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Effect of Benzalkonium chloride (BKC) on the blood cells and immune system of *Clarias batrachus*

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ABSTRACT
The increase in aquaculture production is coupled with an increase in disease outbreaks and to control diseases especially caused by bacteria, antibiotics are being used indiscriminately. The resultant setbacks have focused the attention on the use of immunostimulants in aquaculture practice to withstand the pathogens. To avoid the use of antibiotics, attempts were made to study the immunomodulatory effect of Benzalkonium Chloride (BKC) in *Clarias batrachus.* BKC is a quaternary ammonium compound that can modify non-specific and specific responses and can be used as an immunomodulator in fishes. To determine the immunomodulatory effect and optimum doses to be applied as injection, bath and feed, the catfish *Clarias batrachus* was used as an animal model. BKC was applied in three forms, *i.e.* injection at the rate of 5, 50 and 500 g/kg of the fish weight, bath at 2 and 3 ppm levels for 1 hour and incorporated in feed @ of 1, 5 and 50 mg per kilogram dry weight of feed. White blood cells counts, differential counts, phagocytosis, agglutination assay, NBT assay and serum bacterial activity of the fish was performed for assessing the changes in specific and non-specific responses to BKC. The BKC treated fishes showed increased non-specific and specific responses in comparison to untreated control. The fishes treated with immunostimulant and later challenged with a virulent strain of *Aeromonas hydrophila,* showed delayed and lower mortality than the untreated controls. The present study suggests that BKC can be used as an immunostimulant and can be administered in different forms, like injection, 3 ppm as bath and 50mg/kg dry weight of BKC in feed.

Keywords: *Clarias batrachus*, BKC, Immunostimulation, Blood cells

1. Introduction
Synthetic chemicals and antibiotics have been used to prevent or treat fish diseases and have achieved partial success. However, several hazards are associated with their excessive use like immunosuppression, nephrotoxicity, growth retardation, development of drug resistance, environmental problems and residues in flesh [7, 14]. Vaccination against specific pathogens is an excellent method to prevent diseases and some vaccines have been developed, but with varying degrees of success. Such success depends on the particular disease and particular bacteria and unfortunately, with a few exceptions [8, 26], the systematic use of vaccines in fish is long way off. Problems with the present antibiotic, drug and chemical treatments to prevent diseases in fish set the stage for a new concept in disease prevention – immunostimulation. In this context, much attention is deviated towards improving the immune status of the fish by taking advantage of both the non-specific and specific immune responses.

Different immunostimulants like levamisole, glucans, lipopolysaccharides, various extracts from animals etc. have been tried in fishes and have shown encouraging results particularly in enhancing the non-specific immune responses. Using immunostimulants with fish vaccines is an attractive method for increasing the protective abilities of fish. By boosting the potency of the vaccine, smaller doses can be used. Injection of oil-based adjuvants such as Freund's complete adjuvant (FCA) have been shown to stimulate higher antibody titres upon injection [12, 9]. Other compounds, often called biological response modifiers or immunostimulants, may act more directly on the immunopoietic cells themselves. For instance, levamisole as used in the following studies, is known to be a T lymphocyte stimulator in mammals [11].

Quaternary ammonium compounds are extensively used as disinfectants in fish culture, and for treating bacterial diseases. Most of these compounds are wetting compounds and may have some detergent action on cell membranes. The work on the immunostimulatory properties of these compounds are relatively few [3, 18].

Administering immunostimulant via, injection or by treating the cell lines with the immunostimulant have advantage in that the exact dose of the drug needed for maximum stimulation can be found out. But these tests have drawbacks like the exact doses that will reach the organism once it is administered in water is not known. Since these compounds are highly water soluble, the chances that the required quantity not reaching the animal is common. Also the efficacy of injection method in field conditions is questionable. In view of this an experiment was conducted with an aim to find the efficacy of BKC as an immunostimulant in *Clarias batrachus* either by administering it as a bath treatment or through feed, since these are the easy and practical methods of administration.

