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Immunomodulatary potential of Chitosan and Levamisole against bacterial diseases of *Penaeus monodon*

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Effective eco-friendly disease management strategies are emerging as important criteria for sustainable shrimp farming due to problems such as development of resistant microbial strains by application of antibiotics and other chemo-therapeutants. Vaccine development for the crustaceans is impractical due to lack of specific immune system. However, the non-specific immunity can be enhanced using immunostimulants. They enhance the microbicidal activity of haemolymph and phagocytosis of the cells. Studies were made to evaluate the immunomodulating effects of chitosan and levamisole. Different parameters such as growth and survival stimulating potency, hematological index, phagocytic index and bacterial clearance were studied. Significant increment in the growth and immune index were noted in the shrimps fed with levamisole 150 groups. Challenge experiments were made with commercially important shrimp pathogens *Vibrio harveyi* in both control and experimental shrimps and 100% mortality observed in the control group, but in the experimental diet administered group showed protection in a considerable manner.

Key words : Shellfish, Disease-management, Immunostimulants, levamisole, Chitosan

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INTRODUCTION

The world production of shrimp has declined drastically due to dreadful viral and bacterial epizootics, environmental crisis and consequent legal restrictions. In terms of economic loss in the aquaculture industry, disease has been cited as the single largest factor. In shrimp, viral diseases are the most devastating followed by bacterial outbreaks. Viral outbreaks cause very high mortality, reaching 100% within 3-10 days of onset of clinical signs. According to several reports, the majority of bacterial infections are attributable to *Vibrio* species (Lightner, 1993). Vibriosis in shrimp also causes death ranging from trivial to 100% (Lightner, 1988). There have been no remedies developed so far for such outbreaks.

The attempts made for controlling/preventing such devastating outbreaks using conventional antimicrobials and other chemotherapeutants were mostly unsuccessful and created severe environmental consequences. The uncontrolled and repeated use of antibiotics causes a major setback in the successful treatment of bacterial infections due to the development of antibiotic resistant pathogens. Developing specific bacterins/vaccines are also impractical in crustaceans because of their poorly developed specific immune system. In Crustacea, the first lines of defense are elicited by haemocytes through phagocytosis, encapsulation and nodule formation. The phagocytic activity is enhanced considerably by the activation of prophenoloxidase (Pro-PO) system localized in the semigranular and granular haemocytes. The pro-PO inturn is activated by the immune enhancers. Considering these aspects, the disease resistance capacity could be enhanced successfully through the activation of such non-specific defense factors. Among the factors concerning the humoral defense system, the phenoloxidase (PO), the Pro-PO activating system, bactericidin and lectins are considered as important ones (Azad *et al.*, 1995).

Immunostimulants are delate substances, which enhance the non-specific defense mechanism and provide resistance against the invading organisms. Perusal of

literature indicates that immunostimulants are proven very successful in treating/preventing microbial diseases in cultured shellfishes. Crustaceans treated with immunostimulants such as glucan (Chang *et al.,* 1999), levamisole (Huxley *et al.,* 2000), chitin/chitosan (Huxley, 1998) usually show enhanced phagocytic cell activities. Hemocytes are also activated by immunostimulants. In addition, they enhance the clotting activities and produce bactericidins. Administering immunostimulants can also increase antibacterial activity of haemolymph. In tiger shrimp *Penaeus monodon,* increased bacterial clearance was noted after injection with glucan (Sung *et al*., 1996).

RESEARCH METHODOLOGY

Collection of shrimps:

Forty days old tiger shrimp, *Penaeus monodon* were collected from extensive farm near Rajakamagalm area, Kanyakumari district, Tamil nadu. The collected shrimps (200 nos) were acclimated to laboratory conditions in one tonne fibre reinforced plastic (FRP) tanks. The tank was filled with seawater and maintained with adequate aeration and optimum water quality (salinity = 35$\pm$2%; temperature = 28$\pm$2^{0}C). The shrimps were fed with pelleted feed (CP Nova, Kochi).

