

The effect of viral infection on the relationship between the LOS cells and moulting stages of the black tiger prawn (*Penaeus monodon*)

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Abstract. The formation of spheroid cells in the lymphoid organ of penaeid prawns has been suggested to be associated with infectious diseases either bacterial or viral. In this study, the abundance of lymphoid organ spheroid (LOS) cells, termed spheroid to total tissue (STT) ratio, in association with the moulting stages in *Penaeus monodon* that were experimentally infected with presumptive gill associated virus (GAV) was investigated. The experimental prawns were divided into 2 groups: a control group and a GAV infected group. The day post-injection was classified in three categories: (1) 1 – 5 dpi; (2) 6 – 10 dpi and (3) 11 – onward dpi. The results showed that there were no significant effects of days post-injection, moult stages, lunar phase, and the size of the animals on the STT ratio ($P > 0.05$). The relationship between the LOS cells and lunar phase was being masked by the significant interaction between lunar cycle and the treatments ($P < 0.05$). In the control group, the ratio of STT was lowest at the new moon and highest at the full moon. On the other hand, in the infected (GAV) group, the ratio of STT was lowest at first quarter moon and highest at last quarter moon. The STT ratio was significantly higher in infected group than in the control group ($P < 0.05$). In this study, sex also had a significant effect on the spheroid to total tissue ratio of *P. monodon* ($P < 0.05$).

Key Words: penaeid prawns, lymphoid organ, spheroid cells, lunar phase, gill associated virus.

Introduction. One of the most catastrophic impacts on the world aquaculture industry was viral diseases in cultured penaeid prawns. At least 20 viruses of penaeid prawns have been identified since 1995 (Lightner 1996; Lightner & Redman 1998) and the number has increased in recent years as new viruses were found. In Australia alone, viral infections such as hepatopancreatic parvovirus (HPV), monodon baculovirus (MBV), lymphoidal parvovirus (LPV), infectious hypodermal and haematopoietic necrosis virus (IHHNV), spawner-isolated mortality virus (SMV), lymphoid organ virus (LOV) later named gill associated virus (GAV), bennetae baculovirus (BBV), parvo-like virus of *Penaeus japonicus* (P-PJ), penaeid haemocytic rod-shaped virus (PHRV) and gut nerve syndrome have emerged in wild and farmed penaeid prawns (Owens 1997; Spann & Lester 1997; Munday & Owens 1998).

Since 1994, viruses became a major concern when mass mortality of 12–15 g prawns occurred and was named mid-crop mortality syndrome, MCMS (Owens 1997). Predominantly 2 viruses were identified as involved in MCMS, a parvo-like virus, SMV and a yellowhead-like virus, GAV (Cullen & Owens 2003). Currently, the infestations of these two viruses are still significant to the Australian prawn industry. Owens *et al* (2003) reported that approximately 24% of spawners and 44.7% of *Penaeus monodon* ponds were infected with SMV. Meanwhile, the prevalence of GAV in *P. monodon* was much higher; around 95% (Munro & Owens 2005). Therefore, it is not surprising that GAV

received plenty of attention from those who are involved in the aquaculture industry (farmers, scientists and policy makers).

Gill associated virus has been studied by applying routine histology; transmission electron microscopy (TEM); reverse transcriptase nested polymerase chain reaction (RT-nPCR); *in situ* hybridization (ISH); immunohistochemistry; real-time quantitative RT-PCT (qRT-PCR) and haemoagglutinin assay using chicken erythrocytes ((Spann & Lester 1997; Cowley et al 2000a; Cowley et al 2002b; Callinan & Jiang 2003; Spann et al 2003; de la Vega et al 2004; Munro & Owens 2005). The GAV genome has also been sequenced (Cowley et al 2000b; Cowley et al 2002a). Data from experimentally infected prawns showed that GAV could be transmitted vertically from broodstock to their progeny and that *P. monodon* was the natural host of the virus (Spann et al 2000; Cowley et al 2002b). Histopathologically, GAV infected prawns had spheroid cells in the lymphoid organ (LO) (Spann et al 1997; Spann et al 2003).

This report examines the abundance of lymphoid organ spheroid (LOS) cells within the lymphoid organ in association with the moulting stages in *P. monodon* that were experimentally infected with presumptive GAV.

Material and Method

Experimental animals. *P. monodon*, were transported from a commercial farm in northern Queensland to aquaria at The College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, Australia. The prawns were reared in two 1,000 L plastic bins with a recirculating system and two aerators in each bin. Salinity was held at 35 ppt and temperature varied between 26–28°C.

