

Mixed Aetiological Agents Of Nodular Form Disease In Marine Fish (*Argyrops spinifer*)

Maha A El-Hady and Nahla R El-Khatib

Fish Diseases Department, Animal Health Research Institute, Dokki, Giza.

ABSTRACT

Ninety *Argyrops spinifer* with body weight range, 50-300g, were examined for isolation and identification of bacterial and parasitic causes of nodular disease. Experimental infections of *Oreochromis niloticus* with two isolated *Nocardia asteroides* strains were done by two routes I/P and I/M for pathogenicity. Sensitivity test of isolated *Nocardia asteroides* strains to different chemotherapeutic agents was conducted *in-vitro*.

The results revealed that 46 out of 90 examined fish (51.1%) showed nodular disease in musculature, from which 40 fish (44.4%) caused by mixed infection with myxozoan parasite and bacterial agent *Nocardia asteroides* while only 6 fish (6.7%) caused by single infection with myxozoan parasite. The spores of *Kudoa spp.* were stellate in shape, with 4 valves and 4 polar capsules. Post mortem soft flesh syndrome was recorded in the highly infected fish. Clinical signs and post-mortem lesions of nodular disease were recorded. *N. asteroides* caused 20% mortality in experimented *O. niloticus* and showed sensitivity to chemotherapeutic agents.

INTRODUCTION

Argyrops spinifer is one of the most important fish species in Mediterranean Sea. Myxozoans of the genus *Kudoa* parasitize a wide variety of fish species (1). Most *Kudoa* and many myxozoan species in general, have high host specificity. The majority of *Kudoa* species infect the somatic muscle of marine and estuarine fish establishing cysts, which contain many spores. As the parasite grows, it produces proteolytic enzymes (2,3) that break down the filaments of the muscle fibre (4). Post-mortem flesh quality of farmed Atlantic salmon has been severely impacted by *K. thyrssites* infections due to a protease that digests host muscle tissue causing different levels of myoliquefaction depending on the severity of infection (5). Although it is not known to cause host mortality, *K. thyrssites* is responsible for causing economic losses to the fisheries sector to such an extent that the fish becomes unmarketable (6). In Greek mariculture a case of kudoasis was previously reported in juvenile gilthead seabream, associated with high mortalities (7).

Nocardiosis is a systemic chronic granulomatous disease of fish caused by several species of the bacterium *Nocardia*. Nocardiosis

is a disease of both salt and freshwater fish. Severe emaciation, inactivity and skin discoloration are the clinical signs of the disease (8). Signs of Nocardiosis are nearly similar to mycobacteriosis in affected fish. All age groups may be infected with lesions, manifested as small white spots present in the dermis, muscle, gills and internal organs (9). It presents itself as an underlying debilitating factor in many types of fish. Affected fish often have other concurrent or secondary infections (10). Heterogeneity within *Nocardia asteroides* taxon has been recorded (11) where variations may include morphology, colonial form and colour, presence of aerial as well as substrate mycelium, phenotypic characters and pathogenicity. Infection of fish with *Nocardia asteroides* develops slowly and the incubation period is apparently long. Mode of transmission and carrier state is unknown. Morbidity and mortality rates are quite low (12, 13). The internal pathology of nocardiosis is easily confused with other 'white-spot-forming' diseases, such as mycobacteriosis, photobacteriosis and myxosporidiosis especially if mixed infections exist (10). In Egypt, (11) *Nocardia asteroides* was isolated from *Oreochromis niloticus* at lake Qarun and lake

Manzala with prevalence rates of 24 and 64%, respectively.

So, this study was planned for isolation and identification of the mixed aetiological agents of nodular form disease in *Agyrops spinifer*. *In-vitro* sensitivity test of isolated *Nocardia* against various chemotherapeutic agents besides a pathogenicity study was made on apparently healthy *Oreochromis niloticus* to monitor the pathological changes made by such bacteria.

MATERIALS AND METHODS

A. Naturally infected fish

Ninety *Agyrops spinifer* with body weight range, 50-300g, were collected from fishermen at Mediterranean Sea in Alexandria Governorate. They were kept in ice plastic bags and transported immediately after capture to the Fish Disease Department lab in Animal Health Research Institute, for bacteriological and parasitological examinations.

1. Clinical picture

Clinical signs and post-mortem lesions of naturally infected fish were recorded.

2. Parasitological examination

External and internal examinations of the collected fish for any abnormalities were done. Muscle tissue from such fish was also examined macroscopically for any nodular lesions and by wet mount preparations for the presence of myxozoan spores (15). Positive impression smears for myxozoan spores were air dried, fixed in methyl alcohol and stained with Geimsa's stain. Spore descriptions were made (16).