Preparation of immunostimulated feed:

For immunostimulation of *P. monodon* two immunostimulants *viz.*, Levamisole and Chitosan were used at different doses. The methodology of preparation of the feed and its incorporation is described below:

Levamisole feed:

Levamisole @300 mg/kg shrimp body wt (as Feed A) and @50 mg/kg shrimp body wt (as Feed C) were incorporated in the shrimp grower feed (CP feeds, Cochin). The medical grade levamisole (from MBT Lab CMFRI, Vizhinjam) was finely ground in a mortar and pestle. The required quantity of fine powder was initially dissolved in a small quantity of 1% acetic acid and subsequently in 4% gelatin solution prepared in phosphate buffered saline (PBS) at pH 7.2. The resultant aliquot was sprayed on shrimp feed so as to get the required concentrations of the immunostimulants. The feed thus prepared was dried at 40^{0}C in a hot air oven for 24 hours.

Chitosan feed:

Chitosan @300 mg/kg shrimp body wt (as Feed B) and @50 mg/kg shrimp body wt (as Feed D) were incorporated in the shrimp grower feed (CP feeds, Cochin). The chitosan (manufactured by: CIFT, Kochi) powder was initially dissolved in a small quantity of 1% acetic acid and subsequently in 4% gelatin solution prepared in phosphate buffered saline (PBS) at pH 7.2. The resultant aliquot was sprayed on shrimp feed so as to get the required concentrations of the immunostimulants. The feed thus prepared was dried at 40^{0}C in a hot air oven for 24 hours.

Growth parameters:

The initial (Day 0) and Final (Day 30) live wet weight of all the experimental group of shrimps was recorded. The growth rate was calculated from the results obtained:

$$\text{Absolute growth rate (AGR) (g/body wt/day)} = \frac{\text{Final body wt - Initial body wt}}{\text{Total no. of days}}$$

$$\text{Relative growth rate (RGR) (\%)} = \frac{\text{Absolute growth rate}}{\text{Initial body wt}} \times 100$$

Haemolymph sampling:

Haemolymph was obtained from the ventral part of the haemocoel of the second abdominal segment using a 25-gauge needle and a 1 ml syringe (disposable type; DISPO VAN, India) filled with 0.2 ml sodium citrate 12.5% (by dissolving 12.5g of sodium citrate in 100 ml of distilled water) as anticoagulant. The puncture procedure prevented extraction of tissue particles during the haemolymph sampling. The haemolymph was stored in 1.5 ml Eppendorf cups and kept on ice until analyzed (within 1 h of sampling). All the experiments were carried out using pooled haemolymph.

Dilution fluid for haemocyte count:

The diluting fluid suggested by Stewart *et al.* (1967) was used in order to prevent cell rupture, agglutination and plasma clot formation. The composition of diluting fluid was as follows:

Sodium chloride (NaCl)	-28.4g
Magnesium sulphate ($MgSO_4 7H_2O$)	-08.7g
Magnesium chloride ($MgCl_2 6H_2O$)	-05.5g
Calcium chloride ($CaCl_2 2H_2O$)	-01.5g
Potassium chloride (KCl)	- 0.7g
Distilled water	-1000ml

The pH was adjusted to 7.6 using 0.1N NaOH. One ml of 0.4% formaldehyde solution was added to prevent microbial growth.

Total haemocyte counts (THC):

THC was determined using haemocytometer. 0.5 ml of the haemolymph was directly taken into the WBC pipette. Then the diluting fluid was drawn into pipette up to the 11th mark to get 20 times dilution. The inner surface of the WBC pipette was rinsed several times, with the diluting fluid before drawing the haemolymph. At least one minute of gentle stirring was required for complete dispersion of cells to prevent agglutination and plasma clot formation. Naubauer's hemocytometer (ROHEM, India) was loaded immediately with the haemolymph taken in WBC pipette. The cells in all four 1.0mm squares were counted. The total hemocytes counts per mm^3 of haemolymph were calculated as described below:

$$(THC) = \frac{\text{Hemocytes in four } 1mm^3 \text{ x depth factor x dilution factor}}{\text{Number of squares counted}}$$

(where, depth factor = 10 and dilution factor = 20)

Phagocytic assay:

Bacterial strain obtained from the Marine Biotechnology laboratory Vizhinjam research centre of CMFRI, was used for the phagocytic assay. A drop of freshly collected haemolymph was mixed with one drop of formalin killed bacterial strain (1x 10^7 cells /ml) in PBS (pH 7.2) on a glass cover slip and placed in a humidified chamber for 30 minutes at 20^0C. After incubation the cover slips were washed with PBS. Excess liquid was drained from the cover slips by placing one edge against a paper towel and the cells were fixed with methanol for 5 minutes. They were stained with May Grunwald-Giemsa (HiMedia, India,Code: SO39) for 15 min and washed 3 times with distilled water. The cover was turned upside down on to a drop of mounting fluid on a glass microscope slide, and the results were read via oil immersion light microscopy (1000x magnification). Phagocytic cells (engulfing more than three bacteria) were counted over the whole slide, and the number was compared with the counts from control shrimps.

