

Infestation of Red Sea Cultured *Plectropomus Areolatus* Broodstock with *Benedenia epinepheli* (Yamaguti1937) Parasite in Saudi Arabia with Some Treatment Trails

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Abstract Grouper (*Epinephelus* spp.) is one of the most economically important cultured marine fish in Saudi Arabia. Over 10 species of grouper fish have been cultured there. Grouper fish, Taradi, *Plectropomus areolatus* is gaining importance as a candidate species for marine and coastal aquaculture. Captive broodstock of grouper, Taradi (size range, 20-30 cm; body weight 0.5-2.5 Kg) collected from Red Sea coast, Jeddah demonstrated clinical infection with a monogenean parasite affect in 76% fishes. Prevalence of infection, taxonomy of the parasite, histopathology and trials for treatment were recorded. The parasite was visible measured 3.17-3.95×0.89-2.07 mm. It was found that the parasite has tissue tropism to the gills, fins and skin of Taradi fish. However, fishes in advanced state of infestation showed flashing, erratic swimming behavior, restlessness and off food, with development of small focal hemorrhages on the body surface. In some cases, sloughing of the skin and excessive mucus secretion were seen. A description of the parasite treatment and its histopathology of skin and gills of infested Taradi fish was discussed. Successful treatment of monogenean infection was carried out using formalin, praziquantal and freshwater bath which found to be effective in dislodging the parasite from the fish. While bath treatment using neem leaf water extract was found to be less effective. Histopathological alterations for naturally infested Taradi fish were discussed.

Key words: Grouper *Epinephelus* sp., Taradi, *Plectropomus Areolatus*, Saudi Arabia, Prevalence, Treatment, Formalin, Praziquantal, Neem Leaf Water Extract, Freshwater Bath

INTRODUCTION

Monogenean trematodes (Pectobothrii) are predominately true or obligatory ectoparasites on specific sites on the external body surface mainly on gills, skin, fins or surface of the nasal epithelium of agnatha, condrichthyes and osteichthyes. These monogeneans are common parasites of both freshwater and marine fishes. Their presence on stocks at a farm is an indication of poor husbandry. Furthermore, these parasites may be considered as mechanical vectors and a predisposing factor for the infection by some viral, bacterial, mycotic and protozoal pathogens of fish [1].

Monogenetic trematodes (Monogenea) are parasitic flatworms. They vary in size. They hold onto their hosts via a combination of hooks, anchors and suckers at their posterior end (opisthaptor) and use the anterior end

(prohaptor) for feeding and assisting in moving to other locations on the host. They are hermaphroditic and lay eggs or give birth to live young. Their life cycle does not involve more than one species of host [2].

The grouper, Taradi, *Plectropomus areolatus* is an important cultured fish species especially in Kingdom of Saudi Arabia, where there is a considerable interest in its spawning and farming *Plectropomus areolatus*. However, maintenance of a healthy fish population depends on knowledge of the disease causing agent and the implementation of an effective disease control program [3, 4]. Parasitic diseases pose great problem in the breeding program of these fishes. Ectoparasites, especially monogenean infection causes serious damage to the fishes in captivity [1]. Parasitic problems of grouper in Malaysia, Kuwait, Indonesia and India cause much serious problems [5, 6]. Thus the present study aimed to

report the first occurrence of monogenean infections, *B. epinepheli* in Taradi, *Plectropomus areolatus* fish in Jeddah, Saudi Arabia. The prevalence, pathology of the infection and trials to control the parasitic infestation using formalin, praziquantal as chemical treatment and Neem leaf water extract *Azadirachta indica* as medicinal plant.

MATERIALS AND METHODS

Naturally Infested Fish: A total number of 280 broodstock Taradi, *Plectropomus areolatus* fishes (0.5- 2.5 kg weight, 20-30cm, length) were collected from the Red Sea of Jeddah coast, Saudi Arabia in June 2011, maintained in 4 concrete ponds 100 m³ each provided with filtered recycled sea water (Temp. 29-33°C, salinity: 30-32ppt) for culturing broodstock. The fish were examined for clinical signs and post mortem lesions. Fish were examined thoroughly for ectoparasites. Wet mounts of scrapings were taken from the skin, fins and gills, examined under microscope.

Clinical and Postmortem Examination: Fish were observed alive in the pond for any clinical abnormalities. Gills were dissected out and gill filaments were examined using stereo binocular microscope. Gills and all the visceral organs in dead fish were also dissected out and examined for any abnormal lesions and internal parasites Eissa [1].