For experimental study, the prawns were transferred and individually kept in recirculating aquaria (60×60×30 cm) with a constant water level. Prior to use, the filter system and experimental aquaria were chlorinated with liquid chlorine (100 g/L) at 30 ppm overnight. The next day, the equipment was rinsed with fresh water overnight and once again the following day. During the experiment, photoperiod was maintained at 12 h light: 12 h dark, salinity varied from 28 to 33 ppt and temperature ranged from 26 to 30°C. The experimental animals were fed with commercial prawn pellets at 10% total body weight divided into 2 daily feedings. Siphoning was conducted once every two days to remove the waste from the aquaria. Prawns with a mean (\pm SD) body weight of 13.25 \pm 3.82 g (6.91–26.31 g) and total length of 11.8 \pm 1.0 cm (9.8–14.2 cm) were used in the experiment.

Viral extraction. Frozen prawns from northern Queensland infected with presumptive gill associated virus (GAV) were thawed in a 37°C water bath. Viral extract was prepared from 5 g of head soft tissues from 3 prawns after the hepatopancreas was removed. The tissues were placed in stomacher bags and homogenised in 20 mL double strength phosphate buffer saline (PBS). The homogenized tissues were clarified by centrifugation at 1,500 rpm for 10 minutes and the supernatant was further centrifuged at 5,000 rpm in an Eppendorf Centrifuge 5804 for 15 minutes. To the extract, was added 1% Bovine Donor Serum (BDS) and filtered through 0.45 μ m filter using a 5 mL syringe to form a cell-free extract. Furthermore, the extract was divided into 70 μ L aliquots in 1.5 mL Eppendorf tubes and stored at –80°C prior to use.

Viral injection. The experimental prawns were divided into 2 groups: a control group and a GAV infected group. Prior to use, aliquots of virus were rapidly thawed in a 37°C water bath. For each group, prawns on one day after moulting (intermoult stage) were individually injected into the first abdominal segment, either with 50 μ L double strength phosphate buffer saline for control, or 50 μ L cell-free viral extract for viral infected group using a 0.5 mL Terumo insulin syringe with a needle (29 gauge \times 1/2").

Sampling procedure and moult staging. Prawns were sampled on 1, 2, 3, etc. days post-injection (dpi). Three prawns was sampled every sampling time from each group. The number of samples for each treatment depended on the duration of the moult cycle.

The day post-injection was classified in three categories: (1) 1 – 5 dpi; (2) 6 – 10 dpi and (3) 11 – onward dpi. The stage of moult was determined by using the setal development of new setae and the withdrawal of epidermis from the base of setae (Rusaini & Owens 2011). Four moult stages could be determined during the experiment, stage A/B, C, D0, and D1/D2. Stage A and B were categorized as one stage because stage A was only found in one prawn and morphologically and physiologically stage A and B were almost similar and presented after moulting. Similarly, stage D2 was also only found in one prawn and expressed as a single stage with D1.

Histology. Prawns were anaesthetized by placing them in iced water for a few minutes, prior to histological examination. The prawns were immediately fixed by injecting 0.5 mL of Davidson's fixative into the hepatopancreas and adjacent area of the cephalothorax and then immersed in the fixative at a ratio of tissue to fixative 1:10 for 48 hours. The cephalothorax was cut in midsagittally, placed in a histocassette, stored in 70% ethanol and then processed for routine histological examination using standard paraffin embedded procedure (Bell & Lightner 1988). Sections were cut at 5 µm and stained with haematoxylin and eosin (H & E).

Analysis of the lymphoid organ. Quantification of the lymphoid organ spheroid (LOS) cells was conducted by using a modified transect method (Rusaini & Owens 2007). This method was based on the abundance of LOS as assessed by using light microscopy and a micrometer eyepiece with magnification 100× (10×10). Along a transect, the perpendicular bars of the eyepiece were used to count (sample) the number of the normal tubules and the LOS. Only the normal stromal matrix tubules and the LOS cells that come into contact with the bands at 10 divisions on either side of the scale line were counted. The sampling was carried out along the longest diagonal of the LO. Care was exercised to keep the sampling process on the right track and direction. The ratio of the lymphoid organ spheroid cells to the total tissue or spheroid to total tissue (STT) ratio was determined (Rusaini & Owens 2010a) as follows:

$$\text{STT ratio} = \frac{\text{The count of intersections of the eyepiece on the LOS}}{\text{The count of intersections of the eyepiece on the total tissue}} \times 100\%$$

Data analysis. To examine the effect of each treatment, moult stages, lunar cycles, day post- injection, sexes, and the size of the animals (weight and total length) on the spheroid to total tissue ratio, the prevalence of vacuolated spheroids and the number of vacuoles in spheroid, the data was analyzed using univariate analysis of variance with SPSS program version 11. Post-hoc analysis was performed using least significant differences (LSD) to examine the differences between individual mean of the variables. Differences between means were considered significant at P<0.05 level.

