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Efficacy of Benzalkonium Chloride as an Antibacterial and Immunostimulant in *Macrobrachium rosenbergii* (de Man)

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Abstract

During the last decade many improvements have taken place in aquaculture, especially in prawn and shrimp farming. The shift from extensive to intensive and semi-intensive farming has brought about an increase in disease outbreaks, especially by bacteria. To control these diseases, antibiotics have been used indiscriminately. To avoid the use of antibiotics and the development of resistant strains of bacteria, we studied the efficacy of benzalkonium chloride (BKC), a quaternary ammonium compound as an antibacterial and immunostimulant in *Macrobrachium rosenbergii* (de Man). Efficacy was evaluated *in vitro* by minimum lethal concentration (MLC) and found to be 1.0 ppm for *P. fluorescens* and 1.5 ppm for *E. tarda*, *V. alginolyticus*, *S. aureus* and *A. salmonicida*. The 24 hr LD_{50} of BKC for two month old *M. rosenbergii* was 10.0 ppm. Its immunostimulant effect was evaluated by challenging BKC treated *M rosenbergii* (1.5 ppm BKC bath for 15 days) with *P. fluorescens* and *V. alginolyticus*. The NBT (nitroblue tetrazolium) assay showed that BKC treatment stimulated nonspecific immune response by the activation of granular cells. It also protected *M. rosenbergii* from disease when compared to untreated controls. A single treatment of BKC was effective for *P. fluorescens* for 49 hours and 46 hours for *V. alginolyticus*. Bath treatment at 2.0 ppm for one hour showed successful control of bacterial infection in *M. rosenbergii* previously infected with the bacteria. The results showed that BKC could be used as an effective antibacterial and immunostimulant in *M. rosenbergii*.

Introduction

The ever increasing demand for prawns in the international and domestic markets has stirred tremendous interest among aquafarmers, entrepreneurs, and industrialists to experiment with new culture methods. The adoption of an intensive aquaculture system in an unplanned and unscientific manner has caused additional stress on shrimp and prawns. The indiscriminate use of antibiotic for controlling bacterial disease has led to the development of an antibiotic resistance that is transferable to nonresistant bacteria by R-plasmids (Aoki and Kitao 1985). Quaternary

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ammonium compounds like BKC are used as antibacterials for controlling bacterial diseases in aquaculture (Anderson and Conroy 1969). BKC is a nonspecific antibacterial chemical used for the treatment of columnaris disease in juvenile freshwater fish and bacterial gill disease in juvenile salmonids at 1 to 2 mgl^{-1} bath for one hour (Anderson and Conroy 1969; Amend 1970; Bullock and Conroy 1971). Kongkaew (1988) recommended BKC at 1.0 to 1.25 ppm for the treatment of vibriosis. BKC and hyamine benzethorium chloride have been used for the treatment of bacterial black spot and red gill disease of juvenile and adult shrimps (Baticados et al. 1990). Jeney and Anderson (1993) developed a method to enhance the immune response in rainbow trout for protection against *Aeromonas salmonicida* by using a bacterin after prior immersion for 30 min in an immunostimulant solution containing levamisole, quaternary ammonium compound and ISK, a short chain polypeptide of fish extract. This elevated both the specific and nonspecific immune responses.

In our study BKC (Lysotan a 10% preparation of BKC, manufactured by Schuke and Mayr GmbH, Germany and kindly provided by M/s Agrivet Farm Care, Mumbai) was tested for its efficacy in controlling bacterial infections and in stimulating immunity in *M. rosenbergii*. It is a quaternary ammonium compound comprising a mixture of alkyl dimethyl benzyl ammonium chlorides. It is a white or yellowish white amorphous powder, aromatic in odor, and easily soluble in water.

