

Experimental Infection of Tenacibaculosis and a Trial for Treatment by Plant Extract Carvacrol in Surge Wrasses Fish (*Thalassoma Purpureum*)

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Abstract: The experimental infection of surge wrasses fish by *Tenacibaculum maritimum* was successfully conducted through immersion bath for 18hrs in 1.5×10^6 suspension, the infected fish exhibited skin ulcers, stomatitis, tail rot, signs of respiratory distress as gasping and accumulation at air source site in association with 60% mortality. Carvacrol is a major compound of oregano and thyme and has antimicrobial activity against wide range of microorganisms. The *in vitro* susceptibility assay proved strong effect of carvacrol on *T. maritimum*. 100ppm of both carvacrol and its precursor cymene for 14 days as food additives controlled the tenacibaculosis in surge wrasses fish and no clinical signs or mortality could be recorded in the treated fish. 50ppm of them prevented the disease clinical signs and reduced mortality to 10%.

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Key words: Tenacibaculosis, *T. maritimum*, treatment, Surge wrasses fish, carvacrol, cymene.

1. Introduction

Surge wrasses fish (*Thalassoma purpureum*) is a valuable coral reef living fish in the red sea and used as food and ornamental fish. Marine Tenacibaculosis is a serious bacterial disease affecting a great variety of marine fish especially cultured species (Toranzo *et al.*, 2005). It caused massive mortalities and severe economic losses in marine fish cultures worldwide including Japan, Scotland, Spain, France and North America (Wakabayashi *et al.*, 1986; Bernardet *et al.*, 1990; Alsina and Blanch 1993; Bernardet *et al.*, 1994; Ostland *et al.*, 1999). It is caused by *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*) and this pathogen directly and primarily attacks skin, mouth, fins and tail of fish, causing severe necrotic and ulcerative lesions on the body surface (Toranzo *et al.*, 2005).

Tenacibaculosis has been reported among wide range of marine fish species including sole, *Solea solea* (L.) (Bernardet *et al.*, 1990); Senegalese sole (*Solea senegalensis*) (Cepeda and Santos, 2002); Japanese flounder (*Paralichthys olivaceus*) (Baxa *et al.*, 1986); turbot (*Psetta maxima*) (Avendaño-Herrera *et al.*, 2004a). Also, it is recorded in Atlantic salmon (*Salmo salar* L.), Rainbow trout (*Oncorhynchus mykiss*), Striped trumpeter (*Latris lineata*), Greenback flounder (*Rhombosolea tapirina*) (Soltani *et al.*, 1996; Handlinger *et al.*, 1997), sea bream and sea bass (Toranzo *et al.*, 2005) and Picasso tiger fish (*rhinecanthus assasi*) and Black damselfish (*Neoglyphidodon nesiotes*) (Abd El-Galil and Hashiem, 2012).

Up to now, most treatments proposed for the tenacibaculosis outbreaks are based on the

administration of antibiotics through feed. Oxytetracycline, amoxycillin, trimethoprim and enrofloxacin are an effective antimicrobial therapy against *T. maritimum*, (Soltani *et al.*, 1995; Avendaño-Herrera *et al.*, 2008). The uses of antibiotics in aquaculture may introduce potential hazards to public health and to the environment by the emergence of drug-resistant *T. maritimum* within the population and further diminished the effect of chemotherapy (Tsoumas *et al.*, 1989; Avendaño-Herrera *et al.*, 2008). Furthermore, the normal microbial flora in the digestive tract, which is beneficial to fish are also killed or inhibited by oral antibiotic chemotherapy (Gerald and Jane, 1966; Sugita *et al.*, 1990).

Plant extract carvacrol had strong antimicrobial activities against both gram-positive and gram-negative bacteria and was generally recognized as safe by the FDA (Davidson and Saxton, 2011). Carvacrol had antimicrobial activity against wide range of fish pathogenic bacteria such as *Bacillus cereus* (Ultee *et al.*, 2000) *Salmonella typhimurium* (Kim *et al.*, 2006 and Tohamy, 2006) *Staphylococcus* and *E. coli* (Gholam and Mohammad, 2007) *Streptococcus* (Botelho *et al.*, 2007), *A. hydrophila* (Zheng *et al.*, 2009) and *E. tarda* (Rattanachaikunsopon and Phumkhachorn, 2010). Carvacrol, but not cymene was able to inhibit many bacterial strains and the synergistic effect between carvacrol and cymene against drug resistant bacterial strains was reported Ultee *et al.* (2000) and Rattanachaikunsopon and Phumkhachorn (2010).

