Studies on Mycoplasmosis among Some Fishes

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Abstract
Mycoplasma organisms were isolated from naturally infected
Oreochromis niloticus, Clarias lazera, Cyprinus carpio and to
our knowledge for the first time from Anguilla anguilla. The
isolated strains were antigenically different from those of
poultry, humans and terrestrial animals. Antibiogram for the
isolated Mycoplasmas was carried out in-vitro against different
chemotherapeutic agents as enrofloxacin, norfloxacin and
ciprofloxacin as the most active drugs. Pathogenicity studies
were done by experimental infection via gill scarification and
subcutaneous inoculation with the isolated mycoplasma strains.
Clinical signs and lesions were described. Immune response
was detected in the infected fish sera using growth inhibition,
growth precipitation and metabolism inhibition tests.
O. niloticus were experimentally infected via gill scarification
with Mycoplasma gallisepticum strain isolated from chickens.
The organism could pass through the fish and was re-isolated
from their gills and internal organs for 10 days post infection.

Introduction
Many authors investigated the relationship between mycoplasma as
a pathogen and fish as a host. The first approach to this field of study
was done by Kunze et al. (1972) who isolated mycoplasma from fish
cell line, which draw the attention to
further investigations.
Mycoplasma was isolated from a
ten fish, Tincia tinca for the first
time by Kirchhoff et al. (1984). The
isolated mycoplasma was designated
as Mycoplasma mobile 163K. Other
authors as Rosengarten and
Kirchhoff (1984); Kirchhoff and
Rosengarten (1984); Fisher and
Kirchhoff (1987); Kirchhoff et al.
(1987) and Stadlander and
Kirchhoff (1988) succeeded to
isolate mycoplasma from fish. In
Egypt, El-Shabiny et al. (1989)
isolated mycoplasma from Tilapia
nilotica, Solea solea and Synodontis
shall. Again El-Shabiny et al. (1996)
isolated mycoplasma from Clarias
lazera and Husien et al. (1998) isolated mycoplasma from Nile carp (Labeo niloticus). Morphological and biochemical characteristics of the fish mycoplasma were studied by Kirchhoff and Rosengarten (1984); Kirchhoff et al. (1987); El-Shabiny et al. (1989); El-Shabiny et al. (1996) and Husien et al. (1998). In addition, other properties of fish mycoplasma as agglutination and adsorption to sheep RBCs, hemolysis and adherence to glass or plastic surfaces and gliding motion were studied by Kirchhoff et al. (1987); El-Shabiny et al. (1989) and El-Shabiny et al. (1996).

Pathogenicity of fish Mycoplasma was described by Kirchhoff and Rosengarten (1984) who found general hemorrhages and skin inflammation; El-Shabiny et al. (1996) showed hemorrhages on body surface, inflamed and sloughed skin and fin destruction and Husien et al. (1998) detected hemorrhages on skin, fin rot and gray patches on gills. Moreover, the internal lesions were investigated in the form of congested spleen, yellowish discoloration of liver (El-Shabiny et al., 1996) and gill and fin necrosis (Husien et al., 1998).

Humoral antibodies against Mycoplasmas were detected in the sera of infected carp and tench by using ELISA, indirect hemagglutination and complement fixation tests (Eggebrecht, 1986) and by using indirect immunofluorescence, growth inhibition and metabolism inhibition tests from sera of infected Tinca tinca L. (Kirchhoff et al., 1987).

The sensitivity of fish mycoplasma to chemotherapeutic agents was described by Frerichs (1996) who used ciprofloxacin for the control of fish cell line contaminated with mycoplasmas while, Husien et al. (1996) found that the isolated strain was sensitive to enrofloxacin and norfloxacin.

The aim of the present study is to: 1) Survey the presence of Mycoplasmosis in various fish species obtained from different localities; 2) To study the immunogenicity of the isolated strains of mycoplasma and 3) To investigate if Mycoplasma gallisepticum from chickens can infect or stored in the fish as a new reservoir.

Material and methods

Naturally infected fish

A total of 65 fish were collected from natural sources (El-Rah el Tawfiki, Abbasa fish hatchery and Manzalla Lake) and Kaluobia fish markets. The fish samples included 30 Oreochromis niloticus (70-200 g body weight), 15 Clarias lazera (150-300 g body weight), 13 Cyprinus carpio (200-400 g body weight) and 7 Anguilla anguilla (200-500 g body weight). The obtained fish were sent to the laboratory under all accurate methods of transportation according to Brown and Gratzek (1980). The fishes were examined clinically as described by Amlacker (1970). Clinical
signs and post-mortem findings whenever present were summarized and presented in results.