Bacterial clearance:

To determine how rapidly bacteria were cleared form the haemolymph, shrimp were injected with 0.1 ml *V. fischeri* suspension (containing 10^5 cells/ml) and haemolymph were sampled from the animal after 10 and 90 min. One hundred ml of haemolymph diluted with sterile saline was spread on to triplicate TCBS agar (HiMedia, India, Code: M870) plates. The samples were immediately mixed with 7 ml of melted TCBS agar, poured into Petri dishes, and incubated at appropriate room temperature for 18 h. Numbers of bacterial colonies per plate were counted and divided by the volume of haemolymph extracted to determine the number of colony forming units (CFU) per milliliter of haemolymph. The number of per milliliter divided by the number of bacteria per CFU milliliter injected times 100% give the percentage of bacteria still in circulation.

Challenge experiments:

Vibrio fischeri strain for challenge experiments was obtained from the Marine Biotechnology Lab, CMFRI Vizhinjam. On the 30th day, three groups each of healthy and immunostimulated shrimps (10 individuals per group) were challenged with the median lethal dose (LD_{50}) of *Vibrio fischeri*. After the administration, the shrimps were transferred to 60-liter glass aquaria. They were observed for a period of 4 days for mortality and infections. Parallel controls (10 no's) received 0.2 ml normal saline (0.85%) only. The chosen concentration was inoculated intra-muscularly at the ventral part of second segment. The mortality/infectivity percentages were estimated by the following formula:

$$\% \text{ of mortality} = \frac{\text{No. of dead/infected shrimp}}{\text{Total no. of injected shrimp}} \text{ x } 100$$

$$PRP = 1 - \left\{ \frac{\% \text{ Mortality in the treated group}}{\% \text{ Mortality in control group}} \right\} \text{ x } 100$$

RESULTS AND ANALYSIS

The results obtained from the present investigation have been discussed under following heads:

Growth of shrimps fed with immunostimulants:

The entire immunostimulated group showed the higher absolute growth rate (AGR) over the control. However, the feed C fed shrimp showed high AGR value 0.106 g/body wt/day followed by feed D 0.076-g/body wt/day. The relative growth rate (RGR) also had the same trend. Feed C fed shrimp showed high RGR value 0.96% followed by Feed D, which gave 0.70%. The detailed results are given in Table 1.

Haemogram:

THC's of shrimp haemolymph increased in the experimental animals from the control group as shown in the Table 2. The THC of the feed C increased to 24.64% over the control group on the day 1 with a drastic increase

Table 1 : Absolute growth and relative growth rate of *P. monodon*

Experiments	Absolute growth rate (AGR) (g/body wt/day)	Relative growth rate (RGR) (%)
Control	0.0086	0.077
Feed-A	0.0090	0.079
Feed-B	0.0320	0.286
Feed-C	0.1060	0.961
Feed-D	0.0760	0.701

in the day30 to the extent of 50.13%. The values recorded for the intermittent days were 19.80% for 10th day and 26.92% for 20th day. More or less same trend was noted in all other experimental groups such as feed A, B and D. Interestingly, the drastic increase of THC was noted in the feed B group on the 10th day to the extent of 63.50%. However, it declined considerably to 47.39% in the 30th day after treatment (Fig. 1).