This study was designed to investigate the susceptibility of Surge wrasses fish to tenacibaculosis

and the efficacy of the plant extract carvacrol in combination with its precursor cymene to control the disease.

2. Materials and Methods

Fish

One hundreds of apparently healthy Surge wrasses fish were collected from the red sea coral reef and transported alive to the indoor aquaria of The National Institute of Oceanography and Fisheries (NIOF) at Hurgada, the fish were acclimated and accustomed to the commercial fish ration containing 25% protein (ZooControl Company, Ismailia desert road, Egypt) for two weeks in indoor aquaria and used in this experiments.

Tenacibaculum maritimum inoculum

T. maritimum strain was obtained from previous work (Abd El-Galil and Hashiem, 2012) and cultivated on plates of Flexibacter maritimus medium (FMM) (Pazos *et al.*, 1996), Pure colonies of the *T. maritimum* isolates were picked up and the strain was passed in small group of bird wrasses fish for reactivation, and reisolated and identified again then used for other studies.

Experimental infection

Twenty of the acclimated Surge wrasses fish were subdivided into 2 equal groups each of 10 fish and each group was reared in a separate aquarium. The fish of the first group were experimentally infected by *T. maritimum* suspension containing 1.5×10^6 cell mL^{-1} in a bath immersion for 18 hrs (Avendaño-Herrera *et al.*, 2006a). The second group was submitted to the same procedure without bacteria and used as control. Each fish group was preserved separately at water temperature $24 \pm 2^\circ\text{C}$ and observed for 14days, the clinical signs and numbers of dead fish were recorded.

Determination of median lethal dose (LD₅₀)

Fifty of the acclimated Surge wrasses fish were subdivided into five groups each of 10 fish and overnight cultures of *T. maritimum* were adjusted to densities 1.5×10^6 , 1.5×10^5 , 1.5×10^4 and 1.5×10^3 . The 1st, 2nd, 3rd and 4th fish groups were subjected to 18hrs immersion bath in the previous dilution respectively and the 5th group was used as control. The five fish groups were closely observed for 2 weeks. Mortalities and clinical signs were recorded daily and the internal organs (Livers and kidneys) were aseptically streaked on FMM for *T. maritimum* reisolation.

Table (1): Determination of median lethal dose (LD₅₀) of *T. maritimum*

Fish groups	No. of fish	Dose/fish	Route of injection
Group 1	10	1.5×10^6	Immersion bath for 18hrs
Group 2	10	1.5×10^5	
Group 3	10	1.5×10^4	
Group 4	10	1.5×10^3	
Group 4 (control)	10	-----	

Susceptibility assay

Sensitivity of *T. maritimum* to combination of carvacrol and cymene was evaluated by agar diffusion susceptibility test on FMM plates, which was prepared using seawater as diluents (Pazos *et al.*, 1996). 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10ppm of both carvacrol and cymene (mixed solution) were tested. Bacterial suspension turbidity was prepared, equalized and matched with the MacFarland 0.5 standard, this suspension then spread over a FMM plates and left for minutes to dry then by sterile glass pipette four wells (3mm Ø) were made in each plate. 100µL of a single mixed solution dilution was added to a single well and each plate had control well (had sterile saline). The plates were incubated at 25°C for 72hrs and the diameter of inhibition zones (area at which no growth was visible) were read at right angles by measuring to the nearest millimeter.

Medicated fish diets

The fish diets were prepared according to attanachaikunsopon and Phumkhachorn (2010).

Diet-1: The commercial fish ration was supplemented with 100 ppm of both carvacrol and cymene.

Diet-2: The commercial fish ration was supplemented with 50ppm of both carvacrol and cymene.

Diet-3: The commercial fish ration was used as control diet and prepared by the same process without carvacrol and cymene additives.

Treatment trial

Thirty acclimated Surge wrasses fish were subdivided into 3 equal groups each of 10 fish and each group was reared in a separate aquarium. The three groups were fasted for 24hrs and experimentally infected with *T. maritimum* suspension containing 1.5×10^5 cell mL^{-1} in a bath immersion for 18 hrs (Avendaño-Herrera, *et al.*, 2006a). 12hrs later, the fish of 1st group were fed on diet-1, the 2nd group were fed on diet-2 and the 3rd group were fed on diet-3 (control). Each fish group was preserved in glass aquarium at water temperature $24 \pm 2^\circ\text{C}$, fed at feeding rate 3% of its body weight daily. During the observation period

(14days), the clinical signs and numbers of dead fish were recorded.