Materials and Method

Bacterial species used

The bacteria utilized to induce experimental infection for minimum lethal concentration (MLC) and for challenge studies were *Edwardsiella tarda, Pseudomonas fluorescens, Vibrio alginolyticus, Aeromonas salmonicida,* and *Staphylococcus aureus*. These cultures were obtained from the Fish Health Management Lab., C.I.F.E., Mumbai, India and maintained in BHI slants (Hi Media Laboratories Pvt. Limited, Mumbai - 400 086, India) subcultured every fifteen days and stored at 4ºC. The tests employed for the identification of the bacteria followed the method of Kreig (1991). Composition of BHI agar is as follows:

Ingredients	gm·100 ml
Calf brain, Infusion from	20.00
Beef heart, Infusion from	25.00
Proteose peptone	01.00
Sodium Chloride	00.50
Disodium phosphate	00.25
Dextrose	00.20
Agar	02.00
Final pH (at 25 °C)	7.4 ± 0.2

Minimum lethal concentration (MLC)

MLC was tested following the procedure of Alder (1970). BKC was mixed aseptically in sterile distilled water or in unchlorinated bore well water such that the final concentration of active ingredients ranged from 0.01% to 0.0001%. BKC was mixed thoroughly and 25 ml/ml of 18 hour broth culture of bacteria (count 3.2 x 10^{11}) was added. The flasks were incubated at 33°C for 48 hours. Appropriate controls were included in the experiment. If the BKC was effective at a particular concentration, it did not allow the multiplication of bacteria and the solution was clear. The solution became turbid in the presence of bacterial growth. Controls consisted of sterile distilled water and unchlorinated bore well water but without BKC. To see whether the effect was bacteriostatic or bacteriocidal, 1 ml of mixture from the flask was transferred to a BHI agar plate and incubated at 33°C for 48 hours. Presence or absence of growth was recorded. The least concentration (highest dilution) at which no bacterial growth was observed was considered as MLC of BKC for that particular bacterium.

Lethal dose 50 (LD_{50})

LD_{50} of BKC was performed as per the procedure of Abel (1989). *M. rosenbergii* (20 at two months old) were reared in 25 l of water kept in a plastic tub of 0.50 meter diameter (50 liters total capacity). All experiments were conducted in triplicate. The required dose of BKC was initially diluted in a liter of water and then added to the water in the tubs with the prawns. The different doses experimented were 0.25 ppm, 0.75 ppm, 1.0 ppm, 1.5 ppm, and 2.0 to 10.0 ppm in increments of 1.0 ppm. The prawns were observed for mortality over 96 hours. The concentration at which 50% of the experimental prawns died was considered as LD_{50}.

Period of activity

To check the period of activity, a series of experiments were conducted using nutrient broth. Media (300 ml) was sterilized in 500-ml conical flasks. To the sterile media, BKC was added at either 1.0 ppm or 2.0 ppm. Hourly samples of 3 ml each were then taken in duplicate and transferred to sterile test tubes to which a loopful of bacterial cultures (*P. fluorescens and V. alginolyticus*) was added. These were incubated at 33°C for 24 hours before samples were streaked on nutrient agar plates, incubated at 33°C for 24 hours to record presence or absence of growth.

Nitroblue tetrazolium (NBT) assay

To determine cellular activity, NBT assay was performed by placing a single drop of hemolymph (0.1 ml) on each of two glass cover slips. The cover slips were incubated for 30 minutes at room temperature (22°C) on damp paper towel before being gently washed with phosphate buffered saline (PBS), pH 7.4

and the edges touched to blotting paper to drain excess solution. A drop of 0.1 ml of 0.2% NBT in PBS was taken and placed on each of two microscope slides. Cover slips were placed on top so that adherent cells could be incubated for another 30 minutes at room temperature with the NBT solution. The activated cells contained bluish granules when treated with NBT dye while nonactivated cells did not contain these bluish granules. The activated granular cells with bluish granules were counted under a microscope at 400 x. Granular cells were mostly spherical and contained large, highly refractive granules which were promptly activated when the cells were exposed to nonself materials.

Challenge studies

Challenge studies were performed to assess the immunostimulant effect and protection level of BKC against disease causing bacteria. Twenty five prawns were treated with 1.5 ppm of BKC for 15 days (i.e. BKC was added to the water @ 1.5 ppm at the start and no addditional BKC was added on subsequent days). Then the prawns were transferred to water free BKC and challenged by immersion with 1 x 10^4 organisms ml^{-1} with either *P. fluorescens* or *V. alginolyticus*

Controls were not treated with BKC before a similar challenge with the bacteria. The prawns were subsequently observed for gross signs of disease and mortality.

Statistical analysis

The mean and standard error of the mean (SEM) were calculated for NBT assay. Validity was determined by the Student's t test at 5% level of significance.

