color of MB-Redox tubes appeared yellow at time of inoculation changed to faint pink color after one day then pink colored medium after 3 days. Colonies appeared as red particles on the surface at the third day. After 15 days, the color of MB-Redox media changed to yellow with red particles on the surface (Photo.3 and Table 5).

Table (2): Results of physical and biochemical identification of the isolated acid fast bacilli from diseased fish and water*

Isolate No.	Growth at different temperature			Usual colonial morphology	Pigmentation		Growth rate	Biochemical tests									Identification of isolates
	25°C	30°C	37°C		Dark	light		Niacin	Iron uptake	Nitrate reduction	Tween 80 hydrolysis	Arylsulfatase 3 days	Urea	Growth on			
														MacConkey	TCH	5 % NaCl	
1	+	+	-	S	Buff	Orange	Slow after 10 days	+	-	-	+	+	+	-	-	-	*M. marinum*
2	+	+	-	S	Buff	Yellow	Slow after 12 days	+	-	-	+	+	+	-	-	-	*M. marinum*
3	+	+	-	S	Buff	Orange	Slow after 7 days	+	-	-	+	-	+	-	-	-	*M. marinum*
4	-	+	+	R	Buff	Buff	Slow after 12 days	-	-	-	+	-	+	-	-	-	*Slow grower unidentified atypical mycobacteria*
5	-	+	-	R	Buff	Buff	Slow after 15 days	+	-	-	+	-	-	-	-	-	
6	-	+	+	R	Buff	Buff	Slow after 7 days	-	-	-	+	-	-	-	-	-	
7	-	+	+	R	Buff	Buff	Slow after 10 days	-	-	-	+	-	-	-	+	-	
8	-	+	-	R	Buff	Buff	Slow after 15 days	+	-	-	+	-	-	-		-	
9	+	+	-	S	Buff	Orange	Slow after 12 days	-	-	-	+	+	+			-	*M. marinum*
11	+	+	-	S	Buff	Orange.	Slow after 7 days	+	-	-	+	+	+	-	-	-	*M. marinum*
12	-	+	-	R	Buff	Buff	Rapid after 3 days	-	+	+	-	+	+	-	+	+	*M. fortuitum*
13	-	+	-	R	Buff	Buff	Rapid after 3 days	-	+	+	-	+	+	-	+	+	*M. fortuitum*
14	-	+	+	R	Buff	Buff	Rapid after 3 days	-	+	+	-	+	+	-	+	+	*M. fortuitum*
15	+	+	-	S	Buff	Yellow	Slow after 7 days	+	-	-	+	+	+	-	-	-	*M. marinum*
16	-	+	-	R	Buff	Buff	Slow after 7 days	+	-	-	-	+	-	-	-	-	Slow grower unidentified field atypical mycobacteria
17	+	+	-	R	Buff	Buff	Slow after 7 days	-	-	-	-	+	-	-	-	-	
18	+	+	+	S	Orange	Orange	Rapid within 3 days	-	+	+	-	-	+	-	+	+	Rapid grower unidentified atypical mycobacteria

+ : Positive - : Negative
S : Smooth R : Rough * : According to Koneman *et al.* (1983)

Table (3): Results of comparison between the recovery rate of mycobacteria by using MB-Redox system and conventional method from 150 processed specimens.

Specimens		AFB in direct smear		Positive culture			
Types	No.	No.	%	MB-Redox system		Conventional culture medium (L-J)	
				No.	%	No.	%
Skin ulcer	15	3	20	4	26.6	3	20
Ascitic fluids	4	2	50	2	50	1	25
Liver and spleen	41	4	9.7	5	12.1	5	12.1
Aquarium water	40	4	10	4	10	4	10
Nile water	50	3	6	3	6	3	5
Total	150	16	10.66	18	12	16	10.66

L-J: Lowenstein – Jensen medium. AFB: Acid fast bacilli

Table (4): Results of comparison of time for detection of different mycobacteria grew on MB-Redox system and Lowenstein – Jensen medium

Specimens with positive culture in:	No. (%) of positive cultures detected by day																				Mean day to detection (Range)	Average
	1		3		7		10		14		17		21		24		28		35			
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%		
MB-Redox system:																						
1. *M. marinum*	0	0	3	50	6	100															3-7	5
2. *M. fortuitum*	1	25	4	100																	1-3	2
3. Other atypical mycobacteria	0	0	3	37.5	8	100															3-8	5.5
L-J medium:																						
1. *M. marinum*	0	0	0	0	3	50	6	100													7-10	8.5
2. *M. fortuitum*	0	0	4	100																	3-6	4.5
3. Other atypical mycobacteria	0	0	1	12.5	4	50	6	75	8	100											3-14	8.5

Table (5): Results of using MB-Redox system for detection of mycobacterial growth at different times.