3. Results

Experimental infection

The experimentally infected Surge wrasses fish showed lesions similar to those of naturally infected fish such as off food, lethargy, skin hemorrhagic ulcers (Photos 1&2), tail rot (Photo -3), ulcerated mouth

(stomatitis) (Photo -4), in addition to respiratory distress as gasping and accumulation at the air source. By the end of observation time (14 days) the mortality of the experimentally infected fish reached 60% comparing to zero % mortality in the control group. *T. maritimum* could be reisolated from the experimentally infected fish.

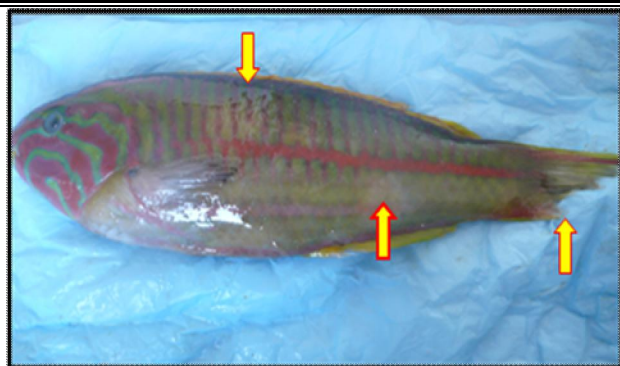


Photo-(1): Surge wrasses fish showed skin ulcers and tail rot

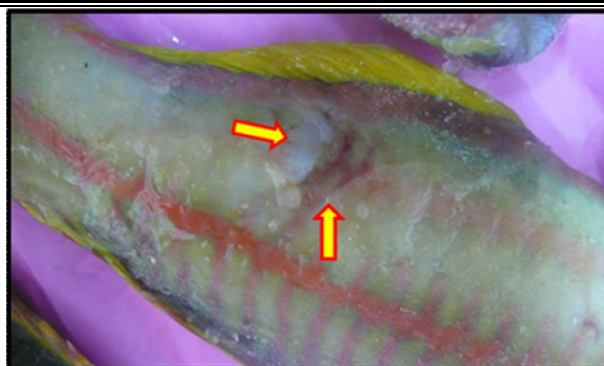


Photo-(2): Surge wrasses fish showed skin ulcer

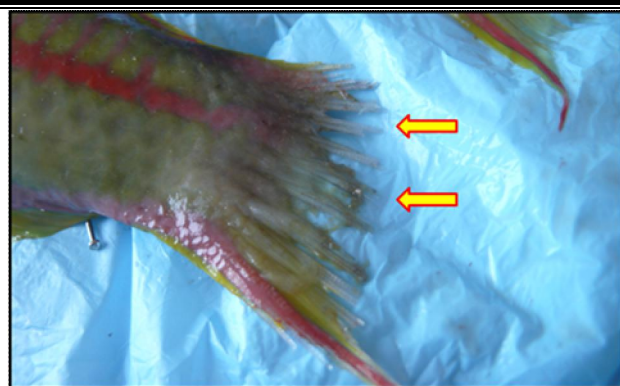


Photo (3): Surge wrasses fish showed tail rot

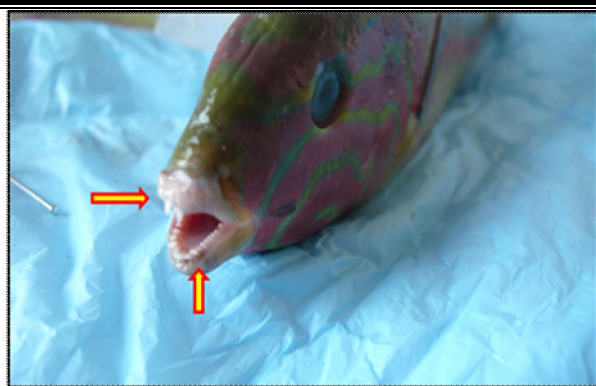


Photo (4): Surge wrasses fish showed mouth ulceration (stomatitis)

Median lethal dose (LD₅₀)

The mortality of the experimentally infected Surge wrasses fish was reported for 2 weeks after immersion bath infection with different concentrations of *T.*

maritimum. The fish death occurred during the 1st week of the experiment. The LD₅₀ of *T. maritimum* for Surge wrasses fish was 1.5×10^5 CFU/mL⁻¹. (Table 2).