2. Material and methods

2.1 Experimental Animals

Clarias batrachus, of average body weight of 150+/-10 gms and 50+/-5 gms were procured from local fish Market of Mumbai. They were transported in open containers and were kept in glass tanks of 500-L capacity in laboratory. Feeding was done with pelleted mixture of Oilcake, rice bran and fishmeal with 30% protein at the rate of 3-4% of body weight daily [10].

2.2 Experimental Design

The experimental dose of immunostimulant was administered by three methods via. injection, through feed and bath treatment. Each treatment dose was done in triplicate and each group had 10 fishes. The fishes were sampled on the second day after administering the experimental dose.

Injection

Three groups of ten fishes each were maintained in separate tanks. Injection doses given were 5µg, 50µg and 500µg per kilogram of fish weight respectively. Injections were given on the 6th, 4th and 2nd day respectively before the sampling. The required doses of BKC for each fish was calculated and mixed in 300µl of Phosphate buffered saline (PBS, pH 7.4) and injected intraperitoneally. Group IV was given injection of only PBS and kept as control. Fishes were sampled two days after the last application of the chemical and checked for various specific and non-specific immune parameters.

Feeding

A purified diet, which contained the required doses of BKC, was prepared with the required ingredients [19].

Fishes of average body weight 50+/-5gms were used for this experiment. BKC was incorporated at three levels, 1mg/kg, 5mg/kg and 50mg/kg of feed. The required quantity of BKC solution was mixed in water and then added to the feed ingredients, which helped in proper mixing and distribution of BKC. A control diet, without BKC was also prepared. The feed was stored in refrigerator, until further use. Fishes were given the experimental diet for 7 days at the rate of 2.0% of the body weight.

Bath treatment

BKC at the rate of 2 and 3ppm respectively were given as one hour bath treatment for seven days continuously. The required dose of BKC was first mixed in one liter of water and then added to required amount of freshwater. Sampling of fishes was done on day 9, to access changes in the various immune parameters.

2.3 Immunization of the experimental animals

The fishes after BKC treatment were immunized with formalin killed *Aeromonas hydrophila*. 0.3ml of the washed bacterial cells in PBS containing 1x10^8 cells per ml was injected intraperitoneally. Booster dose was given on 8th day after the first injection of the bacteria. The agglutination test was performed to find the circulating antibody titres on the 7th day after the booster dose i.e. two weeks after the first immunization.

2.4 Effect of BKC on blood cells

The changes in the blood cells of the experimental fishes were determined to assess the effect of treatment of BKC on the blood cells.

2.5 Preparation of anticoagulant

EDTA (Ethylenediaminetetraacetic acid) or heparin was used as anticoagulant to collect blood. 2.7gms of EDTA salt was dissolved in 100 ml distilled water, sterilized by autoclaving at 15lbs. pressure at 121 ^{0}C for 15 minutes. For Red blood cells (RBC) and White blood cells (WBC) counts, 0.2ml of the sterilized EDTA solution was taken in glass vials of 3 ml capacity and kept them in hot air oven at 60^{o}C until the EDTA solution gets dried completely. After drying, the vials were used for the collection of blood. In each tube approximately 2ml of blood was collected and used for different tests immediately.

2.6 Collection of blood

The fishes were caught gently with minimum stress during the time of handling and blood was collected from heart. The blood is transferred to vial with anticoagulant and mixed well to avoid coagulation.

For collection of the serum, the tubes with blood (without anticoagulant) were kept at room temperature for one to two hours for clotting. The serum was collected with a micropipette and the collected serum was kept overnight and centrifuged the next day at 735 g for 15 min. The supernatant was collected and stored at -20 ^{0}C in screw cap glass vials, till further use.

2.7 Haematological parameters

Total Erythrocyte (RBC) and Leukocyte (WBC) Count

RBC and WBC count was determined as per the method of Schaperclaus, 1986.