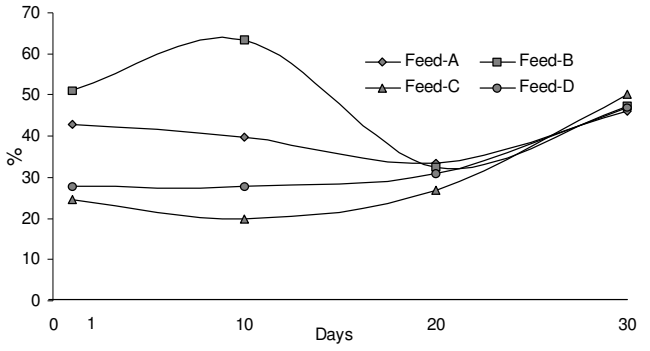

Fig 1: Per cent increment of THC of *P. monodon* over control

Phagocytic index:

Influence of immunostimulants on the *in vitro* phagocytic index (PI) (Table 3) of the experimental groups over the control group is shown in Fig. 2. It is noteworthy that that phagocytic rate also increased parallel to the haemogram. The rate of increase in feed C group was 4.10, 11.20, 20.56 and 14.35 % over the control group on the 1, 10, 20 and 30th day of post-treatment, respectively. In other feed groups, the rate of phagocytosis was comparatively low on the 30th day, but feed D gave an increase in PI (24.53%) on the 20th day.

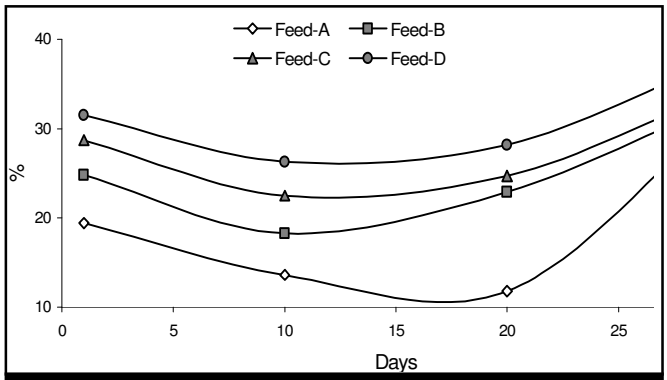

Fig 2: Per cent increment of phagocytic index of *P. monodon* over control

Bacterial clearance

The clearance rate of bacteria (5.5x 10^5 cells) injected in to the haemocoel of the experimental shrimps is depicted in Fig. 3. The bacterial clearance potential was increased considerably in all feed groups in 30th day post treatment. Interestingly feed D treated group had

Table 2 : Total haemocyte count of *P. monodon* in experimental and control group

	Day 1	Day 10	Day 20	Day 30
Control	4562.5 ± 379	4475.0 ±. 095	4550.0 ± 234	4562.5 ± 286
Feed-A	6525.0 ± 132`	6250.0 ± 158	6075.0 ± 155	6662.5 ± 205
Feed-B	6900.0 ± 081	7320.0 ± 298	6075.0 ± 469	6725.0 ± 272
Feed-C	5687.5 ± 175	5362.5 ± 561	5775.0 ± 997	6850.0 ± 216
Feed-D	5825.0 ± 125	5725.0 ± 253	5962.5 ± 085	6712.5 ± 165

Table 3 : Phagocytic index (%) of *P. monodon*

	Day1	Day10	Day20	Day30
Control	43.00 ± 2.58	42.40 ± 2.73	42.80 ± 0.56	42.85 ± 0.77
Feed-A	40.90 ± 0.42	45.35 ± 3.40	45.30 ± 3.72	47.10 ± 0.70
Feed-B	42.10 ± 0.70	45.95 ± 3.41	46.35 ± 1.46	44.80 ± 1.27
Feed-C	44.80 ± 0.98	47.15 ± 2.42	51.60 ± 4.32	49.00 ± 0.56
Feed-D	45.45 ± 0.35	48.50 ± 3.17	53.30 ± 3.43	47.10 ± 0.70

Table 4: Bacterial clearance in the experimental and control groups of *P. monodon*

	Day 1	Day 10	Day 20	Day 30
Control	54.35 ± 1.48	56.80 ± 0.56	56.75 ± 0.77	52.05 ± 0.21
Feed-A	64.90 ± 0.70	64.55 ± 0.21	63.50 ± 0.98	69.00 ± 1.12
Feed-B	67.85 ± 0.49	67.20 ± 0.70	69.75 ± 0.49	69.40 ± 0.20
Feed-C	69.95 ± 0.63	69.60 ± 1.32	70.80 ± 0.56	70.05 ± 0.70
Feed-D	71.50 ± 0.56	71.75 ± 0.63	72.80 ± 0.56	71.95 ±1.32