**Bacteriological examination**

From autopsied fish, samples for bacteriological examination were taken from gills, liver, spleen, swimbladder and intestine. The procedure for mycoplasma isolation was done according to Stadlender and Kirchhoff (1989) using modified Hayflick medium supplemented with horse serum, penicillin and thallium acetate and incubated at 25°C aerobically and anaerobically. Genus determination of the isolates was done using digitonin test according to Tully (1983). Requirements for cholesterol, lack of reversion to bacterium and filter ability through 450nm-membrane filter were studied as described by Kirchhoff and Rosengarten (1984). Biochemical characterization of the isolated Mycoplasmas was carried out according to Aliotto et al. (1970) and results were recorded. Agglutination and adsorption by sheep RBCs was tested according to Sobeslavsky et al. (1968) while, hemolysis was tested as mentioned by Cole et al. (1968).

**Seriological identification**

The isolated Mycoplasmas were examined by growth inhibition test described by Clyde (1964) against different standard mycoplasma antisera found at Mycoplasma Department Animal Health Research Institute Dokki including M. bovis; M. bovirhinis; M. mycoides; M. agalactia; M. gallisepticum; M. fermentans; M. gallinarum; M. pullorum; M. synoviae; M. argininis; M. alkalescens; M. meleagridis and M. anatis.

**In-vitro antibiotic sensitivity test**

The Antibiogram sensitivity test for mycoplasma isolates was carried out according to Cruickshank et al. (1975) against various chemotherapeutic agents as presented and tabulated in results.

**Experimental infection**

**Aquaria**

Five aquaria were used, each measured 1 x 1 x 0.5 m. They were supplied with dechlorinated tap water as the only source of water supply. Sufficient aerators were used and the basal diets were in the form of autoclaved mash.

**Experimental fish**

A total of 65 O. niloticus of average body weight 40±7g were obtained from a private fish hatchery at Kaluobia Governorate and brought alive in a big water tank to the Wetlab. Random samples of 5 fish was picked up and examined bacteriologically to ensure their freedom of mycoplasma. The remaining fish were divided into 5 groups, the first two groups each of 15 fish while the last 3 groups each had 10 fish. The water temperature was adjusted at 29±2°C except for red group it was 35±2°C.
Mycoplasma strains
1- Mycoplasma strain (Fish origin) isolated from naturally infected O. niloticus.
2- M. gallisepticum (well identified strain kindly supplied by Animal Health Research Institute Mycoplasma Dep. Dokki, Egypt)

Experimental design
The design is summarized and presented in table (1).

Table (1): Group number, number of fish, type of inoculated material, dose per fish and route of inoculation

<table>
<thead>
<tr>
<th>Group No</th>
<th>No of fish</th>
<th>Type of inoculated material</th>
<th>Dose / fish</th>
<th>Route of inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>Mycoplasma isolate (fish origin)</td>
<td>2x10^9 CFU</td>
<td>gill scarification**</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>Mycoplasma isolate (fish origin)</td>
<td>2x10^9 CFU</td>
<td>subcut inoculation</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>M. gallisepticum (chicken origin)</td>
<td>2x10^9 CFU</td>
<td>gill scarification**</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>sterile broth</td>
<td>0.5 mL</td>
<td>subcut inoculation</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>sterile broth</td>
<td>0.5 mL</td>
<td>gill scarification**</td>
</tr>
</tbody>
</table>

*The dose was 0.5 mL of 48-hour old mycoplasma broth culture without thallium acetate and penicillin containing 2x10^9 CFU according to El-Shabiny et al. (1996).

**Gill scarification was applied as light scratching for gill filaments. Both infected and control groups were kept under observation for 4 weeks post inoculation. Three fish from the first two groups and two fish from the last three groups were sacrificed at the 3rd, 7th, 10th, 15th day and the remaining fish at the day 28th post-inoculation. Trails for re-isolation of inoculated organisms were done using samples from internal organs and gills and the re-isolated mycoplasma were completely identified as mentioned before. Signs and lesions in experimentally infected fish were recorded.