No. of specimens	At time of inoculation	After one day	3 days	7 days	15 days
13 *	Yellow colored medium	Faint pink colored medium	Pink colored medium with very delicate red particles on the surface	Pink red colored medium with delicate red particles on the surface	Yellow colored medium with delicate red particles on the surface
5**	Yellow colored medium	Faint pink colored medium	Pink colored turbidity of media with fine red particles on the surface	Pink colored media with fine red particles on the surface	Yellow colored media with fine red particles on the surface

*: Identified as ***M. marinum*** (n= 6) and unidentified slow grower (n= 7) atypical mycobacteria
: Identified as ***M. fortuitum*(n= 4) and unidentified rapid grower (n= 1) atypical mycobacteria

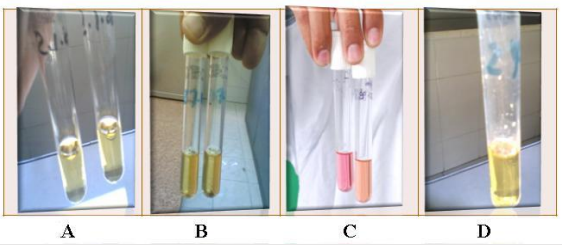

Photo (3): MB-Redox system inoculated with M. marinum. A: MB-Redox tube before inoculation and control, B: One day after inoculation, C: 7 days after inoculation, D: 15 days after inoculation.

Discussion

Piscine mycobacteriosis is a serious and often lethal disease of fish, affecting a wide range of species globally both in culture and wild settings, caused by several species of the genus *Mycobacterium*, the disease has been received considerable attention in recent years because of the discovery of new species in piscine hosts, epizootics in wild fisheries, and the ability of a few species to infect humans (Jacobs *et al.*, 2009).

In the present work, eleven isolates of atypical mycobacteria were isolated from goldfish (18.3 %). Also four isolates were obtained from aquarium water (10%) and three other isolates were obtained from Nile water (6 %), while all Nile tilapia from Nile River in Sohage, Assuit and Qena Governorates were free from mycobacteria.

On applying the morphological, cultural and biochemical studies of the isolated acid-fast bacilli obtained from aquarium fish, aquarium water and water from the Nile (Table 2, Photo. 1 and 2). Six isolates were identified as *M. marinum* and 4 isolates were identified as *M. fortuitum*. Decostere and Hermons (2003) reported that *M. marinum*, *M. fortuitum* and M. chelonae are the main etiological agents of fish mycobacteriosis. The former name is the closest genetic relative of the M. tuberculosis complex (Tobin and Ramakrishnan, 2008) and is a natural pathogen of ectotherms.

The results of comparison between using of the recent MB-Redox system and the conventional L-J medium for recovery of mycobacteria (Table 3) revealed that the recovery rate of MB-Redox for mycobacteria (12%) was higher than that of the conventional L-J medium (10.66 %). These results agree with the findings of Akos and Pal (1999), Emmanuelle *et al.* (1999), Piersimoni *et al.* (1999) and Samra *et al.* (2000) who reported that the recovery rate of AFB by MB-Redox system was higher than the recovery rate of AFB by L-J medium. MB-Redox system gave reliable results, offering the advantages of ready to use MB-Redox tubes in which the antibiotic supplement is already incorporated, easy and immediate reading of the results. Also, as this system does not contain any radioactive substance in comparison with BACTEC 460 TB system so results can be confirmed with acid fast staining and conventional and molecular tests.

The obtained results in Table (4) revealed that the mean time for detection of *M. marinum*, *M. fortuitumand* other unidentified atypical mycobacteria that grew on MB-Redox system was 3-7 days, 1-3 days and 3-8 days, respectively. The mean time for recovery of *M. marinum*, *M. fortuitum* and unidentified atypical mycobacteria that grew on L-J medium in the present work was 7-10 days, 3-6 days and 3-14 days, respectively. The mean times for detection of different Mycobacterium species in MB-Redox system were shorter than that observed with L-J medium. These results agree with that reported by Wolf *et al.* (1998), Abe *et al.* (1999), Akos and Pal (1999) and Emmanuelle *et al.* (1999). Also, the present results agreed with the finding of Heifets *et al.* (2000) who recorded that the time for recovery of NTM in MB-Redox tube was 6.9 days for smear positive specimens and 15.5 days for smear negative specimen. They added that neither MB-Redox system nor other recent techniques (MGIT and BACTEC 460) should be used instead of a solid media (L-J medium) rather than they should be used in addition to it, the combined use of MB-Redox with L-J medium improved the overall level of recovery of mycobacteria from the specimens and provides rapid detection of mycobacterial growth for up to 88% of the culture positive specimens.

The results of detection of mycobacterial growth on MB-Redox tubes at different times (Table 5 and Photo. 3) revealed change in color of medium from yellow to faint pink after 24 hours and pink after 3 days, *M. marinum* grew as delicate red colored particles on the surfaces. *M. fortuitum* and some other mycobacteria grew as fine red particles. After 15 days the medium changed to yellow color. These findings are in agreement with Satio (1998) and Martin *et al.* (2006) who recorded that MB-Redox system is easily detectable by eye on the basis of their formazan (insoluble salt) color (pink-red-violet). The staining of

the particles is stable for 2 weeks after which a decolorization may occur (yellow color).

In conclusion and from these aforementioned results it was found that atypical mycobacteria (*M. fortuitum and M. marinum*) are present in aquarium fish and water as well as in fresh water (Nile water). This is very important due to the mycobacterium infected fish or contaminated water can cause disease in human so that it is very important to avoid contact between the atypical mycobacteria of fish and other mycobacteria (M. tuberculosis complex) to avoid the possible transfer of drug resistance in between. Comparison between conventional method and recent method (MB-Redox system) was done to determine the ideal techniques for recovery of these agents.

Conflict of interest

The authors declare that they have no conflict of interest.

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