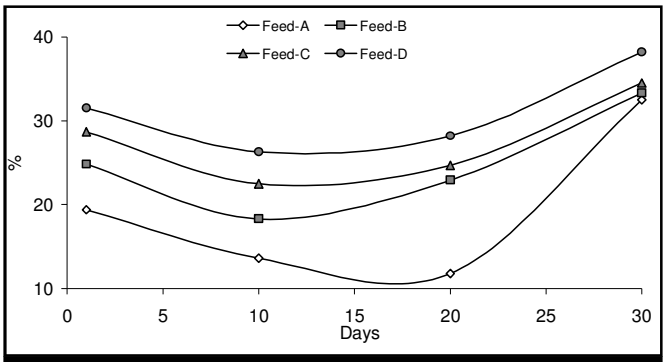

Fig 3: Per cent increment of bacterial clearence of *P. monodon* over control

the peak, to the extent of 38.20% over the control value on the day 30 through 31.5, 26.3 and 28.28 on post treatment days 1, 10 and 20, respectively. However, feed C also had an impressive increment next to feed D on the day 30 (34.58%) via 28.70 on day 1, 22.50 on day10 and 24.75 on 20th day (Table 4).

Challenge experiments:

The survival rate of *P. monodon* after challenging with *V. fischeri* (5.5 x 10^6 cells/ ml) is shown in Table 5. The feed C survival rate of 60% was recorded as maximum. The per cent relative protection given by the immunostimulated feed are also given in the same table (Table 5). The feed C and A gave the protection to the tune of 60.0% and 50.0%, respectively.

Most of the earlier studies on immunostimulation of shrimps have involved injection or immersion. Since oral administration would be more practical in aquaculture situations, the possibility of improving the disease resistance of black tiger shrimp *P. monodon*, by oral administration of different concentration of immunostimulants was evaluated.

Table 5 : Survival and PRP of *P. monodon* after bacterial challenge

Experiment	Survival (%)/ PRP
Control	0.0
Feed-A	50.0
Feed-B	34.0
Feed-C	60.0
Feed-D	34.0

Growth:

The organism's different array of feeding behavior normally reflects the growth rate (Cho *et al.,* 1985). From the results of the dissertation, it is inferred that the orally administered immunostimulants helped the experimental groups of *P. monodon*, to attain increased growth rate over the control group. Shiau and Ping Yu (1998) found that chitin supplementation enhanced the growth of *P. monodon.* Experimental results of Itami *et al.* (1991) indicated that the quality of larvae of *P. monodon* improved after the administration of microencapsulated killed *Vibrio* cells. Observations of Song and Sung-Hung-Hung (1993) confirmed the enhancement of growth in shrimp *P. monodon* by immunostimulation with glucan. However, Boonyaratpalin *et al.* (1993) have reported that higher levels of peptidoglycan in feed had adverse effects in tiger prawn *P. monodon* and were reflected in the growth and survival. In the present experiments, the higher growth in shrimps fed with feed C (50mg/kg body wt of Levamisole) showed safety index than the other groups. The mechanism of better growth might have been possible due to the dose dependent immuostimulation of Levamisole. However, in the chitosan fed groups @50mg/ kg body wt (feed D) also had better growth increment.

THC:

The crustacean haemocyte have been implicated in diverse functions including an active role in the internal defense against invading biotic and abiotic foreign materials (Evans and Brock, 1994). THC could be expected to provide a useful way of assessing the physiological status of the animal (Martin and Graves, 1985). Regarding this aspect, the results of THC obtained in immunostimulants fed group showed higher count over control, which in other way represents the increased health status of the shrimps. The increase of the circulating haemocyte number was due to an increase of

each of the cellular types (Le mollac *et al.*, 2000). Haemogram of *P. homarus* exhibiting a similar pattern of increase in THC was reported by Huxley *et al.* (2000). Higher dose of immunostimulant fed groups @ 300mg/kg body wt (feed A and B) if compared with 50mg/kg body wt (feed C and D) showed minimal increase over control. Smith *et al*. (1984) reported such marked reduction of THC, due to the formation of haemocyte clumps in the gills of *Carcinus maenas*. This was attributed to the over dosage of beta 1, 3-glucan through injection. According to Lipton (DBT report 2001), immense increase in THC of *P. monodon* was noticed, when shrimps were fed with different doses of three immunostimulants. Apart from immunostimulants, the number of circulating haemocyte in the shrimp *P. stylirostrus* could also be related to the protein content in the diet. A significant gain was observed in the shrimps fed with a diet containing 38% of protein.