Results

Clinical signs and histopathology. Two weeks after the experiment started, mass mortality occurred in the stock tanks. After that, it was difficult to find healthy prawns for transfer to the experimental aquaria. As a result, mortality also occurred in the experimental aquaria before treatment and the experiment ended before sampling was completed (one moult cycle). Seven and five prawns were moribund or dead in control and infected group respectively before treatment (injection). One prawn was moribund and five prawns died just after injection in inoculated group, whilst there were no prawns found moribund or dead just after injection in control group. Furthermore, one prawn died in control group 3 dpi and 1 prawn was dead 4 dpi in the infected group. Therefore, only 46 prawns (23 prawns from each treatment) could be assessed during the experiment.

Various histopathological tissue section samples of *P. monodon* with H & E stain are shown in Figure 1.

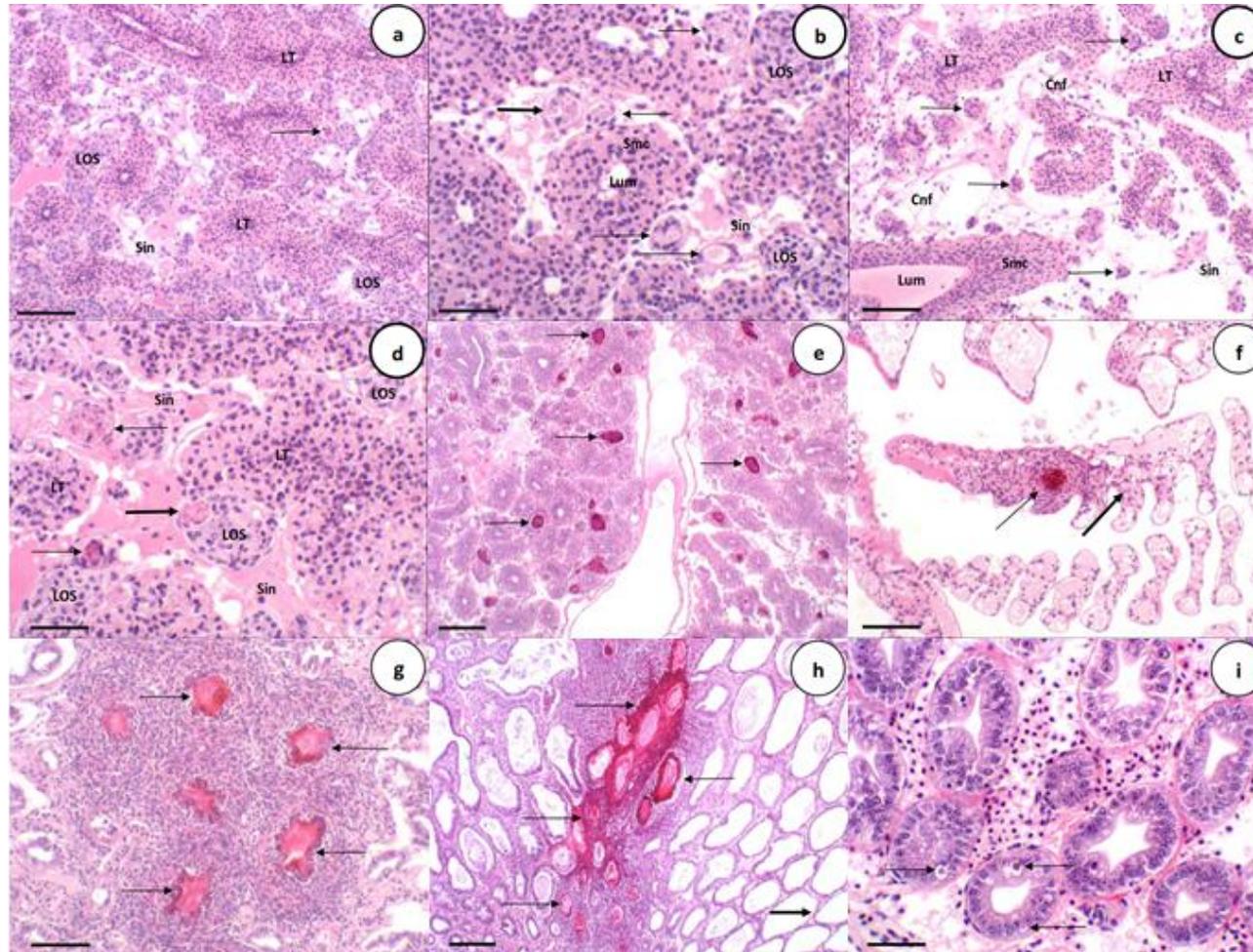


Figure 1. Various tissue section of *Penaeus monodon* with H & E stain. (a) Numerous highly encapsulated LOS cells with fibrocytes in haemal sinuses (Sin) between lymphoid organ tubules and one eosinophilic focus (arrow), scale bar = 100 μ m; (b) highly degenerated LOS cells (arrow) and necrotic eosinophilic foci (bold arrow), scale bar = 50 μ m; (c) abnormal interstitial space (haemal sinuses)/gapping between tubules, note the LOS cells (arrow), scale bar = 100 μ m; (d) eosinophilic foci (arrow) and one focus inside the LOS cell (bold arrow) suggesting that originally these foci developed from the LOS cells, scale bar = 50 μ m; (e) multiple formation of bacterial granulomas (arrow) in the two lobes of lymphoid organ, cross section, scale bar = 200 μ m; (f) melanized nodule in the gill of the prawn with multiple layer of haemocytes encapsulated the nodule (arrow), note the haemocytic infiltration (bold arrow), scale bar = 100 μ m; (g and h) massive melanized nodules (arrow) in the hepatopancreas surrounded by multiple layer of haemocytes resulted in the inflammation of the tissue and atrophy of hepatopancreatic tubules (bold arrow), scale bar = 100 μ m (g) and 200 μ m (h); (i) massive haemocytic aggregations in the haemal sinuses between tubules (bold arrow) and apoptotic cells (arrow) in the hepatopancreas tubules, scale bar = 50 μ m.

Some prawns showed amputated and reddish appendages, red body coloration, pinkish gill, lethargy, anorexia, out of balance (swimming in their side), soft cuticle, poorly developed setae in the inner uropod and have biofouling with ciliates. Histopathologically, every single prawn demonstrated spheroid cells within the lymphoid organ. Some spheroids were highly encapsulated by fibrocytes or flattened epithelial cells (Figure 1a) while some appeared highly degenerated or less dense (lack of granule material inside) (Figure 1b). Abnormal interstitial space (gapping) between tubules was more frequently observed (Figure 1c). Necrotic eosinophilic foci were also often found in the lymphoid organ during the experiment (Figure 1d). Ectopic spheroids were observed in antennal gland, tegmental gland, hepatopancreas, connective tissue, heart and gills.