2.8 Differential blood count

The differential blood count of the fishes was performed [16]. Briefly, blood was collected in vials containing EDTA and mixed properly. A small drop of the blood placed on a clean grease free slide, oil and using a spreader slide, a thin smear was made. The blood smear was air dried and stained with Leishman's stain. For this, the slide were flooded with the stain for 5 minutes and then diluted with distilled water and kept again for 10 min. Then the slides were washed under tap water, air dried and observed under high power magnification (100X).

2.9 Phagocytic Assay

Phagocytosis was performed to assess the non- specific activity [5]. Briefly, 100µl of blood was mixed with equal quantity of bacterial suspension of *S. aurues* in microtitre plates. The density of the bacterial culture was maintained at 10^5 cells/ml in phosphate buffer saline (PBS). The mixture was incubated for 20 minutes at room temperature. After incubation a thin smear was prepared and fixed with absolute alcohol for 5 min. The smear was later stained with Giemsa stain for 5 min. The phagocytic cells the neutrophils and monocytes that have engulfed bacteria were counted as positive. A total of 200 cells were counted and the results, expressed as a percentage.

2.10 Respiratory burst activity

The respiratory burst activity of the neutrophils was assessed by nitro blue tetrazolium (NBT) test [16]. NBT test gives information concerning the neutrophil activation. NBT dye changes from yellow to insoluble dark blue granules of formazan in the cell cytoplasm. This dye can be reduced only in the activated cells, which produce oxygen radicals. Therefore the number of cells reducing the dye gives an idea on proportion of activated and non-activated cells *in vitro*.

Blood was collected from the fish in a heparinized vial (Sigma USA). 0.1ml of freshly prepared NBT solution was mixed with 0.1ml of the blood and 15µl of stimulant incubated at 37 ^{0}C for 10 min and at 26 ^{0}C for another 10 min. 50-70µl of this blood was transferred to a clean microscopic slide and a thick smear was prepared, which avoids the breaking of neutrophils during the preparation of the slide. The dried slide was then stained by Wright's stain and observed under 100X magnification. The neutrophils with dark blue formazan granules in the cytoplasm were counted as positive. A total of 200 cells were counted and the percentage of NBT positive cells was determined.

2.11 Serum bactericidal assay

Aeromonas hydrophila was cultured in brain heart infusion (BHI) broth and the bacteria were pelletized by centrifuging the broth at 3000g at 4 ^{0}C for 15 min. The bacterial pellet was washed thrice with PBS by centrifuging at 10,000 rpm for 15 min. The final suspension of bacteria in PBS was adjusted to an optical density of 0.5 at 540 nm, which gives approximately 10^8 cells per ml. 100µl of this bacterial suspension and 900µl of fresh antiserum or control serum were mixed in sterile eppendorf tubes. A control was also maintained with bacterial suspension (100µl) and normal saline (900µl). The tubes were incubated at 25 ^{0}C for 60 min and subsequently, all incubation mixtures were used to determine the CFU/ml by the spread plate method on BHI agar. The bactericidal activities of the serum was expressed as percentage of the CFU in comparison to PBS control group [24].

2.12 Circulatory antibody levels

Circulatory antibody levels were determined in microtitre plates [3]. 100µl of normal saline was added to wells 1 to 12 of the U-shaped microtitre plate. 100µl of the antiserum was added to the well no. 1 and diluted two fold using separate microtips untill well no. 10. Wells 11 and 12 were kept as controls. 50µl of the antigen (1 x 10^5 cells/ml of *Aeromonas hydrophila*) was added to the wells. The plates were then covered and incubated at 4 ^{0}C for 12 – 14 hours. Formation of the mat at the bottom indicates positive reaction, whereas button formation at the bottom indicates a negative reaction. Highest dilution of the antiserum causing complete agglutination of the bacterial cells is taken as titre of antibody.

2.13 Challenge test

One week after the administration of BKC, the fishes were challenged with *Aeromonas hydrophila*. Each fish received an intraperitoneal dose of 0.5 ml of 1x10^8 cells/ml of the bacteria. Mortalities were recorded daily and mean of the mortality was calculated.

2.14 Histopathology

The fish liver and kidney were immediately fixed in neutral-buffered formalin, embedded in paraffin wax, cut at 5 mµ and stained with haematoxylin and eosin [25]. The prepared slides were examined and photographed under a light microscope.