Parasitological Examination: Scrapings from examined fish were taken from behind of fins, lateral sides of the body on skin and gills placed in Petri dish filled with sea water. Specimens were recovered as a wet mount for primary parasitological examination. Parasites were examined live and details were recorded. A few of the specimens were washed in normal saline and preserved in 70% ethanol, stained with boraxcarmine, dehydrated in graded alcohol series. And finally cleared in clove oil before mounting in Canada balsam according to Luky [7]. The ten of collected (or detected) parasites were measured for the range and the mean±standard deviation (SD). The prevalence of infection was recorded.

Histopathological Examination: Autopsy samples were taken from the skin and gills of naturally infested Taradi fish with *Benedenia epinepheli* and fixed in 10% formalin for twenty four hours. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared

in xylene and embedded in paraffin at 56 degree in hot air oven for twenty four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness using microtome. The obtained tissue sections were collected on glass slides, deparaffinized, stained by hematoxylin & eosin stain for routine examination then examination was done through the light electric microscope Banchroft *et al.* [8].

Statistical Analysis: All data were presented as mean standard error (SE) and significance of differences was estimated using ANOVA test. Morphological measurements of *Benedenia epinepheli* of 10 specimens of parasite were recorded as mean ± standard error (SE) as described by Sendecor [9].

Drugs Used in Treatment: Formalin 40 % formaldehyde: 100 ppm for 30 min [3]. praziquantal (Sigma): 2.5 ppm for 24h. [10].

Neem Leaf Water Extract: *Azadirachta indica* (*A. indica*): green fresh leaves were obtained from the garden of Fisheries Research Center, Jeddah washed with tap water, dried on sun and finely chopped, grounded in blender then amount of 500g was soaked in tap water, (liter of water) for 24 h at room temperature as described by Cruz *et al.* [11]. The mixture was filtered and the extract (500 g/l) was used immediately in the experiments as Neem leaf water extract.

Determination of 96-h LC₅₀ in Taradi for Neem Leaf Water Extract: Static toxicity tests were run to determine lethal concentrations (96-h LC₅₀) of neem leaf water extract to Taradi, fish. Tests were conducted in 50 L glass aquaria, 6 fish per aquarium, containing neem leaf extract diluted in tap water to the following concentrations: 0 (control group), 1, 2, 4, 6, 8, 10, 12, g/l. Each treatment had 3 replicates. All laboratory conditions were maintained constant. Deaths were recorded every 3 h for the 1st day, then every day for other 3 days. The value of 96-h LC₅₀ were estimated [12].

Experimental Design for Treatment Trials: A total number of 150 naturally infested Taradi *Plectropomus areolatus* fish were divided into 5 groups for treatment trial, each was 10 fish, 3 replicates, hold in 20 L³ fiberglass tank for investigation the effect of treatments on parasites affecting the fish. 1st group was subjected for treatment with formalin 40% as a bath 100 ppm for 30 min. [3]. 2nd group was subjected for treatment with praziquantal as a

long bath 2.5 ppm for 24 h. [10]. 3rd group was treated with Neem leaf water extract *Azadirachta indica* as long bath (0.4 ppm for 2 h.) 4th group was subjected to freshwater treatment for 15 min. [3], while 5th group still without treatment as control group. All groups were monitored along the period of treatment for any abnormal signs.

RESULTS

Clinical Signs and Post Mortem: Diseased fishes appear restless, hyperirritable with flashing and rubbing against hard objects. The skin washy peremic with localized multi focal hemorrhages, ulcerations, sloughing, loss of scales and frayed ragged appearing fins. Some affected fishes were dull and inactive, gathering at the water inflow or on the water surface where they gasp for air and suffer from suffocation. In severely affected cases of infestation, fishes were off food, emaciated, dark in body coloration, with very weak reflexes (easily caught). The operculi appear somewhat opened with rapid respiratory movements (Fig. 1). The affected gill filaments are pale or congested, swollen, completely or partially covered with heavy mucous layer with characteristic mosaic appearance. In some cases, grayish coloration of the tips of gill filaments, erosion of the distal parts of gill lamellae and enlargement of the head region were seen.

Parasitological Examination: The prevalence of infestation with *Benedenia epinepheli* in taradi infested fish:

The results showed that, 213 taradi fish was infested with *Benedenia epinepheli* out of total number 280 brood stock fish with percentage of 76% Table 1.