Another pathological feature was multiple formations of bacterial granulomas in the lymphoid organ (Figure 1e). Generally, these nodules consisted of bacterial colonies in the center and surrounded by melanized zones. The melanized zones were encapsulated by multiple layers of haemocytes. This inflammatory type of reaction was also observed in the gills (Figure 1f) and the hepatopancreas (Figure 1g & h). In the hepatopancreas, infiltration of haemocytes around bacterial colonies in tubules (infected sites) resulted in inflammation. Furthermore, haemocytes walled off the invaded tubules and formed granulomatous nodules. Melanization was demonstrated in old granulomatous lesions. Interstitial space between tubules was extensively expanded with enlarged haemal sinuses and haemocytes (Figure 1i). All these histopathological changes were found both in control and in the infected group.

Moult stages and lunar phases. Sometimes, staging the moult was difficult due to poorly developed setae and cuticle in the inner uropod in some prawns. Unsynchronized development between setae including the retraction of the cellular matrix, the formation of setal cones, and the retraction of epidermis from the setal bases was seen during the experiment. The moulting period of the prawns took around 12–20 days (15.3 ± 2.4 days).

The actual moulting activity (ecdysis) of the prawns varied within the lunar phases (Table 1).

Table 1

The percentage of moulting (ecdysis) of *Penaeus monodon* at four lunar phases

<i>Lunar phases</i>	<i>Number of animals</i>	<i>Ecdysis (%)</i>
NM	17	36.96
FQM	9	19.57
FM	9	19.57
LQM	11	23.91
Total	46	100.00

NM - new moon; FQM - first quarter moon; FM - full moon; LQM - last quarter moon.

Most prawns moulted around new moon (36.96%), followed by last quarter moon (23.91%), and first quarter and full moon (19.57% each). The moult stages also varied within the phases of the moon (Table 2) and all the stages could be observed within the moon phases. Stage A/B was highest at full moon, stage C was highest at full and last quarter moons and stage D0 was highest at first quarter and full moons, while stage D1/D2 was highest at last quarter moon. Most of the prawns were sampled around last quarter moon, followed by full and first quarter moons, and new moon was the lowest (Table 2).

Table 2

The percentage of *Penaeus monodon* sampled at different moult stages at four lunar phases

Lunar phases	Moult stages (%)				
	A/B	C	D0	D1/D2	Total
NM	2.17	2.17	2.17	8.70	15.22
FQM	4.35	2.17	8.70	4.35	19.57
FM	10.87	6.52	8.70	4.35	30.43
LQM	2.17	6.52	6.52	19.57	34.78
Total	19.57	17.39	26.09	36.96	100.00

NM - new moon; FQM - First quarter moon; FM - full moon; LQM - last quarter moon.