3. Bacteriological examination:

Samples from internal organs as well as affected musculature of examined *Agyrops spinifer* were streaked onto nutrient agar, trypticase soy agar, trypticase soy agar supplemented with 3 and 7% sodium chloride plates, then incubated at 28°C for 24-72 hr. The growing colonies were picked up in pure form and reinoculated into trypticase soy agar

for further identification. Identification of all isolates was done by cultural, morphological and biochemical characters (14).

4. *In-vitro* sensitivity test of *Nocardia asteroides* isolates

It was carried out against various chemotherapeutic agents and judgment of the obtained results in comparison to interpretive standards was applied (14, 17).

B. Experimental fish

A total number of 75 apparently healthy *Oreochromis niloticus* were collected from private fish farm (Fayoum Governorate) with an average body weight 80.0±10.0g, and transferred alive to Fish Diseases Department lab. Fish were divided into five equal groups and kept in chlorine-free tap water in five full glass aquaria (120×60×40cm). Fish were acclimated to experimental conditions for 2 weeks with continuous aeration and the water temperature was thermostatically controlled and kept at 22±2°C.

5. Experimental infection

Fish were randomly screened for the presence of *Nocardia* species and were found to be free from the risk of natural infection. A total number of 75 clinically normal *Oreochromis niloticus* were divided into five groups each of 15 fish. The fish in the first two groups were experimentally inoculated intraperitoneally (I/P) with two different isolates of *Nocardia asteroides* (isolates number 2 and 4). Another two fish groups were experimentally inoculated intramuscularly (I/M) with the same isolates. Each fish received a dose of 0.5ml of 72 hours viable tryptic soy broth culture (3.9×10^9 CFU). The used protocol was that previously described (11). The fish in the fifth group was kept as control and inoculated I/P and I/M with sterile tryptic soy broth. Fish were kept under observation for one month for gross pathology, mortality and post-mortem lesions. Samples were taken from internal organs of experimental infected fish for re-isolation of the inoculated bacteria.

RESULTS

1. Clinical picture

Naturally infected fish appeared as apparently healthy but in many cases the fish exhibited skin darkening, dullness and sometimes ulceration with hemorrhage on base of the fins.

2. Parasitological examination

Nodular lesions were found in musculature of *Agyrops spinifer* as 46 out of 90 (51.1 %). All affected fish displayed 0.5- to 2-mm (up to 6 mm) whitish, spherical, or oval plasmodia stage restricted to somatic muscle. Wet mount preparations from whitish milky content of cystic plasmodia revealed that spores were multivalvulid myxozoans, stellate in shape from apical view and suboval in side view had four shell valves and four equal polar capsules morphologically consistent with species of *Kudoa* (Fig. 2). The highly infected fish showed postmortem soft flesh.

3. Bacteriological examination

Nocardia asteroides was isolated from nodular lesions of parasitic infected fish at 44.4% (Table, 1). Isolates of *Nocardia asteroides* produced raised, folded, and moist to chalky colonies after 72 hr. incubation. Another isolates produced yellowish colonies with raised center and their margin become undulated 3 weeks post-incubation. A thin aerial mycelium was seen in some isolates. In old culture 3-4 weeks post-incubation these colonies became wrinkled and undulated (Fig. 3). Morphologically, *Nocardia asteroides* showed branched filaments of unequal sized coccoid elements and rod shaped fragments.

4. In-vitro sensitivity test of *Nocardia asteroides* isolates

The sensitivity of *Nocardia asteroides* to different chemotherapeutic agents revealed that the tested isolates were highly sensitive to Amoxycillin, Colistin sulphate, Danofloxacin, Gentamycin, Nitrofurantoin, oxytetracycline, Sulphamethoxazole. While vary in sensitivity to the other antibiotics (Table, 2).

5. Experimental infection

Fish groups inoculated I/P with *Nocardia asteroides* showed a sluggish movement, dark skin discoloration, granuloma formation on both lateral sides of mouth (Fig. 5). While fish groups inoculated I/M with *Nocardia asteroides* revealed fin and tail rot, skin hemorrhages and ulceration. Postmortem examination of experimentally inoculated fish revealed pale gills, small white spots or nodules on liver with patches of necrosis and congestion of spleen and kidney (Fig. 6) with 20% mortality. Reisolation of *Nocardia asteroides* from internal organs of experimentally infected fish was applied with success.