In the present study the occurrence of monogenean parasites belonging to the family capsalidae were recorded in cultured broodstock fishes of grouper. Large number of readily visible, monogenean parasites were found attached to the gill filaments and skin.

Microscopical examination revealed that, large number of readily visible parasites were found attached to the gill filaments and skin of infested fishes. Microscopically, the adult worm of the recovered parasite was dorso-ventrally flattened, elongated in body shape, measuring 3.17-3.95×0.89-2.07 mm in length and width respectively (Table 2), two pairs of eyes were present; anterior pair being smaller than the posterior one. The anterior end possesses one pair of suckers with an average diameter of 0.19 mm. The posterior end was enlarged and armed with disc like opisthaptor with hooks (Fig. 2 & 3). There covered parasite was identified as *B. epinepheli* (Yamaguti 1937).

Histopathological Examination: Parasitic particles of *Benedenia epinepheli* were embedded in between the primary gill filaments, associated with focal hemorrhages and congestion in the blood vessels: (Fig. 4, A&B) Secondary gill lamellae suffered from hyperplasia with sever inflammatory cells infiltrations (Fig. 4,C&D) Necrosis with ulceration and inflammatory cells infiltration were detected all over the epidermal and dermal

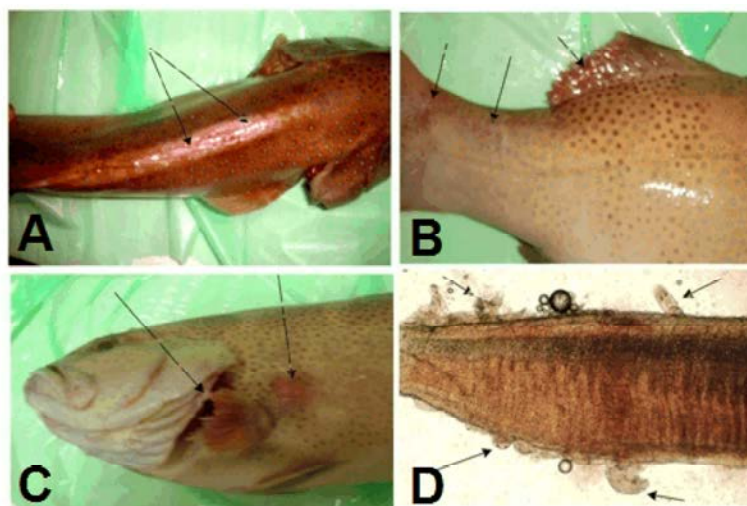


Fig. 1: (A) Taradi, *Plectropomus areolatus* showing hyperemic frayed dorsal fins, (arrows). (B) Taradi, *P. areolatus* showing eroded hyperemic dorsal fins and abrasions in the caudal peduncle region, (arrows). (C) Taradi, *P. areolatus* with semi opened opercula and ulcer on the lateral side, (arrows). (D) wet mount of gill filament infested with large number of *B. epinepheli*.