The abundance of LOS cells. All prawns tested (100%) demonstrated spheroid cells within the lymphoid organ. In the experimental aquaria, the STT ratio ranged from 9.1 to 91.9% ($46.6 \pm 20.0\%$). There were 76.1% of the prawns showed vacuoles within the spheroid cells. The prevalence of vacuolated spheroids varied between 0.0 and 84.6% ($18.8 \pm 22.1\%$). The number of vacuoles per spheroid ranged between 0.0 and 2.3 (0.4 ± 0.6). Moribund or dead prawns before injection in control group had STT ratio of about 5.1–54.6% ($26.5 \pm 17.6\%$) while the GAV infected group before injection had STT ratio around 9.5–37.1% ($24.5 \pm 13.0\%$). Six prawns, which were moribund or dead just after inoculation with GAV, had an STT ratio ranging from 9.1 to 67.9% ($37.9 \pm 15.1\%$). Six prawns from the stock tanks showed the ratio of STT varied from 36.7 to 70.4% ($57.2 \pm 14.2\%$).

Days post-injection (dpi) and the LOS cells. The inconsistency of the STT ratio during the days post-injection (dpi) appeared during the experiment (Figure 2). The STT ratio was lowest at 1–5 dpi, increased and reached a peak at 6–10 dpi and slightly decreased at 11 – onward dpi. While the prevalence of vacuolated spheroid and the number of vacuoles within the spheroids increased with the number of days post-injection.

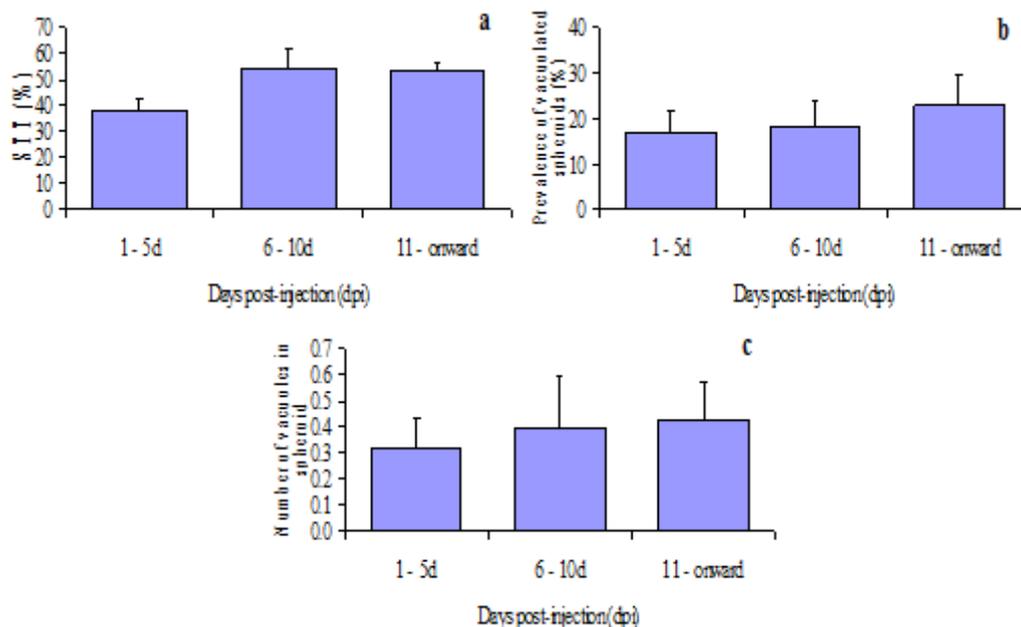


Figure 2. Mean (\pm SE) the spheroid to total tissue (STT) ratio (a), the prevalence of vacuolated spheroids (b) and the number of vacuoles in spheroids (c) of *Penaeus monodon* at various days post-injection (dpi).

It was apparent that the more days post-injection of the prawns, the higher the prevalence of vacuolated spheroids and the number of vacuoles in the spheroids.

However, the univariate analysis of variance demonstrated that there was no significant effect of days post-injection on the STT ratio ($F = 0.228$; $df = 1, 45$; $P > 0.05$), the prevalence of vacuolated spheroids ($F = 0.332$; $df = 1, 45$; $P > 0.05$) or the number of vacuoles in the spheroid cells ($F = 0.212$; $df = 1, 45$; $P > 0.05$). The interaction between the days post-injection and the other independent variables could not be analyzed due to losses of degrees of freedom (df).

Moult stages and the LOS cells. The ratio of STT, the prevalence of vacuolated spheroid and the number of vacuoles in spheroid cells varied within the stage of moult. The ratio of STT, the prevalence of vacuolated spheroids and the number of vacuoles were highest in stage C (Figure 3). The lowest of the STT ratio and the prevalence of vacuolated spheroids were in stage D0, while the lowest number of vacuoles was in stage A/B.