2.15 Statistical analysis

Statistical analysis was done by one-way ANOVA. $P< 0.05$ was considered to be significant. The mean values are expressed as +/- the standard deviation.

3. Results

3.1 Haematology

RBC and WBC Counts

There was no significant difference in the number of RBC, except for small reduction in group injected BKC at 500µg/kg of fish. The RBC count among different groups treated with BKC as bath, did not show any significant difference. Administering BKC through feed at the rate of 1, 5 and 50 mg/kg dry weight of the feed showed no change in the RBC count.

Highest WBC counts were observed in the group that was administered BKC by injection at the dose of 50 µg. The results were significant statistically at 5% level. The group treated at concentration of 3 ppm of BKC in water had the highest WBC count, 52.83 x 10^3 cells/ml of blood. Counts for the 2 ppm and control were 39.82 and 27.98 x10^3 cells/ml of blood respectively. Group fed with BKC at the rate of 50 mg/kg feed had the highest number of WBC/mm^3 i.e. (66.36 x 10^3). This maximum value was followed by the treatment at 5 mg/kg feed, which had a mean WBC count 51.42 x10^3. Both the above treatments were significant at 5% level from the control. But the treatment at1 mg level did not show any significant change. The results of the RBC and WBC counts in different treatment groups are given in Table 1.

Table 1: Mean RBC and WBC counts (± S.E) for the different treatment groups

Treatments	Doses	Mean RBC count (X 10^6 cells/ml)	Mean WBC count (X 10^3 cells/ml)
Injection (µg /kg weight of fish)	Control	2.13 ± 0.05	38.66 ± 1.20
	5	2.16 ± 0.04	68.17 ± 1.30
	50	2.23 ± 0.05	95.17 ± 1.08
	500	1.92 ± 0.04	43.50 ± 1.20
Bath (ppm)	Control	2.03 ± 0.03	27.90 ± 0.42
	2	1.96 ± 0.07	39.82 ± 0.06
	3	2.31 ± 0.04	52.83 ± 0.91
Feeding (mg BKC/kg feed)	Control	1.59 ± 0.05	34.33 ± 0.56
	1	2.07 ± 0.04	35.67 ± 0.71
	5	1.82 ± 0.05	51.42 ± 0.88
	50	2.65 ± 0.06	66.33 ± 1.58

3.2 Differential counts

Injection of BKC mainly increased the percentage of granulocytes and monocytes. The increase in proportion of lymphocytes is less than that compared to increase in phagocytic cells. The percentage increase of neutrophils were the highest in the group given the BKC injection of 50µg/kg body weight of the fish, (22.66), which was much higher than the control percentage of 17.50. Injection at the rate of 500µg decreased the percentage of neutrophils and it was close to that of control fish, which was 17.50%. The relative proportion of phagocytic cells increased in the group treated at 3 ppm level, when compared to the control and the 2 ppm treated groups. The results were significant at 5% level. There was no significant difference between the treatments – 1mg and 5 mg from the control at 5% level of significance. The changes in relative proportion of the different cells were quite significant for the group fed feed containing 50 mg BKC per kg of feed. Results are shown in Table 2.

Table 2: Mean Differential Counts (± S.E) of the different Injection groups

Treatments		Lymphocytes	Monocytes	Granulocytes
Injection (µg /kg weight of fish)	Control	67.33 ± 0.84	6.50 ± 0.61	17.50 ± 0.67
	5	72.83 ± 0.83	7.83 ± 0.31	20.83 ± 0.31
	50	70.00 ± 0.52	9.34 ± 0.43	22.66 ± 0.61
	500	75.50 ± 0.43	8.16 ± 0.31	17.80 ± 0.70
Bath (ppm)	Control	79.00 ± 0.58	8.50 ± 0.34	13.16 ± 0.31
	2	78.67 ± 0.31	7.83 ± 0.31	16.60 ± 0.49
	3	71.83 ± 0.49	9.16 ± 0.31	19.17 ± 0.31
Feeding (mg BKC/kg feed)	Control	75.50 ± 0.99	7.67 ± 0.33	18.00 ± 0.37
	1	78.16 ± 0.31	8.00 ± 0.47	17.16 ± 0.65
	5	71.50 ± 0.43	8.33 ± 0.33	18.66 ± 0.33
	50	70.50 ± 0.56	9.80 ± 0.30	21.66 ± 0.49