Serum samples were collected from fish infected via gills with mycoplasma of fish origin and examined at 1, 2 and 4 weeks post inoculation for the presence of antibodies using growth inhibition (Clyde, 1964), growth precipitation (Kroggaard-Jensen, 1972) and metabolism inhibition tests (Purcell et al., 1966).

Results
From table (2) it is clear that more than 56.9% of the examined fish were clinically diseased. It is also obvious that 14 out of 65 examined fish were positive to mycoplasma isolation (21.5%) From this survey a
reversion to bacterium and their characters of the suspected need for cholesterol in aerobic cultivation. The biochemical (3).

Table (3): Biochemical properties of the isolated mycoplasmae from naturally infected fish

<table>
<thead>
<tr>
<th>Fish spp.</th>
<th>No. examined fish</th>
<th>No. of positive fish</th>
<th>Biochemical properties</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Glucose fermentation</td>
</tr>
<tr>
<td>O. niloticus</td>
<td>30</td>
<td>7</td>
<td>+ve</td>
</tr>
<tr>
<td>C. lazera</td>
<td>15</td>
<td>3</td>
<td>+ve</td>
</tr>
<tr>
<td>C. carpio</td>
<td>13</td>
<td>2</td>
<td>+ve</td>
</tr>
<tr>
<td>A. anguilla</td>
<td>7</td>
<td>2</td>
<td>+ve</td>
</tr>
</tbody>
</table>

From table (3), it is clear that regardless of donor fish all isolates fermented glucose, reduced tetrazolium, failed to hydrolyze urea, positive to phosphatase activity and did not hydrolyze arginin. In addition the isolates hydrolyzed and agglutinated sheep RBCs; adhered to glass and plastic surfaces and showed no inhibition zone when tested by growth inhibition test against the aforementioned available reference antisera. Results of in-vitro Antibiogram for the isolates appear in table (4)
Table (4): In-vitro sensitivity test of mycoplasmae from naturally infected fish to different chemotherapeutics

<table>
<thead>
<tr>
<th>Antibiotic designation</th>
<th>Concentration in µg</th>
<th>O. niloticus</th>
<th>Clarias lazera</th>
<th>Cyprinus carpio</th>
<th>Anguilla anguilla</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Strain</td>
<td>Strain</td>
<td>Strain</td>
<td>Strain</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GI/mm</td>
<td>GI/mm</td>
<td>GI/mm</td>
<td>GI/mm</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>15</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Oxytetracyclin</td>
<td>30</td>
<td>16</td>
<td>15</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>Tylosin</td>
<td>15</td>
<td>20</td>
<td>21</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>20</td>
<td>21</td>
<td>21</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>10</td>
<td>22</td>
<td>23</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>Danofloxacin</td>
<td>30</td>
<td>20</td>
<td>20</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>20</td>
<td>22</td>
<td>21</td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td>Vibramycin</td>
<td>30</td>
<td>20</td>
<td>19</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>20</td>
<td>22</td>
<td>22</td>
<td>21</td>
<td>23</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>10</td>
<td>19</td>
<td>18</td>
<td>18</td>
<td>17</td>
</tr>
</tbody>
</table>

GI/mm = growth inhibition per mm  
R = resistant

From table (4) it is clear that the isolates were resistant to erythromycin; of moderate sensitivity to oxytetracyclin and gentamycin and were highly sensitive to enrofloxacin followed by ciprofloxacin, norfloxacin; danofloxacin; lincomycin and vibramycin.

Experimentally infected O. niloticus with fish mycoplasma via gill scarification showed clinical signs represented by hemorrhages all over the skin, fins and under the base of the fins, and in the late of the experiment the fins become eroded. The fish appeared off food, sluggish swimming and surfacing. While those infected subcutaneously had hemorrhages on skin, on fins, excess mucus, minute erosion on the skin, fin rot especially caudal fin which was completely lost. The superficial layer of the skin was inflamed and sloughed off (Fig. 3). Fish became off food and glance of the bottom. Macroscopic lesions in the fish infected via the gills appeared as, congestion or palness or grayish patches in gill filaments. The liver showed congestion or hemorrhagic streaks or greenish discoloration. The gallbladder appeared enlarged and the spleen was congested and enlarged, slightly congested kidneys. The intestine and swim bladder were normal. While those infected subcutaneously the lesions restricted to the skin and
pale gills as the internal organs had no macroscopical lesions. Re-isolation of the inoculated organism revealed positive results from gills and internal organs of fish inoculated via gills and only from skin of fish inoculated subcutaneously and identified as previously described. Humoral antibodies against the infective organism (Mycoplasma from *O. niloticus*) appeared after one week in sera of infected fish and continued up to 4 weeks post-infection (the last date of observation). The sera gave moderately positive reaction (++) as judged by growth inhibition, which presented by the inhibition zone; growth precipitation that appeared as precipitation line and metabolism inhibition, which recognized by inhibition of color change due to the metabolic effect of mycoplasma owing to the antibodies. Regarding the possibility of fish to harbor *M. gallisepticum* as a reservoir, *O. niloticus* were infected via gill scarification showed no signs of disease and had no internal lesions. The mycoplasmas were re-isolated from the gills and internal organs for about 10 days post-infection while, trials for re-isolation at 15th days and 28th days post-infection gave negative results.
Discussion