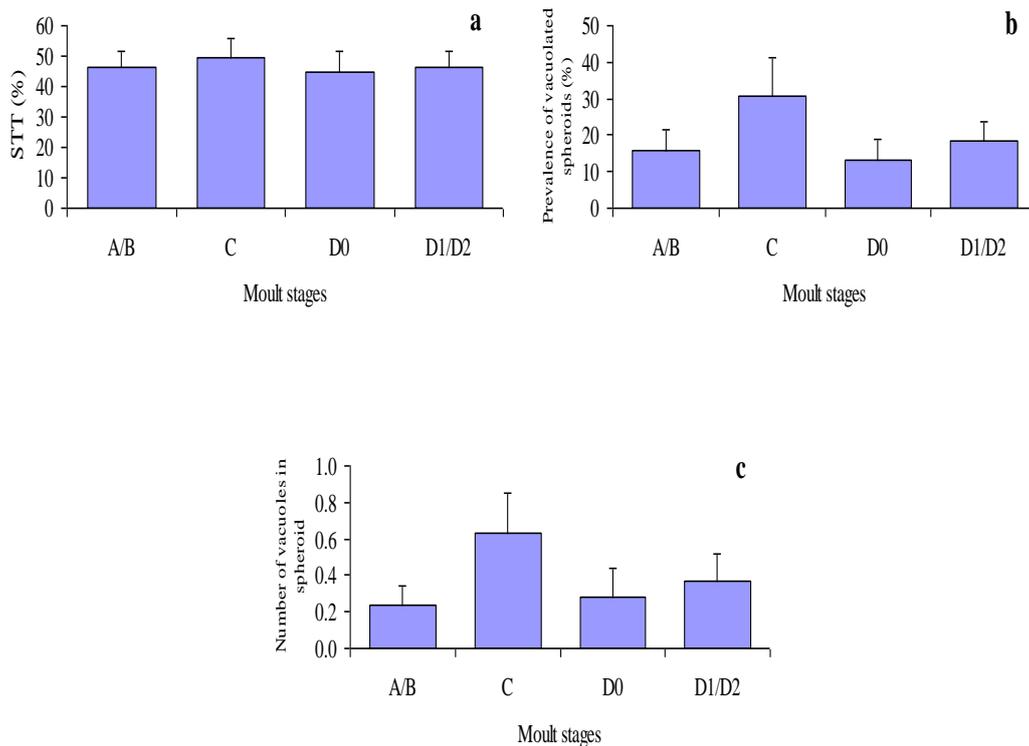


Figure 3. Mean (\pm SE) the spheroid to total tissue (STT) ratio (a), the prevalence of vacuolated spheroids (b) and the number of vacuoles in the spheroids (c) of *Penaeus monodon* at different moult stages.

However, statistical analysis showed that moult stages had no significant effect on the ratio of STT ($F = 1.438$; $df = 3, 45$; $P > 0.05$), the prevalence of vacuolated spheroid ($F = 0.888$; $df = 3, 45$; $P > 0.05$), or the number of vacuoles in the spheroids ($F = 0.974$; $df = 3, 45$; $df > 0.05$). The interaction between moult stages and treatments also revealed no significant effect on the ratio of STT ($F = 0.045$; $df = 1, 45$; $P > 0.05$), the prevalence of vacuolated spheroids ($F = 1.297$; $df = 1, 45$; $P > 0.05$) or the number of vacuoles in spheroids ($F = 2.244$; $df = 1, 45$; $P > 0.05$). However, moult stages interactions with the other independent variables could not be analyzed due to loss of the degrees of freedom.

Lunar phases and the LOS cells. The variability of the STT ratio, the prevalence of vacuolated spheroids and the number of vacuoles in the spheroid within the lunar phases was also evident during the experiments (Figure 4). It was found that the ratio of STT was highest at last quarter moon, while the prevalence of vacuolated spheroids and the number of vacuoles inside the LOS were highest at new moon.