3.3 Immune parameters

3.4 Phagocytosis

The group treated with BKC at the rate of 50µg/kg body weight of the fish had the highest phagocytic activity of the neutrophils. The percentage positive phagocytic cells in this group were 61.66, while that for the 5 µg level was 42.83%. Increasing the dose of BKC greater than 50µg reduced the phagocytic activity of the neutrophils. For the bath treatment group, group treated with BKC at 3ppm level had 59% of positive cells and the control had 36.33% of positive cells. The percentage positive cells for the 2ppm level was 49.83%. The percentage of cells showing phagocytosis was highest in the group given BKC at the rate of 50 mg/kg feed i.e. 57.17. The percentage positive phagocytic cells for the 1 mg and 5 mg level were 32.33 and 41.00 respectively. Results are given in Table 3.

3.5 Respiratory burst activity

The group that received BKC at the rate of 50µg/kg body weight of the fish had highest neutrophil activity. Increasingly the dose, more than 50µg, reduced nitroblue tetrazolium reduction by neutrophils. The results are statistically significant at 5% level. The percentage NBT positive cells for the group treated at the 50µg/kg body weight was 65.50, while that for the 5 and 500µg levels were 42.83 and 50.66 respectively.

NBT assays also show the same trend as that of the phagocytosis. The highest activity of the neutrophils was found to be for the group given bath at the level of 3 ppm. This group had a percentage positive cell of 51.66. The values for 2ppm and control were 41.33 and 35.50% respectively. The NBT assay showed number of NBT positive cells increasing in the group fed BKC at the rate of 50mg/kg feed. The value of which was 50.83%. The value for the 1mg and 5mg level are 32.33 and 41.33 respectively. The results are shown in Table 3.

Table 3: Mean values (± S.E) for NBT positive cells and phagocytic positive cells for different treatment groups

Treatment	Dose	Positive phagocytic cells (%)	NBT positive cells (%)
Injection (µg /kg fish weight)	Control	34.00 ± 1.32	31.50 ± 1.25
	5	42.83 ± 1.40	42.83 ± 1.11
	50	61.66 ± 1.54	65.50 ± 2.09
	500	50.66 ± 1.91	52.50 ± 1.17
Bath (ppm)	Control	36.33 ± 1.12	35.50 ± 1.54
	2	49.83 ± 0.91	41.33 ± 0.88
	3	59.00 ± 0.58	51.66 ± 0.88
Feeding mg/kg feed	Control	30.33 ± 1.09	29.60 ± 0.76
	1	32.33 ± 0.87	32.33 ± 1.02
	5	41.00 ± 0.93	41.33 ± 2.45
	50	57.17 ± 0.83	50.83 ± 1.08

3.6 Serum bacterial activity

Bactericidal activity of the serum was the highest for the group treated at 50µg/kg body weight of the fish where the percentage reduction of bacteria was 72.62 compared to 30.41% of the control fish. Application of BKC at the rate of 2ppm and 3 ppm level showed mean percentage reduction of bacteria to 44.35 and 58.04 respectively. Results of serum bactericidal activity tests corroborates the effectiveness of treatment with 50 mg BKC/kg feed. The bactericidal activity of the serum was the highest at this level, followed by the treatment with 5gm of BKC/kg feed. The decrease in number at 1 mg inclusion level was not statistically significant at 5% level. Results are shown in Table 4.