Results and Discussion

The MLC of BKC for the different bacteria tested were found to be 1-1.5 ppm in sterile distilled water and 1.5 ppm in unchlorinated bore well water (Table 1). *P. fluorescens* was highly sensitive to BKC. Gump (1979) has reported that bacteriostatic activity of BKC against *P. aeuroginosa, V. cholera, S. aureus* was 0.06 ppm, 0.51 ppm and 0.80 ppm respectively.

Table 1. Minimum lethal concentration of BKC for different bacteria.

Serial no	Bacterial species	MLC	
		in water (ppm)	unchlorinated bore well water (ppm)
1.	*P. fluorescens*	1.0	1.5
2.	*E. tarda*	1.5	1.5
3.	*V. alginolyticus*	1.5	1.5
4.	*A.salmonicida*	1.5	1.5
5.	*S. aureus*	1.5	1.5

The LD_{50} of BKC for *M. rosenbergii* (PL_{20}) was 3.0 ppm in 96 hours and 10 ppm in 24 hours. Liao and Guo (1990) reported that the tolerance of *M. rosenbergii* to BKC was 2.0 ppm in 24 hours.

The period of activity of BKC was tested for *P. fluorescens* (Table 2) and *V. alginolyticus* (Table 3) in nutrient broth. It was found to be 48 hours and 46 hours at 1.0 ppm and 53 and 49 hours at 2 .0 ppm, respectively.

Table 2. Period of activity of BKC on *P. fluorescens* in nutrient broth.

Serial no.	Time in hours	Growth	
		1 ppm	2 ppm
1.	Before adding BKC	+	+
2.	Immediately after adding BKC	+	+
3.	1 hour	-	-
4.	6 hour	-	-
5.	12 hours	-	-
6.	18 hours	-	-
7.	24 hours	-	-
8.	30 hours	-	-
9.	36 hours	-	-
10.	42 hours	-	-
11.	48 hours	-	-
12.	49 hours	-	-
13.	50 hours	+	+

Table 3. Period of activity of BKC on *V. alginolyticus* in nutrient broth.

Serial no.	Time in hours	Growth	
		1 ppm	2 ppm
1.	Before adding BKC		
2.	Immediately after adding BKC	+	+
3.	1 hour	-	-
4.	6 hours	-	-
5.	12 hours	-	-
6.	18 hours	-	-
7.	24 hours	-	-
8.	30 hours	-	-
9.	36 hours	-	-
10.	42 hours	-	-
11.	48 hours	+	+
12.	49 hours	+	+
13.	50 hours	+	+

Table 4. Number of activated granulocytes (± SEM) from *M. rosenbergii* challenged with bacteria in combination with BKC.

Group	Activated cells/field
Negative control	2.01 ± 1.01
Positive control	6.05 ± 1.03
BKC and bacteria	12.04 ± 2.45

The positive control (bacteria only) and BKC (1.5 ppm) treated groups were significantly different from the negative control group ($P < 0.05$). The BKC treated group was significantly different from the positive control group ($P < 0.05$).

The NBT assay (Table 4) showed that untreated *M. rosenbergii* (negative control) had a baseline of 2.01 ± 1.01 activated granular cells/field while prawns challenged without immunostimulant had 6.05 ± 1.03. Prawns treated with 1.5 ppm BKC and then challenged showed 12.04 ± 2.45 activated cells/field. The activated cells contain bluish granules when treated with NBT dye while nonactivated cells do not contain these bluish granules. Certain cells like neutrophils ingest and reduce nitroblue tetrazolium (NBT) dye. The yellow dye is taken up by the activated cells and reduced to blue derivative. The blue particles containing cells are counted directly under a microscope. The dye is only reduced in activated cells, therefore the number of cells reducing the dye gives an idea of the proportion of activated cells *in vivo* (Hudson and Hay 1989). The NBT assay was tried to assess the activation of cells, for the first time in crustaceans.

M. rosenbergii treated with BKC and challenged with pathogenic bacteria showed no gross signs of disease and there was no mortality. By contrast, prawns not treated with BKC and challenged with pathogenic bacteria showed signs of disease. Prawns infected with *P. fluorescens* showed dark hepatopancreas and sluggish movement. Prawns infected with *V. alginolyticus* showed degeneration of tissues of the telson. Anderson and Jeney (1992) reported that bathing rainbow trout for 30 minutes in immunostimulant solution before a 2-minute bath in *A. salmonicida* antigen bacteria elevated both the specific and nonspecific defense mechanisms.