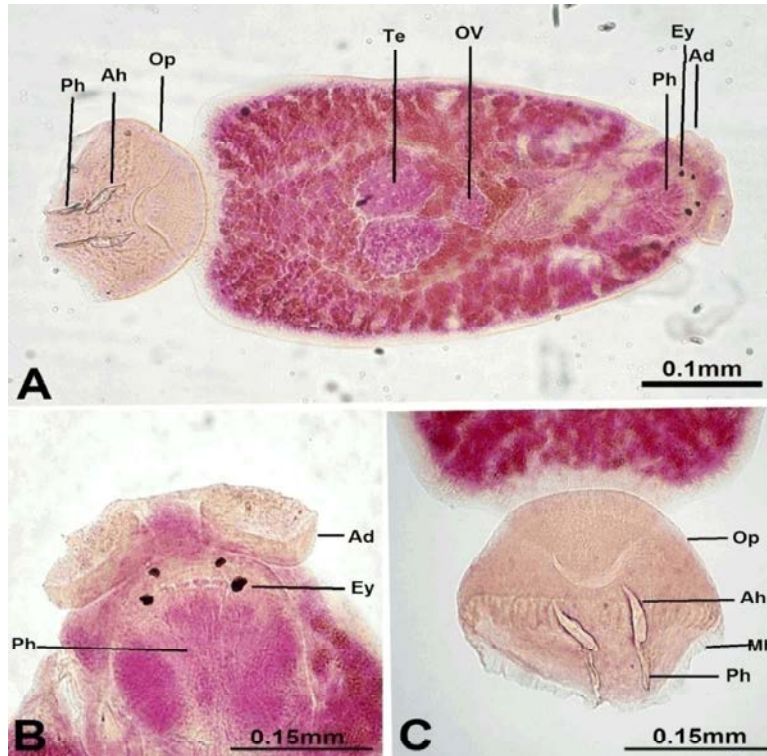


Fig. 2: (A) Whole adult monogenea *Benedenia epinepheli* recovered from fins of taradi, *Plectropomus areolatus* stained with Carmine (Ad) adhesive organs (Ey) eye spots (Ph) Pharynx (Ov) ovary. (Te) testis (Op) Opisthaptor (Ah) anterior hook (Ph) posterior hook (B) Prohaptor, (Ad) adhesive organs, (Ey) Eye spots (Ph) Pharynx (C) Opisthaptor (Ah) Anterior hook (Mh) Marginal hooklets (Ph) Posterior hook



Fig. 3: (A) Wet mount of larva of *Benedenia epinepheli* recovered from skin of Taradi, *Plectropomus areolatus* (B) Adult *Benedenia epinepheli* photographed by phase contrast microscope.

Table 1: Showing prevalence of monogenea is (*Benedenia epinepheli*) in taradi fish

Total No. of examined fishes	No. of infested fishes	Percent of infested fishes %
280	213	76

Table 2: Morphometry of *B. epinepheli*, the monogenean parasite infecting grouper (*Plectropomus areolatus*)

Parameter	Range	* Mean±SD
Body length	3.17-3.95	7.12± 0.356
Body width	0.89-2.07	2.96±0.148
Opisthaptor	0.67-0.89	1.56±0.078
Suckers	0.35-0.45	0.80±0.04
Testes	0.43-0.57	1.00±0.05
Ovary	0.15-0.33	0.48±0.024
Pharynx	0.14-0.31	0.45±0.022

Measurements (mm), n=10 * Mean ± SD

Table 3: Efficiency of treatment trials of grouper, taradi , *Plectropomus areolatus* infested with *B. epinepheli*

Group	No. of fish	Types of Treatment	means of treatment	Dose/ppm	Duration	Treated fish	Curative%
1 st group	30	Formalin40%	bath	100	30 min.	26±1.3*	87
2 nd group	30	praziquental	bath	2.5	24 hr.	30±1.5*	100
3 rd group	30	Neem leaf water extract	bath	0.4	120 min.	11±0.55	37
4 th group	30	freshwater	bath	---	15 min.	30±1.5*	100
5 th group	30	control	bath	---	---	---	---

* Significance

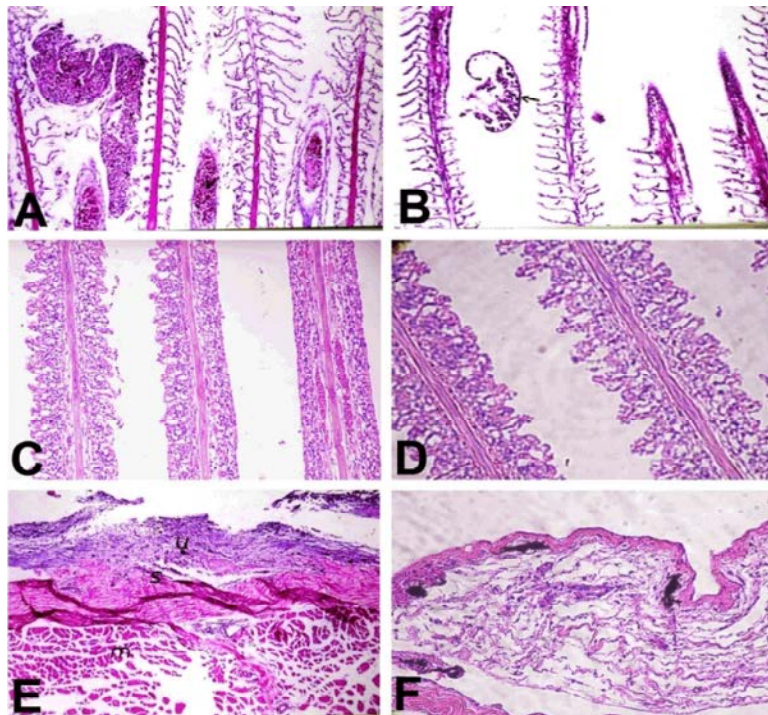


Fig. 4: (A&B)Parasitic particles of *Benedenia epinepheli* were embedded in between the primary gill filaments, associated with focal hemorrhages and congestion in the blood vessels(C&D) Secondary gill lamellae suffered from hyperplasia with sever inflammatory cells infiltrations (E)Necrosis with ulceration and inflammatory cells infiltration were detected all over the epidermal and dermal layers,(F) there is focal aggregations of melanocytes with diffused edematous fluids in between the muscle bundles (H&E)