Table 4: Serum Bacterial Activity, mean values (± S.E) for different treatment groups

Treatments	Doses	Mean Bactericidal activity (%)
Injection(µg /kg fish weight)	Control	30.41 ± 4.60
	5	39.26 ± 7.67
	50	72.62 ± 5.88
	500	36.13 ± 5.02
Bath (ppm)	Control	33.12 ± 3.81
	2	44.35 ± 2.91
	3	58.04 ± 5.33
Feeding(mg BKC/ kg feed)	Control	29.33 ± 4.89
	1	32.68 ± 2.32
	5	46.24 ± 8.03
	50	68.38 ± 2.26

3.7 Agglutination titre

The agglutination titre increased in the groups once the dose is increased from 5µg /kg body weight of the fish. Doses above 50µg reduced the antibody titres. Antibody titre was the highest for the group treated at 3 ppm dose of BKC in water, which was 1:64 and that for the 2 ppm group the mean titre was 1:32. Circulatory antibody titre against *Aeromonas hydrophila* was the highest for the group fed the feed containing the highest dose of BKC (50mg/kg feed). The value was 1: 64, while that for the 5mg and 1mg levels were 1: 32 and 1:16.00 respectively. All the results were statistically significant at 5% level of significance (Table 5).

Table 5: Mean agglutination titres (± S.E) for the different treatment groups

Treatments	Doses	Mean antibody titre
Injection (µg /kg fish weight)	Control	1: 8 ± 1.33
	5	1 : 16 ± 1.68
	50	1 : 64 ± 5.33
	500	1 : 16 ± 1.33
Bath (ppm)	Control	1 : 8.00 ± 1.69
	2	1 : 32 ± 2.66
	3	1 : 64.00
Feeding (mg/kg feed)	Control	1 : 10.28 ± 1.59
	1	1: 12.00 ± 1.79
	5	1 : 14.67 ± 1.33
	50	1 : 26.67 ± 3.37

3.8 Challenge studies

The fishes that received the dose of 50µg/kg body weight of the fish had the highest survival rate. The mean death time for this group was 58.33 hours, while that for the 500µg and 5µg BKC treated fishes, the mean death times were 47.50 and 33.16 hours respectively. The mean death time for the control fish was 20.66 hrs after the antigen injection.

Treating the fishes at the rate of 3 ppm of BKC in water highly delayed the mortality time. The group treated at concentration of 3 ppm had a mean death time of 50.62 hrs compared to the 36.50 and 23.16 hrs for the group treated at 2 ppm level and control.

Feeding the fishes with BKC at 50 mg level increased the mean death time to 36.50 hours, which was high compared to the other treatment levels and the control. Inclusion of BKC at 1 mg level did not show any significant increase in mortality time. The results for the 5 mg and 50 mg BKC/kg were significant at 5% level (Table 6).

Table 6: Mortalities rates in hours (± S.E) for different treatment groups

Treatments	Groups	Mean Death Time (hrs).
Injection (µg /kg fish weight)	Control	20.66 ± 2.03
	5	33.16± 1.35
	50	58.33± 1.67
	500	47.50 ± 1.80
Bath (ppm)	Control	23.16 ± 1.08
	2	36.50 ± 1.31
	3	50.62 ± 3.81
Feeding (mg/kg feed)	Control	17.67 ± 2.74
	1	22.42 ± 2.43
	5	25.75 ± 1.53
	50	36.50 ± 2.06

3.9 Histopathological studies

No cellular changes were observed in liver and kidney of the control and BKC treated fishes.

4. Discussion

4.1 RBC and WBC count

The average RBC counts of the control fish was found to be 2.2 x 10^6 cells/mm^3 of the blood. The RBC counts agree with the values earlier reported [1]. In the group injected at the rate of 500µg/kg of fish weight, there was a reduction in the number of RBC. This can be due to the haemolytic effect of BKC at high concentration.

The total WBC count gives idea regarding the immune status of the fish, as they are the main components of the immune system. There was a significant increase in the no. of total WBC's following the administration of the drug. The fishes given the 50µg dose had the highest number of WBC's followed by the group treated with the 5µg dose. The results for the group given the highest dose (500µg) was more or less same as the control fish. In the group treated at the concentration of 3ppm, the number of WBC were the highest and in the group fed at the dose of 50 mg/kg feed, the WBC counts were the highest. The results obtained are in conformation with the results of earlier works [3], who reported an increase in the total WBC counts following the administration of QAC. Works, using immunostimulants like Levamisole, ESK etc. have also shown that immunostimulants increase the number of WBC in the experimental fish.