Bath treatment of prawns challenged with *P. fluorescens* and *V. alginolyticus* with 2.0 ppm BKC for one hour was found to be successful and the prawns were clinically healthy for 7-10 days thereafter.

Each group of prawns treated with BKC followed by a challenge with P. *fluorescens* and *V. alginolyticus* showed increased nonspecific defenses when compared to prawns given the bacterin alone. The mode of action of BKC in increasing the protection was not known.

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References

Abel, P.D. 1989. Water Pollution Biology: The toxicity of pollutants to Aquatic Organisms. Ellis Horwood, New York. pp 120-125

Alder 1970. Disinfection and Antiseptics. In Microbiological Methods. (C. H.Collins and P. M. Lyne, eds). University Park Press, Baltimore.

Amend, D.F. 1970. Myxobacterial infections of Salmonids: Prevention and treatment. In A Symposium on Diseases of Fishes and Shell fishes (S.F. Snieszko, ed). American Fisheries Society, Washington D.C., U.S.A.

Anderson, J.I.W. and Conroy, D.A. 1969. The pathogenic myxobacteria with special reference to fish disease. Journal of Applied Bacteriology, 32 (30):9.

Anderson, D.P., Siwicki,A.K., Dixon, O.W. and Lizzio ,E.F. 1989. Immunostimulation by Levamisole in rainbrow trout (*Salmo gairdneri*) *in vivo.* In Viruses of lower vertebrate (W. Ahne and E. Kurstak, eds.) pp 469-478, Springer- Verlag, Germany.

Anderson D.P. and Jeney, G. 1992 Immunostimulants added to injected *Aeromonas salmonicida* bacteria enhance the defense mechanism and protection in rainbow trout (*Oncorhynchus mykiss*). Veterinary, Immunology and Immunopathology. 34 (3-4): 379-389.

Aoki, T. and Kitao, I. 1985. Detection of transferable R-Plasmids in strains of the fish pathogenic bacterium, *Pasteurella piscida*. Journal of Fish Diseases. **8**(4): 345-350.

Baticados, M.C.L., Cruz-Lacierda, E.R., Cruzdela, M.C., Dyremdez Fernandes, D., Gawtan, R.C., Lawilla, R.Q., Pilogo, R.C., and Lio- Po, G.D. 1990. Diseases of Penaeid shrimps in the Phillipines. Aquaculture Extension Manual No. 16 Aquaculture Department, South East Asian Fisheries Development Centre (SEAFDEC), Iloilo, Philippines.

Bullock, G.L. and Conroy, D.A. 1971. Bacterial gill disease. In :Bacterial Diseases of Fishes (S.F.Snieszko & H.R.Axelrodm, eds.), pp 77-87. TFH Publications Inc, Jersey City.

Epple, A. 1967. A staining sequence for A, B and D cells of pancreatic islets. Staining Technology 35: 139-152.

Gump, W. 1979. Disinfectants and Antiseptics. In: Encyclopedia of Chemical Technology (Kirk-Othmer, ed) Wiley-Interscience, New York, 7 :815-818 pp.

Hudson, L. and Hay, F. C.1989. Practical Immunology, Blackwell Scientific Publication, London pp 188-189, 449

Jeney, G.L. and Anderson, D.P. 1993b). An *in vitro* technique for surveying immunostimulants in fish, Aquaculture, 112(2-3): 283-287.

Kongkaew, H. 1988. Intensive tiger shrimp culture. Thai Fish Gazzette, 41:381-385.

Kreig, N.R. 1991. Bergey's Manual of Systematic Bacteriology vol. 1 Williams and Wilkins, USA.

Liao, I.C. and Guo, J.J, 1990. Studies on the tolerance of postlarvae of *Penaeus monodon, P. japonicus, P. semisulcatus, P. penicillatus, Metapenaeus ensis* and *Macrobrachium rosenbergii* to benzalkonium chloride, benzethorium, Coa-Fish-Ser. no. 24, 95-99 pp.

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