

NOTE

Bacterial clearance rate and a new differential hemocyte staining method to assess immunostimulant activity in shrimp

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ABSTRACT: New methods were developed to assess immunostimulant efficacy in the black tiger shrimp *Penaeus monodon*. Test shrimp were fed with 2 or 4 % yeast extract (YE)-coated feed while controls were fed non-coated feed. After 4 wk of feeding, individual shrimp were assessed for total hemocyte counts (THC), the number of granular hemocytes (GH) and rate of bacterial clearance. For hemocyte counts, formalin-fixed hemolymph was stained with 1.2 % Rose Bengal in 50 % ethanol for 20 min at room temperature. Some of this mixture was used for THC with a hemocytometer while some was smeared on a microscope slide and left to dry before counterstaining with hematoxylin for GH counts. By this technique, high quality smears were obtained for accurate differential counts. Bacterial clearance assays were used to assess the sum effect of humoral and cellular defense mechanisms. *Vibrio harveyi* was injected intramuscularly at 1×10^8 cells per shrimp and hemolymph was collected in anticoagulant at 0, 15, 30 and 60 min post-injection for quadruplicate drop counts (20 μ l) on TCBS agar. Total hemocyte counts for shrimp fed with 4 % YE were significantly higher ($p < 0.05$) than those for shrimp fed with non-coated feed. The percentage of granular cells and the rates of bacterial clearance for the YE-fed shrimp were higher than those for shrimp fed the control diet. These 2 methods provide a simple and rapid comparison of shrimp groups for differences in anti-bacterial defense capacity.

KEY WORDS: Black tiger shrimp · *Penaeus monodon* · Hemocytes · Bacterial clearance · Immunostimulant

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INTRODUCTION

One approach to overcome disease problems in shrimp aquaculture has been the development of feed additives called immunostimulants which may increase shrimp defense against potential pathogens (Smith et al. 2003). Immunostimulant efficacy is usually measured by changes in parameters such as phenoloxidase activity, phagocytotic index or level of superoxide dismutase (Campa-Cordova et al. 2002, Lopez et al.

2003). The related assay procedures are often sophisticated and time consuming and none have yet been universally accepted as standard measures of shrimp defense potential.

Despite the variety of shrimp defense responses, many of them originate from hemocytes. Shrimp hemocytes are involved in defense processes such as phagocytosis, encapsulation, melanization and coagulation (Johansson et al. 2000). Thus, hemocyte number is sometimes used as an indicator of shrimp health sta-

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tus (Perazzolo et al. 2002). Many humoral defense molecules such as prophenoloxidase (proPO), antimicrobial peptides and clotting factors are contained in hemocyte granules, the release of which is stimulated by pathogen invasion (Söderhäll & Cerenius 1998, Sritunyalucksana & Söderhäll 2000). Consequently, the granular cell number may be used as a more specific measure of the potential defense capacity related to these molecules. It was shown in one study that increased phenoloxidase activity in response to an immunostimulant coincided with an increase in total hemocyte count, while the activity per hemocyte was more or less constant (Supphantharika et al. 2003).

Since defense against bacterial pathogens in shrimp depends on a number of cellular and humoral activities interrelated in a complex way, we reasoned that following the rate or efficiency of bacterial clearance after a severe challenge would be a simple method of measuring the summative potential of these activities. It would require no special equipment or reagents and would constitute a direct assessment of host ability to inactivate a bacterial pathogen. In preliminary tests (unpublished), we found that clearance of several million bacterial cells injected into 10 g shrimp was extremely rapid, with 99% removal occurring within 60 min. Challenge tests over such a short interval would save time and cost in tests for immunostimulant activity. Thus, we carried out the tests described herein to determine whether bacterial clearance rate could be used to examine the efficacy of a purported yeast extract (YE) immunostimulant. Simple, differential hemocyte counts were used as a confirmatory measure since they had previously been reported to be affected by immunostimulants (Supphantharika et al. 2003).