layers, there is focal aggregations of melanocytes with diffused edematous fluids in between the muscle bundles (Fig. 4, E&F).

Results of 96-h LC₅₀ of Neem Leaf Water Extract in Taradi, *Plectropomus Areolatus*: The 96-h LC₅₀ of neem leaf water extract in Taradi, *Plectropomus areolatus* was determined as 4 ppm the dose which make the half number of the fish died so the used dose for treatment was determined as $\frac{1}{10}$ of LC₅₀.

Trials of Treating Infested Fish with Some Chemotherapeutic Agents: Trials of treatment of infested fish using formalin, praziquantal, neem leaf water extract and freshwater bath revealed that, formalin and praziquantal were found to be efficiently affect *B. epinepheli* dislodging the parasite from skin and gills with curative percents 87% and 100 % respectively with survival percents 62 and 75 respectively while bath treatment in neem water extract resulted in less curative percent (37%) but of higher survival percent (100%). In contrast freshwater bath efficiently dislodge the parasite with high curative percentage 100% and high survival percentage 100% Table 3.

DISCUSSION

Monogeneans are dorsoventrally flat (no body cavity), hooked suction elongated worms having a spindle or cigar shaped. There are many species of monogenetic trematodes that parasitize fish but only some species make frequent and definite injuries. The most economically important groups from the pathological viewpoint are in the suborder Monopisthocotylea especially the families of gyrodactylidae and dactylogyridae that are significant in both cultured freshwater and marine fishes [1].

Monogenea can reproduce and multiply so quickly that they can overpower their hosts. They damage the host through ingestion of mucus, skin and blood so that much of the fish's protective coating is destroyed and then potentially dangerous infections can set in, by injury to gill tissue that leads to fusion and hyperplasia of gill tissues and the subsequent decrease in gill surface area and thus a decrease in efficiency of respiration. Benedeniide monogeneans belonging to the family Capsalidae are pathogenic to marine fish under culture conditions [4, 13].

Regarding the clinical and post mortem signs, the present study showed that naturally infested taradi fish appear restless, hyperirritable with flashing and rubbing against hard objects. The skin becomes hyperemic with localized multi focal hemorrhagic areas, ulcerations sloughing and loss of scale; skin and frayed ragged appearing fins. then become dull and inactive, gathering at the water inflow or on the water surface where they gasp for air and suffocation may be observed. In heavy infested fish, it is off food, emaciated, dark body coloration and the fish reflexes are very weak. The operculi appear somewhat opened with rapid respiratory movements. These signs may be attributed to the pathogenesis of benedenia. The parasites insert the central hooks (hamuli) as well as the marginal hooks of opisthaptor deeply into the skin and gill epithelial tissues. Pronounced proliferation occurs then swelling, fusion and deformation of gill lamellae with collapse of the capillaries occur due to the pressure from the propagating epithelial tissue. This may lead to the formation of long thin processes, prolongation or to the coalescence of adjacent gill filaments and lamellae. Extensive degeneration of gill epithelia and blood vessels may also occur causing mosaic appearance of gill tissue [1, 2]. Skin flukes feed primarily on mucous, epithelial tissues as well as blood. Feeding injures the epithelial tissue of the skin and method of attachment as skin and fins get destroyed. The parasites regularly relocate around the fin margin and frequently cross over the body surface to another fin, the caudal, pectoral and pelvic ones. The results nearly agree with the results obtained by [3, 14, 15].