Recognition and identification of fish Mycoplasmas is justified by data presented in tables 2&3. Mycoplasmas were isolated from *O. niloticus*, *C. carpio*, *C. lazera*, and *A. anguilla*. These findings supports and is supported by El-Shabiny et al. (1989 and 1996) who isolated Mycoplasmas from *O. niloticus*, *Solea solea* and *Synodontis schall* and *C. lazera*. It is also supported by Husein et al. (1998) who isolated Mycoplasmas from Nile carp. However, to the best of our knowledge, isolation of mycoplasmas from Eels is recorded for the first time in the present study. It is interesting that this kind of fish can move between marine and freshwater. Under the present circumstances it is difficult to postulate from where the infection came.

The isolated Mycoplasmas fulfilled the criteria of class Mollicutes related to genus mycoplasma as they fermented glucose, reduced tetrazolium, had phosphtatase activity, formed film and spots, grow at 25°C on modified Hayflick media containing horse serum. Moreover they agglutinated and adsorbed sheep RBCs and adhered to glass and plastic surface. This findings agreed with that reported by Kirchhoff and Rosengarten (1984); kirchhoff et al. (1987); El-Shabiny et al. (1989 and 1996) and Husein et al. (1998).

Concerning the in-vitro sensitivity test, the antibiogram for the isolated Mycoplasms against common chemotherapeutics agents revealed that enrofloxacin, norfloxacin and ciprofloxacin may be considered the most effective drugs. These results coincided with that of Frerichs (1996) who used ciprofloxacin for elimination of mycoplasmas from contaminated fish cell lines and Husein et al. (1998) who described that mycoplasms isolated from Nile carp were sensitive to enrofloxacin and norfloxacin.

Regarding the pathogenicity of fish Mycoplasms (isolated from *O. niloticus*) the fish infected via gills showed signs of hemorrhages allower the skin and fins and fin erosion. The post-mortem findings revealed gill congestion or presence of gray patches, liver discoloration, enlarged and congested spleen. The same findings were observed in *C. lazera* by El-Shabiny et al. (1996) and nearly similar with those reported by Husein et al. (1998). While fish infected subcutaneously revealed minute hemorrhages on the skin and fins, erosions of skin, inflammation and sloughing of the superficial layer of skin and fin rot and palness of gills. These findings is supported by Stadtländer and Kirchhoff (1988 and 1990) who considered the fish Mycoplasmas as a surface parasitic agent causing damage to epithelium and by Husein et al. (1998) who reported that the
external surface of gills and fins as the predilection seat for the organism. Moreover, the sera of fish infected with mycoplasma of fish origin proved to have antibodies as detected by growth inhibition, growth precipitation and metabolism inhibition tests. In the same respect Eggebrecht (1986) found antibodies in the sera of mycoplasma infected carp and tench using ELISA, indirect haemagglutination and complement fixation, and Kirchhoff et al. (1987) detected antibodies in sera of mycoplasma infected Tinca tinca L. by using indirect immunofluorescence growth inhibition and metabolism inhibition tests. The present work is the first approach to investigate the role of fish as a new reservoir for M. gallisepticum passed through the infected fish for about 10 days post-infection. So, fish can be added as a carrier host for transmission of M. gallisepticum especially in countries that used wet chicken manure as pond fertilizer or feeding carcasses of dead chickens to C. lazera and similar carnivorous fish or as a way of attraction during seining. This finding is supported by Olah et al. (1972) who found Mycoplasmas in the water sediment in some freshwater lakes. This point needs more investigations.

References