Table 1. Percentage of aetiological agents of nodular form disease in *Agyrops spinifer* (n= 90)

Fish pathogen	infected fish		single infected fish		Mixed infected fish	
	No.	%	No.	%	No.	%
<i>Kudoa spp.</i>			6	6.7		
<i>Nocardia asteroides</i>	46	51.1	-	-	40	44.4

Table 2. *In-vitro* sensitivity of *Nocardia asteroides* isolates to different chemotherapeutic agents.

Chemotherapeutic agents	Concentration per disc	<i>Nocardia asteroides</i> isolates			
		1	2	3	4
Amoxycillin	10 µg	S	S	S	S
Cephalothin	30 µg	S	R	S	R
Colistin sulphate	25 µg	S	S	S	S
Danofloxacin	5 µg	S	S	S	S
Erythromycin	15mcg	S	I	I	S
Gentamycin	10 µg	S	S	S	S
Lincomycin	10 µg	S	R	R	R
Nalidixic acid	30 µg	R	R	S	S
Nitrofurantoin	300 µg	S	S	S	S
Oxolonic acid	2 µg	R	R	S	S
oxytetracycline	30 µg	S	S	S	S
Sulphamethoxazole 23.7ug/ Trimethoprim 10.25 ug	25 µg	S	S	S	S

S: Sensitive

I: Intermediate

R: Resistant

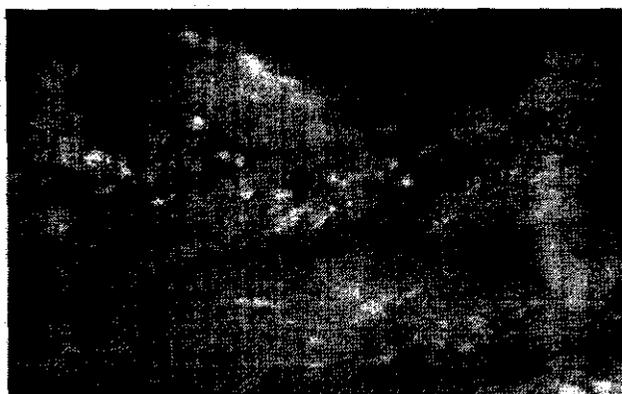
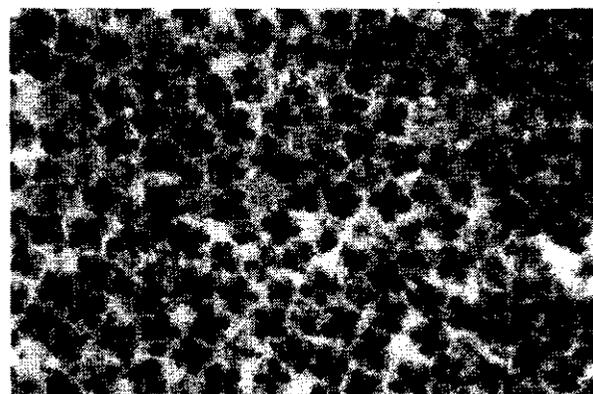
Fig.1. heavily infected musculature of *Agyrops spinifer* with multiple nodules caused by *Kudoa*.Fig.2. Giemsa stained *Kudoa* spores X400.Fig.3. Colonies of *Nocardia asteroides* isolated on Tryptic Soy Agar r (4weeks post-incubation).Fig.4. Experimentally infected *Oreochromis niloticus* with *Nocardia asteroides* showing fin rot, hemorrhages and skin ulceration.



Fig.5. Experimentally infected *Oreochromis niloticus* with *Nocardia asteroides* showing bilateral masses in oral mucosa, redness of eye.



Fig.6. Experimentally infected *Oreochromis niloticus* with *Nocardia asteroides* showing pale gills, white nodules on liver with necrotic patches.

DISCUSSION

The number of studies concerning *Agyrops spinifer* diseases in Egypt is very limited. Myxozoans are economically important fish parasites with over 2,180 described species (18). Genus *Kudoa* is a myxosporean parasite of marine fishes. It has worldwide distribution and infects wide range of host species (1). In the present study, examined fish were infested with Myxozoans cyst (44.4 %). The structural features of the present species proved that the spore have four polar capsules with four shell valves that are the main criteria for genus *Kudoa* (18). *Kudoa* is not known to cause host mortality, it is responsible for causing economic losses to the fisheries sector by causing postmortem "myoliquefaction," softening of the flesh to such an extent that the fish becomes unmarketable (6).