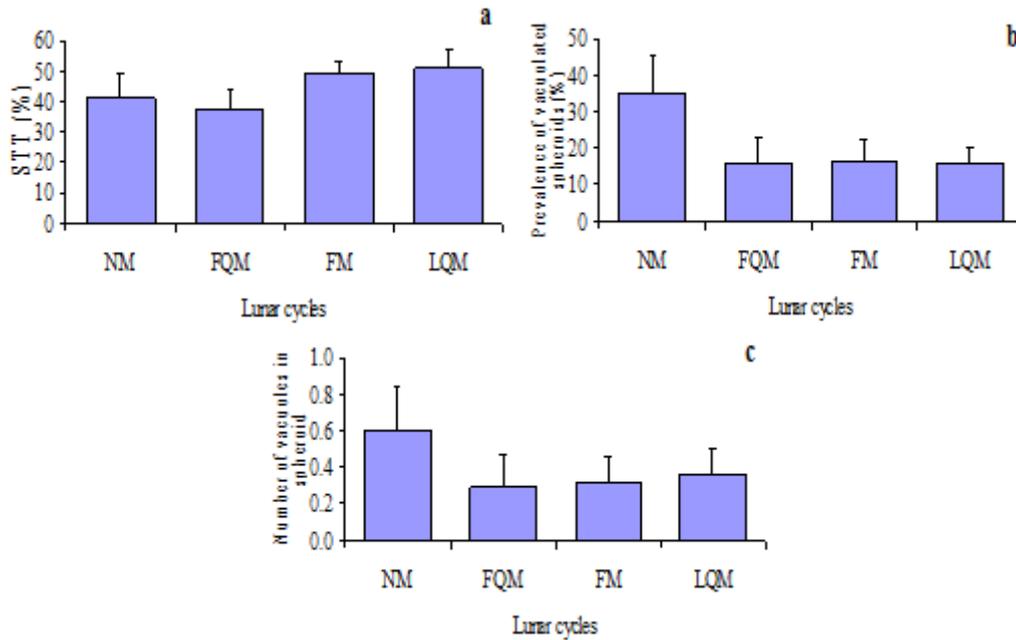


Figure 4. Mean (\pm SE) the spheroid to total tissue (STT) ratio (a), the prevalence of vacuolated spheroids (b) and the number of vacuoles in spheroids (c) of *Penaeus monodon* at four lunar phases. NM, new moon; FQM, first quarter moon; FM, full moon and LQM, last quarter moon.

As a single factor, lunar phase had no significant effect on the ratio of STT ($F = 2.434$; $df = 3, 45$; $P > 0.05$), the prevalence of vacuolated spheroids ($F = 1.302$; $df = 3, 45$; $P > 0.05$) or the number of vacuoles in the spheroid ($F = 1.385$; $df = 3, 45$; $P > 0.05$). However, as it is illustrated in Figure 5, the relationship between the LOS cells and lunar phase was being masked by the interaction between lunar cycle and the treatments. In the control group, the ratio of STT was lowest at the new moon and highest at the full moon. On the other hand, in the infected (GAV) group the ratio of STT was lowest at first quarter moon and highest at last quarter moon. This interaction between lunar phases and treatment on the ratio of STT was significant ($F = 6.938$; $df = 1, 45$; $P < 0.05$), but not on the prevalence of vacuolated spheroids ($F = 0.599$; $df = 1, 45$; $P > 0.05$) or the number of vacuoles in spheroids ($F = 0.487$; $df = 1, 45$; $P > 0.05$).

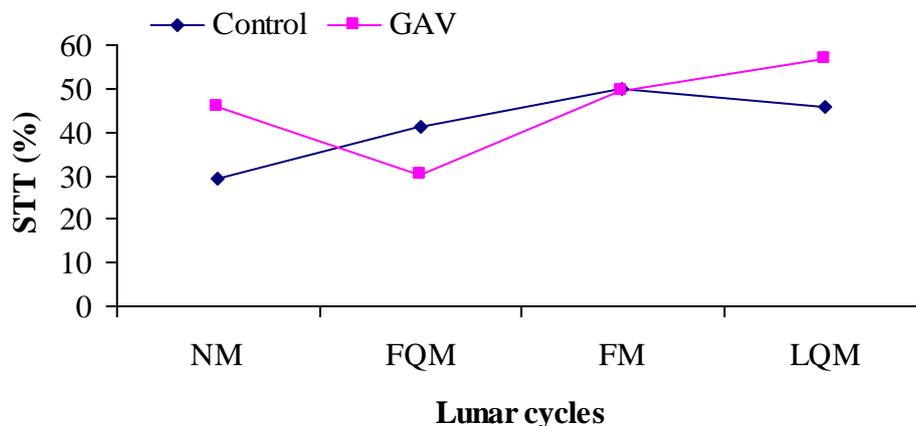


Figure 5. The spheroid to total tissue (STT) ratio of *Penaeus monodon* at four lunar phases with two different treatments.

The STT ratio ($F = 3.010$; $df = 1, 45$; $P > 0.05$), the prevalence of vacuolated spheroids ($F = 0.077$; $df = 1, 45$; $P > 0.05$) and the number of vacuoles in spheroids ($F = 0.204$; $df = 1, 45$; $P > 0.05$), the lunar phase interaction with sexes were not statistically significant. Again, due to loss degrees of freedom, the lunar phase interactions with the other independent variables could not be analyzed.

Treatments and the LOS cells. The ratio of spheroid cells, the prevalence of vacuolated spheroids and the number of vacuoles in the spheroid cells was different in each treatment (Figure 6). ANOVA demonstrated that there was a significant difference between treatments on the ratio of STT ($F = 6.032$; $df = 1, 45$; $P < 0.05$). The STT ratio was significantly higher in infected (presumptive GAV) group than in the control group. The prevalence of vacuolated spheroids and the number of vacuoles in spheroid were also higher in GAV infected group than control group, but not significantly for the prevalence of vacuolated spheroids ($F = 0.041$; $df = 1, 45$; $P > 0.05$) or the number of vacuoles in spheroids ($F = 0.005$; $df = 1, 45$; $P > 0.05$). Treatment interactions with the other independent variables could not be analyzed due to loss of degrees of freedom.