Differential staining of shrimp hemocytes is difficult since their cell membranes are easily disrupted. To avoid this problem, fixative or anticoagulant must be held in the syringe or injected prior to hemocyte removal with a needle (Nash et al. 1995). Even with these precautions, smears give poor staining results since cell membranes or intracellular granules and cytoplasm are indistinct. For example, hematoxylin and eosin staining of hemolymph smears results in intense eosinophilic staining of the background hemolymph protein and this masks the staining of the cell cytoplasm and cell membranes, making it difficult to recognize cell types. However, results in an earlier report by Chang et al. (2000) on the use of Rose Bengal to prepare shrimp blood agar suggested that this stain might be adapted for improved differential staining of shrimp hemocytes in smears. Here we describe a technique for using Rose Bengal and hematoxylin to obtain differential staining of shrimp hemocytes of a consistently high quality. For penaeid shrimp, hemocytes are often classified into large-granular, small-granular (or

semigranular) and agranular (or non-granular) hemocytes according to Martin & Graves (1985). In this study, large-granular and small-granular hemocytes were grouped together as total granular hemocytes.

MATERIALS AND METHODS

Experimental animals and feeding trial. A total of 350 black tiger shrimp *Penaeus monodon*, with a fresh weight of approximately 3 g, were obtained from a shrimp farm in Chachoengsao province, Thailand. They were reared from post-larvae derived from captured, wild broodstock and were therefore of unknown pedigree. On arrival at the wet laboratory, the shrimp were acclimatized in a large 1500 l covered aquarium (60 × 60 × 60 cm) containing recirculating artificial seawater over a period of several days at a salinity of 10 ppt with water temperature ranging from 25 to 29°C. During this interval, 1 gill filament and 1 pleopod were removed from each of 20 shrimp. Each pleopod was assayed for white spot syndrome virus (WSSV) by PCR (IQ2000 WSSV detection kit, Farming Intelligene) and each gill filament for yellow head virus (YHV) by RT-PCR (IQ2000 YHV/GAV detection kit, Farming Intelligene) using the kit extraction and PCR protocols. The negative results with this sample assured freedom from WSSV and YHV at a level of 15% with 95% confidence (Cameron 2002). No tests were done for other pathogens since the shrimp were grossly normal. After acclimatization, shrimp were randomly divided into 3 groups with 5 replicates of 20 in each group (i.e. n = 100 for each group) in 150 l plastic aquaria (60 × 60 × 60 cm) under the same rearing conditions. Mean weights from each aquarium were averaged to give mean weights for each test group of 5 replicates. There was no significant difference in mean weight ± the standard error of the mean (SEM) (p > 0.05) for the 3 groups at the start of the test (control group: 3.3 ± 0.7 g, 2% YE group: 3.6 ± 0.8 g and 4% YE group: 3.4 ± 0.7 g). The control group was fed with commercial feed and 2 experimental groups fed with the same commercial feed top-dressed with YE at 2 and 4% (Netropro, Alltech Biotechnology), respectively. Feed was given 2 times daily in excess for a period of 4 wk. Survival and weight gain were recorded after 4 wk as means from each aquarium, giving 5 means for each group to be used in statistical comparisons. Next, the surviving shrimp from each group were pooled and 12 each were selected for bacterial clearance tests. The remainder from each group were used for hemocyte counts.

Hemocyte counts. At the end of the trial, hemolymph was collected individually and prepared for total hemocyte counts (THC) and granulocyte counts (GH) that included both large-granular and small-granular