Concerning parasitological examination, morphological characteristics of recovered trematodes resembled that of the monogenean, *Benedenia* sp. Described by [16] *B. epinepheli* was first reported in 1937 from wild fishes of Japan [17] and [18]. Redescription of *Benedenia epinepheli* (Yamaguti, 1937) Meserve 1938 (Monogenea: Capsalidae) from cultured and aquarium marine fishes of Japan. Since then, the parasite has been reported worldwide from subtropical and tropical areas with low host-specificity infest several species of grouper [5, 6, 13, 14]. However, pathobiological information on grouper and their parasite is fragmentary in India [3]. Monogeneans are generally highly host specific [19] and the wide range of host species with low specificity is quite exceptional in monogenea. Among the members of the genus *Benedenia*, *B. epinepheli* and *B. hawaiiensis* seem to be the only 2 species with a very high ostrange [3].

Regarding the prevalence of infection, the present study revealed that the prevalence reached 76 % confirming the similar results that obtained by Morsy Kareem [4] high prevalence of *B. epinepheli* infection may be due to that monogenea has direct simple life cycle, don't need intermediate host and transmitted quickly by direct contact especially when there are stress factors in the farm [1].

The present study showed that the general morphology of the present *Benedenia* sp. resembles that of *B. epinephali* previously described in Malaysia, Kuwait, Indonesia and India respectively from grouper [5, 6]. Jithendran *et al.* [3] recovered *B. epinephali* from Captive Broodstock of Grouper, *Epinephelus tauvina* in India. The body dimensions of the parasite are more or less similar to that recorded in previous studies.

High intensities of monogeneans resulting in secondary infections [20]. Conditions of overcrowding and adverse ambient conditions promote epizootic diseases. Further, the population of flukes is often much greater in confined fishes and hence maintenance of fish health in captivity is a major concern. Under suitable environmental conditions the monogeneans multiply very fast as the organisms are hermaphrodite and life cycle is simple and direct without intermediate host and can spread to new host by direct contact from one host to another [2].

The fish diagnostician should attempt to identify the species of monogenean flukes; firstly, some fluke species are not susceptible to the usual therapeutic procedures and hence attempts to treat them with a useless chemical may be time consuming, expensive and of little help to the fishes; secondly, to have a pathogen inventory in a locality and also knowledge of the environment of each species (temperature, pH, salinity and other physical factors) may assist in controlling these organisms [3, 21].

Concerning the histopathological alterations of naturally infested Taradi, present study revealed that there is parasitic particles of *Benedenia epinepheli* were embedded in between the primary gill filaments, associated with focal hemorrhages and congestion in the blood vessels. Secondary gill lamellae suffered from hyperplasia with sever inflammatory cells infiltrations. Necrosis with ulceration and inflammatory cells infiltration were detected all over the epidermal and dermal layers, there is focal aggregations of melanocytes with diffused edematous fluids in between the muscle bundles, these results nearly agree with the results obtained by Osman [15].

Present study revealed that treatment of monogenean infested fish by short bath treatment with 100 ppm formalin for 60 min. long bath with 2.5 ppm praziquental for 24 h. freshwater bath for 15min. found to be effective in dislodging the parasite from the gills and skin the results were nearly with agreement of the results obtained by Jithendran *et al.* and Sharp *et al.* [3, 10]. The efficacy of neem leaf water extract bath treatment was the lowest compared to formalin, praziquental and freshwater bath treatments [22 and 23] reported that, One of the most promising natural compounds is Azadirachtin (AZA), an active compound extracted from the neem tree (*Azadirachta indica*), whose antiviral, antibacterial and antifungal properties have been known for several years. Biswas *et al.* [24] added that, the chemistry and biological activity of both neem extracts and purified AZA have been investigated in various countries.

Freshwater bath treatment was found to be more effective and safer on infested grouper, while formalin and praziquental were found to be more effective but more dangerous on treated fish. Formalin decreases dissolved oxygen in water displaying respiratory stress in fish especially when the temperature and humidity are high at Saudia Arabia. Also, praziquental found to be toxic to both the parasite and fish causing paralysis of infecting parasite especially at the region of attachment (opisthaptor). Osman [15] A detailed investigation about treatment using other chemicals and medicinal plants of taradi, fish infected with *B. epinepheli* must be carried out.

From present study it was concluded that monogenean parasite *B. epinepheli* was recovered from grouper, Taradi, *Plectropomus areolatus* for the first time, in Saudia Arabia. The morphological measurements of *B. epinepheli* parasite recovered from Taradi fish nearly similar to that of the previous studies. Present study revealed that the treatment of choice for *B. epinepheli* in taradi was freshwater bath followed by praziquental bath and formalin but freshwater bath is more safe on fish than the other drugs.

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