Table (2): Showed the median lethal dose (LD₅₀) of *T. maritimum* of Surge wrasses fish

Fish group (10 fish for each)	Bath conc.ml ⁻¹	No. of dead fish / day										Total number of dead Fish	Mortality rate %
		1	2	3	4	5	6	7	8	9	10 to 14		
Group 1	1.5×10^6	2	2	1	-	-	1	-	-	-	-	6	60
Group 2	1.5×10^5	2	1	1	-	1	-	-	-	-	-	5	50
Group 3	1.5×10^4	1	1	-	-	-	1	-	-	-	-	3	30
Group 3	1.5×10^3	1	1	-	-	-	-	-	-	-	-	2	20
Control group	No	-	-	-	-	-	-	-	-	-	-	-	-

Susceptibility assay

The results of the agar diffusion test obtained for different carvacrol and cymene concentrations against *T. maritimum* strain demonstrated that there were some variations in the zones size produced by different concentration. Among the tenth concentrations evaluated the largest inhibition zones diameter were detected for the 10, 9, 8, 7, 6 and 5 ppm and they were 31, 30, 29, 29, 28 and 28 mm respectively and the other concentrations (4, 3, 2 and 1 ppm) gave narrower inhibition zones (20, 13, 7, 4 mm, respectively) comparing with 0 mm inhibition zone around the control well (Table - 3).

Table (3): The susceptibility of *T. maritimum* to different concentrations of carvacrol and cymene combination

Carvacrol and cymene concentration (ppm)	Inhibition zone (mm)
0 (control)	0
1	4
2	7
3	13
4	20
5	28
6	28
7	29
8	29
9	30
10	31

Treatment trial

No clinical signs and mortality could be reported among the fish of the 1st group and the fish were active with good appetite and appearance. No clinical signs could be observed on the fish of the 2nd group but 10% mortality was recorded between them. On the other hand, off food, lethargy, hemorrhagic ulcers on the skin, ulcerated mouth, tail rot and 50% mortality were reported among the fish of the 3rd group (control).

4. Discussion

This study reported the susceptibility of Surge wrasses fish to experimental infection with the marine pathogen *T. maritimum* which was isolated in previous study from the Picasso tiger fish (*Rhinecanthus assasi*) and Black damselfish (*Neoglyphidodon nigrifrons*) of red sea at Hurghada, Egypt (Abd El-Galil and Hasheim, 2012). The infected fish showed the classical clinical signs of tenacibaculosis such as off food, lethargic, skin hemorrhagic ulcers, ulcerated mouth (stomatitis) and tail rot, in addition to respiratory distress in the form gasping and accumulation at the air source site and these signs were associated with 60% comparing with no clinical signs and 0 % mortality in the control

group. Similar lesions were noticed by Baxa *et al.* (1986); Santos *et al.* (1999); Suzuki *et al.* (2001); Toranzo *et al.* (2005); López, *et al.* (2009); Abd El-Galil and Hashiem (2012) in many different marine fish species.

The susceptibility of *T. maritimum* to combination of the plant extracts carvacrol and its precursor cymene was investigated in the laboratory by using agar diffusion test. The most effective concentrations were 10, 9, 8, 7, 6 and 5 ppm which reported the largest inhibition zone diameter documenting the antibacterial effects of carvacrol and cymene mixture solution on the *T. maritimum* and these results were confirmed by Davidson and Saxton (2011) who stated that carvacrol had strong antimicrobial activities against gram positive and gram-negative bacteria, Zheng *et al.* (2009) who pointed out the susceptibility of *A. hydrophila* in channel catfish to the plant extract carvacrol and Rattanachaikunsopon and Phumkhachorn (2010) who reported the susceptibility *E. tarda* in *O. niloticus* to carvacrol.

The treatment trial of tenacibaculosis in Surge wrasses fish was achieved by using the plant extracts carvacrol in combination with its precursor cymene as fish food additives for 14 days (Rattanachaikunsopon and Phumkhachorn, 2010). Equal amounts (100ppm) of both carvacrol and cymene completely controlled the disease where no clinical signs or mortality could be reported between the treated fish, in addition to, the treated fish were alert (react well to the stimuli) and had good appetite and appearance. 50ppm of them reduced the mortality to 10% and no clinical signs in the treated fish could be noticed. On the other hand, typical clinical signs and 50% mortality were detected in the control infected fish group. These findings cleared out the efficacy of carvacrol and its precursor cymene as tenacibaculosis treatment and these finding were confirmed by Ultee *et al.* (1998); Zheng *et al.* (2009; Rattanachaikunsopon and Phumkhachorn (2010); who reported antimicrobial activity of carvacrol against *A. hydrophila*, *B. citreus* and *E. tarda* and the synergistic action between carvacrol and cymene.

In conclusion, the marine surge wrasses fish is susceptible to tenacibaculosis. The carvacrol in combination with its precursor cymene is effective treatment for tenacibaculosis in surge wrasses fish at 100ppm of each for 14 days as food additives. Further studies are required for the treatment duration and dose reduction.

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