(also called semigranular) hemocytes. The numbers of shrimp sampled were 49, 55 and 57 from the control, 2%YE and 4%YE treatments, respectively. Due to technical difficulties, 3 slides from the control group and 2 from the 4%YE group were unreadable. Hemolymph (0.1 ml) was withdrawn from the ventral sinus of the first abdominal segment into a syringe containing 0.1 ml fixative (10% formalin in 0.45 M NaCl) and transferred to an eppendorf tube. After 10 min, 20 μ l portions of the fixed hemocyte suspension were mixed with 20 μ l Rose Bengal solution (1.2% Rose Bengal in 50% ethanol) and incubated at room temperature for 20 min before being used to determine THC by hemocytometer or to prepare smears on microscope slides for measuring %GH. Hemocytometer (improved Neubauer bright line) counts were made for 5/25 squares (volume of 1 square = $0.2 \times 0.2 \times 0.1 \text{ mm}^3$) to calculate THC ml^{-1} hemolymph ($5 \times \text{count} \times 10^4 \times \text{dilution factor}$). For GH counts, smears were completely dried before counterstaining with hematoxylin solution (50 g aluminium or potassium alum, 1 g hematoxylin crystals, 0.2 g sodium iodate, 1 g citric acid, 50 g chloral hydrate and distilled water to 1 l) for 7 to 10 min. The slide was then rinsed with tap water for 10 min followed by dehydration with 95% ethanol (10 dips) and 100% ethanol (10 dips). After dehydration, the slide was submerged in xylene (3 times for 3 min each) before being mounted with Permount (Fisher Scientific) and covered with a coverglass. The proportion of GH in 200 total hemocytes was recorded and this proportion was used to calculate the total number of GH (i.e. $\text{count}/200 \times \text{THC}$).

Bacterial clearance. To determine bacterial clearance rate, 12 shrimp from each of the 3 feed-treatment groups were each injected intramuscularly at the 6th abdominal segment with 10 μ l of a suspension of *Vibrio harveyi* VH1039 (Ruangpan et al. 1999) containing 10^{10} cells ml^{-1} . The bacterial cells were prepared by growth from cryopreserved stocks streaked on tryptic soy agar (Difco Laboratories) containing an additional 1.5% NaCl (TSA). After overnight growth at 30°C, a single colony was transferred to tryptic soy broth (Difco) also containing an additional 1.5% NaCl (TSB), and incubated overnight on a rotary shaker (200 rpm) at 30°C. Next, 0.5 ml of this culture was added to 49.5 ml TSB and incubated for 5 h followed by centrifugation of 10 ml and resuspension of the pellet in 1 ml artificial sea water (ASW, 400 mM NaCl, 100 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mM KCl and 20 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) to yield a suspension of approximately 1×10^{10} cells ml^{-1} .

Three shrimp from each feed-treatment group were removed immediately after injection and then another 3 each at 15, 30 and 60 min post-injection to determine the number of bacterial cells ml^{-1} in hemolymph. Hemolymph (50 μ l) was collected from the ventral

sinus of the first abdominal segment into a syringe containing 100 μ l of anticoagulant solution AC-1 (0.45 M NaCl, 0.1 M glucose, 30 mM Na-citrate, 26 mM citric acid, 10 mM EDTA, pH 7.0) (Söderhäll & Smith 1983) and added immediately to a tube containing 350 μ l of TSB. This was serially diluted 10 fold in Tris-buffered saline (TBS, 50 mM Tris-HCl pH 7.3, 100 mM NaCl) before 20 μ l quadruplicate aliquots were dropped onto plates of thiosulphate, citrate, bile-salt (TCBS) agar (Difco) to obtain bacterial counts (CFU) after incubation at 30°C for 10 h. Only serial dilution plates giving individual drop counts in the range of 20 to 40 colonies were used. Counts were calculated according to dilution and recorded as the mean CFU count \pm 1 SD for the quadruplicate counts.

Statistical analysis. Statistical analysis of the results was carried out using 1-way ANOVA with SigmaStat computer software (Jandel Scientific Software). When normality tests failed, a Kruskal-Wallis 1-way ANOVA on ranks was carried out. Individual differences were determined using Student-Newman-Keul's method and differences were considered significant when $p \leq 0.05$.