4.2 Differential count

The percentage of different cells (differential count), among the WBC is also an important parameter, since it gives idea regarding the activation of the leucocytes. The differential counts of the control fish agree with the counts reported for *Clarias batrachus* [1, 2].

Injection of BKC at two levels, 5 and 50µg /kg of fish increased the percentage of granulocytes (neutrophils, basophils and eosinophils) and Monocytes in the respective groups. The relative percentage of granulocytes in 5 and 50µg treated fishes were 20.83 and 22.26% respectively. The results were statistically significant at 5% level, when compared to the control and 500µg BKC treated fish. The increase in percentage of monocytes (9.34) also was statistically significant, when compared to the control, 5 and 500µg BKC injected fish. The relative percentage of lymphocytes in the treatment groups were lower when compared to the control. This can be due to the increase in the proportion of the granulocytes and the monocytes and not an actual reduction in the number since the agglutination titres showed increased circulating antibody titres in the experimentally treated fish, when compared to the control group.

The highest mean percentage of granulocytes and monocytes, among bath treated fish was for the 3ppm level. The mean percentages being 19.17 and 9.16 respectively. The results of this corroborate with other test results like phagocytosis, which showed an increased percentage of positive cells in the group treated at 3ppm level. The relative percentage decrease in the lymphocytes was not considered as significant.

Administering BKC at the rate of 50mg/kg in feed highly increased the number of granulocytes, the mean percentage value was 21.66 and the value for control was 18.00. The increases in percentage for other groups were not statistically significant. The percentage of monocytes was also the highest in the group fed BKC at the rate of 50mg in per kg feed. The changes in the percentage of lymphocytes were not considered significant.

4.3 Phagocytosis

Phagocytosis is an important mechanism of non-specific defence. The ability of the phagocytes to engulf the bacteria, *Aeromonas hydrophila* was tested and the results were in confirmation with the work done [3], where there was an increase in percentage of phagocytosis, after treating the fishes with a quaternary compound (dichloro-bis {N,N-di methyl – N – carbo deoxymethyl N- ethylene- ammonium} sulphate). Injection at the rate of 50µl of BKC per kg weight of the fish had the highest percentage of positive phagocytic cells and their number reduced once the treatment dose was increased to 500µl of BKC per kg weight of the fish.

The results for phagocytosis assays showed gradual increase when the spleen cells were treated with higher doses of QAC [18] but in this experiment, there was reduction in phagocytic ratio when the doses were raised above 50µg /kg body weight of fish. The present results agree with the findings of [3], who reported decreased phagocytic ratio once the treatment doses were increased more than 50µg/kg body weight of fish. So it is evident that once the levels of BKC injected goes higher than 50µl there is a reduction in the response. However the percentage of phagocytic cells in all the experimental groups were higher than the controls. The reduction in non-specific responses after a particular dose of immunostimulant was reported [18, 3]. Same effect have been reported with the case of other immunostimulants like levamisole and the exact mechanism behind this not known.

In the group that had been given the bath treatment with the immunostimulant, the highest percentage of phagocytosis was found to be that for the 3ppm level. It can also be noted that the percentage of positive cells for 3ppm level was much lower than that obtained for the group treated with 50µl of BKC. Since the bath treatment was done only till the dose of 3ppm, the possibility that increasing the concentration of BKC in water or increasing the time of treatment, may still increase the activity of the phagocytes. Increasing the concentration may not be a good choice since BKC is reported to be toxic at levels higher than 3ppm [23].