From the point of our work, *Nocardia asteroides* was isolated from affected fish with a % of 44.4. Nearly similar results were recorded (11). As with mycobacteria, it has been argued that infected fish should be destroyed so as to prevent any human health hazard. However, some success may result with chemotherapy, in particular with sulphonamides (9). In this study, the all the occurrence of *Nocardia* infection in *Agyrops spinifer* was concurrent with *kudoa* myxosporean parasite. Infestation fish

affected with *Nocardia* infection often have other concurrent or secondary infections (10).

According to our results, the sensitivity of *Nocardia asteroides* to different chemotherapeutic agents revealed that the tested isolates were highly sensitive to Amoxycillin, Colistin sulphate, Danofloxacin, Gentamycin, Nitrofurantoin, oxytetracycline, Sulphamethoxazole. While vary in sensitivity to the other antibiotics. In this concern, it has been recorded that *Nocardia asteroides* isolates had moderate sensitivity of to Nitrofurantoin and Amoxycillin as well as weak sensitivity to Lincomycin and oxytetracycline (11). The development of nocardiosis in fish probably results from environmental stress and so the best method of disease control is by providing good husbandry in the culture system (19).

Regarding the experimental infection of *Nocardia asteroides* in healthy *O. niloticus* fish, by I /P inoculation resulted in pathological clinical alterations such as sluggish movement, dark skin discoloration, granuloma formation on both lateral sides of mouth, while I/M inoculated fish showed fin and tail rot, skin hemorrhages and ulceration with 20% mortality. These results seemed to agree with the results obtained from Chinook salmon where a large oral granulomata was caused by *Nocardia asteroides* (20). On the basis of present study, postmortem

examination of experimentally inoculated fish revealed pale gills, small white spots or nodules on liver with patches of necrosis and congestion of spleen and kidney. Similar lesions were described previously in Egypt (11). Typical granulomatous lesions by *Nocardia asteroides* in both Formosa snaked head and large mouth bass through intraperitoneal and intramuscular inoculation were recorded (21).

Our study clearly demonstrates that the majority of occurrence of *kudoa* myxosporean parasite in *Agyrops spinifer* was concurrent with *Nocardia* infection. There are no exist in the literature or records of *Kudoa* spp. have concurrent infections Basically, nocardiosis is debilitating factor in many types of fish and the affected fish often have other concurrent or secondary infections without determination of certain causative agents (10). In our opinion, the infection of musculature by of *Kudoa* can produce a slow necrosis, without major damage to living fishes as supported recently (22) *kudoa* is a debilitating factor for infected fish. On the other hand, nocardial infections appear to progress more quickly during the summer months when water temperatures reach 24°C (10). Myxosporidian developmental stages variation is usually due to the influence of temperature on the host, the parasite, or both (1). Also, possible role of the parasites in enhancing infections of fish with bacterial pathogens was suggested (23).

In conclusion, we have shown that nodular forming diseases of *Nocardia* and *Kudoa* are having possible relationship and need further studies to clarify the relationship and better understanding the concurrent infections by using experiments and molecular new techniques.

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المخلص العربي

المسببات المرضية المختلطة لمرض الشكل العقدي فى اسماك المرجان

مها عبد العظيم الهادى ، نهلة رمزى الخطيب
قسم أمراض الأسماك - معهد بحوث صحة الحيوان - الدقى - جيزة.

تم عمل فحص بكتيرى و طفيلى على اسماك المرجان ووجد ان ٥١ % من الأسماك مصابة بعقد فى العضلات. وكان سبب هذه الاصابة مسبيين مرضين احدهما طفيلى والاخر بكتيرى .
الإصابة الطفيلية كانت بطفيل من مجموعة الميجزوسبورديا وهو الكودوا. تم فحص عدد ٩٠ سمكة مرجان وقد وجد ان عدد ٤٦ (١,٥١%) منها مصابة بعقد فى العضلات. وبوغيات طفيل الكودوا يشبه النجوم الفضائية وله ٤ صمامات ويحتوى على ٤ حويصلات طرفية .
وميكروب النوكارديا استوريدس المسبب لمرض العقد فى الأسماك تم عزله ايضا من ٤٠ سمكة مرجان (٤,٤٤%) من نفس الحويصلات المصابة بالطفيل . تم عمل عدوى صناعية بميكروب النوكارديا استوريدس فى مجموعتين من اسماك البلطى النيلى بالحقن فى العضلات وفى الغشاء البرتونى. تم عمل اختبار حساسية على العترات المعزولة من ميكروب النوكارديا استوريدس ضد العديد من مضادات البكتيريا.