RESULTS AND DISCUSSION

Shrimp growth and survival

After the 4 wk feeding trial, there were no significant differences ($p > 0.05$) in the final mean fresh weight \pm SEM of the shrimp among the treatment groups: 4.7 ± 1.0 g for the 2%YE, 4.3 ± 1.0 g for the 4%YE and 4.3 ± 1.1 g for the control. Nor was there any significant difference ($p > 0.05$) in survival rate (\pm SEM) amongst the groups (survival rates of control, 2%YE, and 4%YE groups were 61 ± 2 , 67 ± 7 and $69 \pm 7\%$, respectively). This indicated that the tested immunostimulant had no effect on growth and survival rate over the period of the test. We cannot exclude the possibility that a longer period of administration would give different results. Survival and growth were somewhat lower than normal for aquarium trials with *Penaeus monodon*, possibly due to stress from the high stocking density (equivalent to 55 ind. m^{-2}) and the fact that ambient temperature during the tests was sometimes 4°C lower than mean optimum temperature for juvenile *P. monodon* growth (i.e. optimum range 27 to 33°C, mean 30°C) (Lester & Pante 1992). Dead shrimp revealed no gross signs of WSSV or YHV infection and were usually found in the morning partially cannibalized. They may have been attacked during the vulnerable period of molting. Despite these constraints, the surviving shrimp were grossly normal, and growth and survival values for the 3 groups were statistically equivalent.

Total hemocyte and granulocyte counts

At the end of the 4 wk feeding trial, mean THC (Fig. 1) in the 4%YE group ($n = 55$) (1.1×10^7 cells ml^{-1}) was 1.3 times higher ($p = 0.03$) than that in the control group ($n = 46$) (8.3×10^6 cells ml^{-1}). However, there was no significant difference between the mean count for the 2%YE group ($n = 55$) (9.1×10^6 cells ml^{-1}) and those of the 4%YE and control groups. Comparisons of THC are difficult because of the wide variation amongst individual shrimp, as is evident from the standard deviation bars in Fig. 1. The normal counts fell in the range and degree of variation reported for *Penaeus monodon* by van de Braak et al. (1996) and Owens & O'Neill (1997) (i.e. $5 \times 10^7 \pm 2 \times 10^7$ and $2.3 \times 10^7 \pm 1.4 \times 10^6$, respectively).

Staining of hemocytes with Rose Bengal facilitated their counting by hemocytometer and their differentiation in smears (Fig. 2). With respect to granulocyte counts (Fig. 3), there was a 2.6 \times increase in the mean granulocyte count ($p < 0.001$) for the 2%YE ($n = 55$) and 4%YE ($n = 55$) shrimp groups when compared to the control group ($n = 46$). There was no significant difference in the number of granulocytes between the 2 YE-fed groups.

These results show that inclusion of YE in the diet of aquarium-reared *Penaeus monodon* can result in a significant increase in THC and in the proportion of granulocytes. This could be called immunostimulant activity. Since YE is a complex material containing basic nutrients, vitamins, minerals and cell-wall components, it was not possible to assign its immunostimulant effect to any particular ingredient. However, the yeast cell-wall component β -glucan has been reported

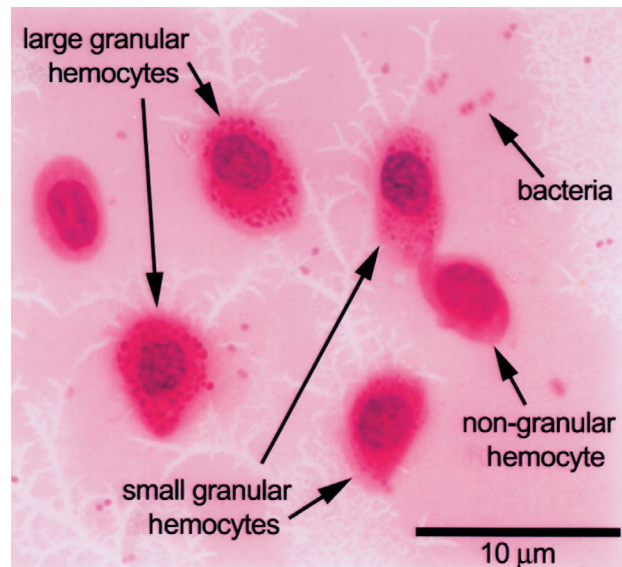


Fig. 2. *Penaeus monodon*. Shrimp hemocytes stained with Rose Bengal and hematoxylin solution