Phagocytic index:

Phagocytosis is the important cellular defense mechanism. Most of the foreign particles in the crustacean haemocoel are removed by phagocytosis (McKay and Jenkin 1970). From the phagocytic index, it was evident that the rate increased over control among the immunostimulant fed group. Interestingly the feed C (50mg/kg body wt levamisole) had a notable increase of about 14.35%. The provision of better increment might be due to the dose-dependent immuostimulation of levamisole. The *in vitro* phagocytosis of erythrocytes by hemocytes of the crayfish, *Parachaeraps bicarinatus*, required specific opsonins (Fontaine and lightner, 1974; Paterson *et al.,* 1976, Smith and Ratcliffe, 1978; Goldenberg *et al.,* 1984). Opsonins appeared to be hemagglutinins, which enhanced adhesion of erythrocytes to hemocytes (McKay *et al.*, 1969). According to Takahashi *et al.* (1995) high phagocytic activity in crustaceans was noticed through the administration of β 1-3 glucan and peptidoglucan.

Bacterial clearance:

Decapod crustaceans are able to rapidly clear a variety of exogenous materials, which may enter into their haemolymph including viruses (McCumber and Clem, 1977, Johnson *et al.*, 1981), bacteria (Cornick and Stewart, 1968; McKay and JenKins *et al.*, 1970; White and Ratcliffe, 1982; Adams 1991) and even dyes and latex particles (Merrill *et al.,* 1979; Mullainathan *et al.*, 1984; Factor and Beekman, 1990). The bacterial clearance of the present findings also showed the increased activity in the immunostimulated group over control within 90 minutes. Specifically the feed D and C (50mg/kg body wt of chitosan and 50mg/kg body wt of levamisole) showed comparable results than the other groups, which indicated the dependency of optimal dose. In the case of lobsters some antibacterial factors found in plasma such as bacteriocydins, lectins and agglutinins were reported as being effective against a variety of bacteria (Cornick and Stewart, 1968). Sung *et al.* (1994) studied the clearance ability of hemolymph of *P. monodon* immersed in a viable cell suspension of *V. vulnificus*, which showed that *Vibrio* cells were largely eliminated from shrimp hemolymph within 12 h following invasion and completely at 24 hours.

Challenge experiments:

The beneficial effects of feed C (50mg/kg body wt of levamisole), which manifested the high PRP as well as survival value (60%) against *V. fischeri* over control was recorded. The findings of Takahashi *et al.* (1995) after oral administration of immunopotentiators, such as beta -1, 3-glucan and peptidoglycan, to shrimp also indicated increased resistance against bacterial infection. Previous data from Robertson *et al*. (1990) indicated that the use of beta-glucan into Atlantic salmon resulted in a marked increase in resistance to both vibriosis and enteric red mouth disease. Based on their results and the present observations on the duration of enhanced protection in shrimp, levamisole apparently had the potential to be used prophylactically as a short-term immunostimulant for shrimp. According to Lipton (DBT report 2001) the immense increase in PRP as well as survival of *P. monodon* when fed with different doses of immunostimulants (levamisole 150 mg/kg body wt shrimp) gave survival up to 70 %. According to Chang *et al.* (1999) shrimp fed with experimental diets of beta -1, 3-glucan for post larvae and juvenile and when challenged with WSSV showed less mortality ($p < 0.005$) in all the glucan-fed groups than in the respective non-glucan control groups. Liao *et al*. (1996) performed challenge experiments in grass prawn fed with four doses of beta-1, 3-glucan by intra muscular injection with *V. damsela.* Their results showed that the beta-1, 3-glucan @0.5-1.0 g/kg diet significantly ($p<0.05$) increased the resistance of the prawn against vibriosis. Thus, the protection of the host against invading microbial pathogens can be achieved by using the required quantity of immunostimulants.

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