4.4 Respiratory burst activity

NBT test gives idea regarding the ability of the neutrophils to kill the microorganisms, once it is inside the cell. The higher percentage of NBT positive cells have direct relationship with the overall mechanism of phagocytosis since the killing of the organism by production of oxygen radicals is the end result of a chain of reactions in the cells. The group given the immunostimulant at the rate of 50µg per fish had the highest percentage of NBT positive cells and similar result have been shown [3] by administration of QAC in rainbow trout and in *Macrobrachium rosenbergii* [6]. At higher levels, 500µg/kg fish weight the drug was found to reduce the nitroblue reduction activity [17]. Observed the same result, when the rainbow trout spleen cells were incubated with a QAC. Doses higher than 0.1µg/ml in the culture medium of spleen reduced the NBT positive cells. But the results show deviation from the results of [3] in which the higher doses of BKC (100µg/fish) increased the percentage of NBT positive cells in rainbow trout. Among the bath treated fishes, the group treated at the rate of 3 ppm showed higher percentage of NBT positive cells compared to the other groups. There was no significant difference between the groups treated at 2ppm level and the control. The percentage positive cells are lower than the group given the injection of 50µg level. This again highlights that the optimum level of application of BKC as bath can higher than 3ppm. Among the groups fed with BKC, the highest percentage of positive cells were found in the group given feed containing BKC at the rate of 50mg/kg. BKC also has an effect on activating the cells in prawns treated with 15ppm when determined by NBT assay [20].

4.5 Serum bactericidal activity

C-reactive proteins (CRP) are found to increase in concentration following immunization with Vibrio anguillarum in rainbow trout, *Onchorhynchus mykiss* [21, 13] showed increase in concentration of lysosome post immunization. Compliment system by its classical pathway plays a crucial role in humoral defence against microbial pathogens [29].

[15,24] have reported the antibody mediated killing of *Aeromonas salmonicida* and *Vibrio anguillarum* strains in rainbow trout, *Onchorhynchus mykiss*[22] reported that the bactericidal activity of Channel catfish (*Ictalurus punctatus*) antiserum dose not show any change even with increase in the agglutination titre.

In the present study, serum from the immunostimulant treated fish was used and there was marked reduction in the number of the bacterial cells in the treatment group than the untreated fish. This results confirms the findings of [30] that reating the fish with immunostimulants can elevate the response of complement system.

4.6 Agglutination test

The agglutination titres are a fair representation of the level of specific defence in the animal once it is immunized with a particular bacterium. The results confirm that QAC treatment prior to the injection greatly enhanced the specific response against the antigen. The levels of antibody against *Aeromonas hydrophila* in the fishes given a dose of BKC at the rate of 50µl were quite higher than the control and other treatment groups. This results obtained closely follow the results obtained by [3], where they reported a high level of antibody titre in the fishes treated with QAC prior to immunization.

The group that was given treatment at the rate of 3ppm as bath had the highest level of antibody titre compared to the other treatment groups. Here the antibody titre of 3ppm group is same as that of the fishes given the dose of 50µl as injection. This shows that the treatment at 3ppm level may be the optimum for enhancing the specific immune response but in case of non-specific responses this treatment dose was found to be less than the dose required for maximum stimulation of the non-specific response.

The exact mechanism by which the immunostimulant BKC acts on the immune system is not known [4]. Also there are conflicting reports regarding uptake of the immunostimulants in fish [26] reported that oral ingestion is the principal route of antigen uptake in the bath-immunized fish [31] gave evidence of antigen uptake by salmonid gill cells after bath immunization with bacterin.

Both these mechanisms can help in taking up immunostimulants, like the case of antigens. This is evident by comparing the result obtained for bath and the feeding groups. Even though the inclusion of BKC in feed was very high, the elevations of specific and non-specific responses were very high for the bath treated group at 3 ppm. The reason can be

that in groups which were bath treated, immunostimulant might have been absorbed through the gill surface and also through the oral ingestion, compared to only oral ingestion of BKC by the group fed with BKC.

The study also indicated no cellular changes in the fish on treatment with BKC, it can be concluded that BKC injection at the rate of 50µg/kg body weight or 3ppm bath treatment or if included at @ 50mg/kg dry weight of feed is effective as an immunostimulant in *Clarias batrachus*.

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