to have immunostimulating activity for shrimp (Liao et al. 1996, Sung et al. 1998, Chang et al. 1999, Suphatharika et al. 2003). Whether the effect we observed also occurs in pond-reared shrimp would need to be tested in field trials. Previous work has shown that results from aquarium trials may not be confirmed in a field setting (Sritunyalucksana et al. 1999). This may result from the fact that shrimp consume a large amount of natural feed in the pond environment, especially in the first month of culture (Tacon & Akiyama 1997). Since shrimp are detritivores, the natural feed

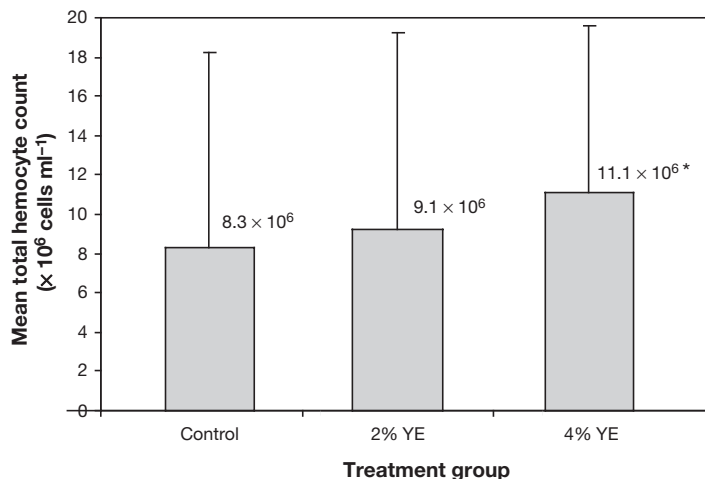


Fig. 1. *Penaeus monodon*. Mean total hemocyte counts (THC) of shrimp following 4 wk feeding with control diet or diet containing 2 or 4% yeast extract (YE). * = significantly different from the control ($p < 0.05$). Error bars show SD

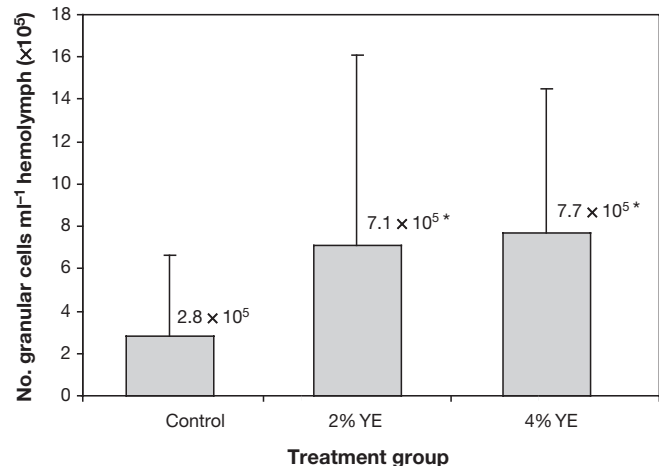


Fig. 3. *Penaeus monodon*. Number of granular hemocytes in total hemocytes after 4 wk feeding the control diet or diets containing yeast extract (YE) at 2 and 4%. * = significantly different from the control ($p < 0.01$). Error bars show SD

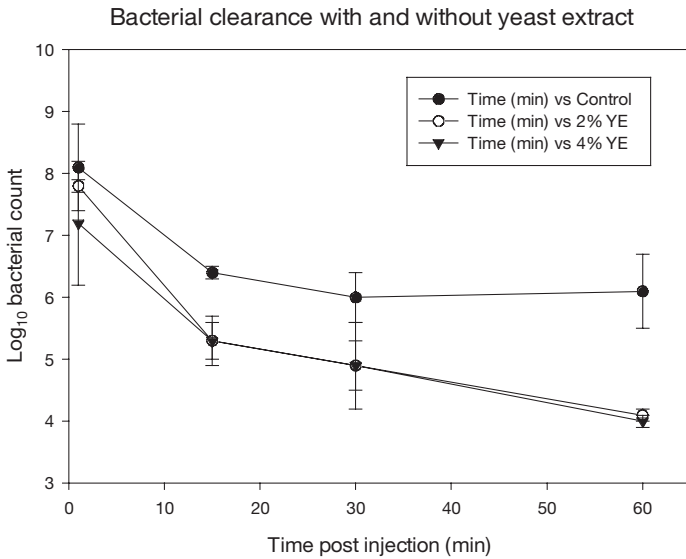


Fig. 4. *Penaeus monodon*. Rate of bacterial clearance by shrimp fed normal diet and diet supplemented with yeast extract (YE) at 2 and 4%. Error bars show SD

may include a sufficient microbial biomass to reach saturation and mask the effect caused by any added to their dry feed diet. Indeed, we show here that there is no difference between differential granulocyte counts resulting from the addition of 2 and 4% YE to the diet.

Bacterial clearance activity

There were no significant differences amongst the test and control groups in the mean bacterial counts immediately after injection. However, counts were significantly lower ($p < 0.05$) for both the 2% YE ($n = 3$) and 4% YE ($n = 3$) groups when compared to the control group ($n = 3$) at 60 min post-injection (Fig. 4). Differences between the YE-treatment groups and the control were evident from 15 min post-injection onwards, but there was no significant difference between the 2 YE-fed groups. Addition of 2% YE to the diet appeared to be sufficient to achieve an immunostimulation threshold and 4% YE did not result in further stimulation. This result is supported by the GH counts where 2 and 4% YE addition gave equivalent increases.

There was a very rapid decline in bacterial counts during the first 15 min after bacterial injection. Thus, even small differences in the time taken for 'immediate' hemolymph withdrawal after injection led to relatively high variation in the initial counts for all groups. Within 15 min after injection, counts for all groups dropped by approximately 2 logs (i.e. equal to more than 90% clearance) and counts continued to drop in

all groups up to 30 min. From 30 to 60 min, counts remained stable in the control group but continued to drop in the YE-fed groups. It appeared that clearance capacity had been saturated by 30 min in the control group but not in the YE-fed groups. In spite of more than 99% clearance in the control group at 60 min, total counts remained high at approximately 10^6 cells ml^{-1} hemolymph compared to approximately 10^3 cells ml^{-1} hemolymph for the YE-fed groups. These results demonstrate the need and usefulness of a very high challenge dose for determining differences in defense potential of test shrimp groups.

Although injection challenge probably does not represent the normal pathway by which shrimp become infected in a natural environment, Alday-Sanz et al. (2002) have shown that bacterial challenge by injection, oral intubation or immersion results in common clearance pathways. In addition, our results augment that study in showing that shrimp have the capacity to rapidly reduce very high numbers of viable bacterial cells and that this capacity may be increased by dietary components. However, in the normal shrimp pond setting, it is unlikely that individual shrimp would be challenged by doses as high as those used in this study. Thus, we propose that the clearance method would be useful in rapid, preliminary screening of dietary components for potential ability to improve defense capacity. Further efficacy tests would be necessary to determine how those components might perform in a production pond setting.

In conclusion, this work has demonstrated the utility of 2 new methods for studying different aspects of shrimp defenses against pathogens. Both are simple and easy to apply without the need for complex reagents or sophisticated equipment beyond a microscope, centrifuge and steam sterilizer that are normally available in any microbiological laboratory. In our opinion, the bacterial clearance method is particularly useful as a summative measure of humoral and cellular defense capacity.

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