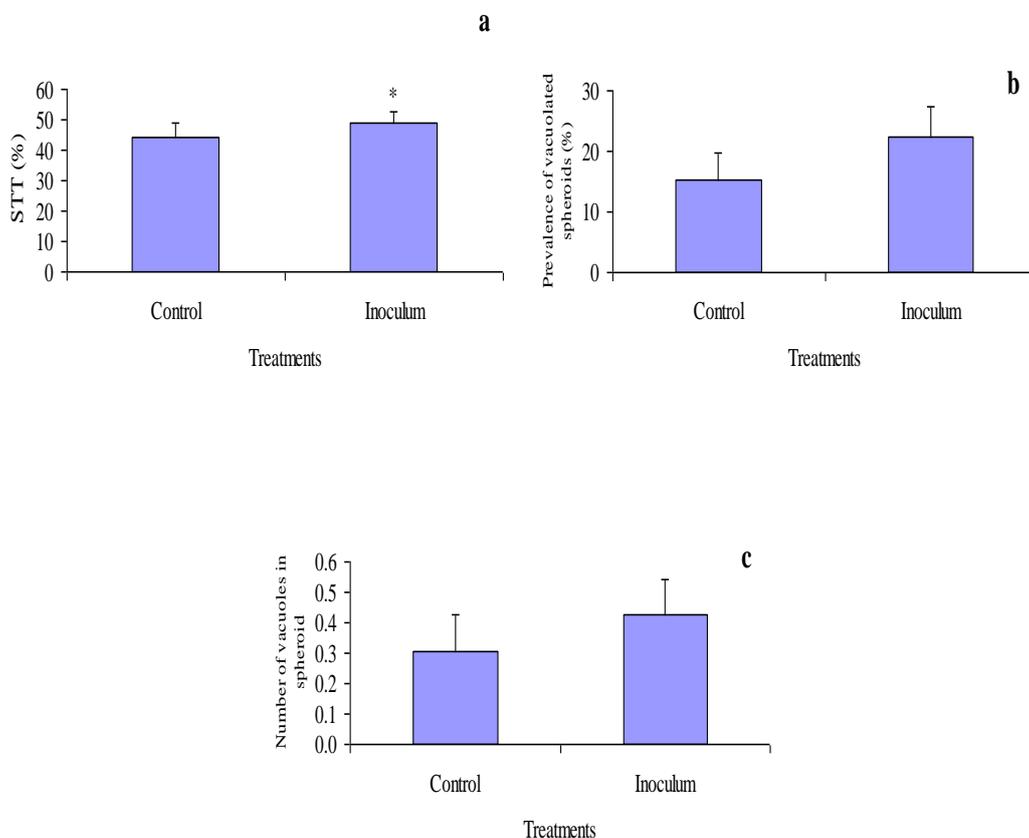


Figure 6. Mean (\pm SE) the spheroid to total tissue (STT) ratio (a), the prevalence of vacuolated spheroids (b) and the number of vacuoles in spheroids (c) of *Penaeus monodon* with two different treatments. * Significant difference ($P < 0.05$).

Sexes (Figure 7) had a significant effect on the spheroid to total tissue ratio of *P. monodon* ($F = 12.798$; $df = 1, 45$; $P < 0.05$) in this study. The STT ratio in females was significantly higher than in males. In contrast, the prevalence of vacuolated spheroids and the number of vacuoles in the spheroid were not significantly different ($F = 0.439$; $df = 1, 45$; $P > 0.05$ and $F = 0.910$; $df = 1, 45$; $P > 0.05$, respectively). Interactions between sex and other independent variables could not be analyzed due to the loss of degrees of freedom.

The size of the animals (weight and total length) had no significant effect on the ratio of STT ($F = 0.000$; $df = 1, 45$; $P > 0.05$ and $F = 0.057$; $df = 1, 45$; $P > 0.05$), the prevalence of vacuolated spheroids ($F = 2.682$; $df = 1, 45$; $P > 0.05$ and $F = 2.231$; $df = 1, 45$; $P > 0.05$) and the number of vacuoles in the spheroids ($F = 1.931$; $df = 1, 45$; $P > 0.05$ and $F = 1.750$; $df = 1, 45$; $P > 0.05$). There was a weak Pearson's correlation between the STT ratio and the prevalence of vacuolated spheroids ($r = 0.318$, $P = 0.031$). A weak correlation also appeared between the ratio of STT and the number of vacuoles in LOS cell ($r = 0.318$, $P = 0.008$). However, there was a strong correlation ($r = 0.933$, $P = 0.000$) between the prevalence of vacuolated spheroids and the number of vacuoles in the spheroids.

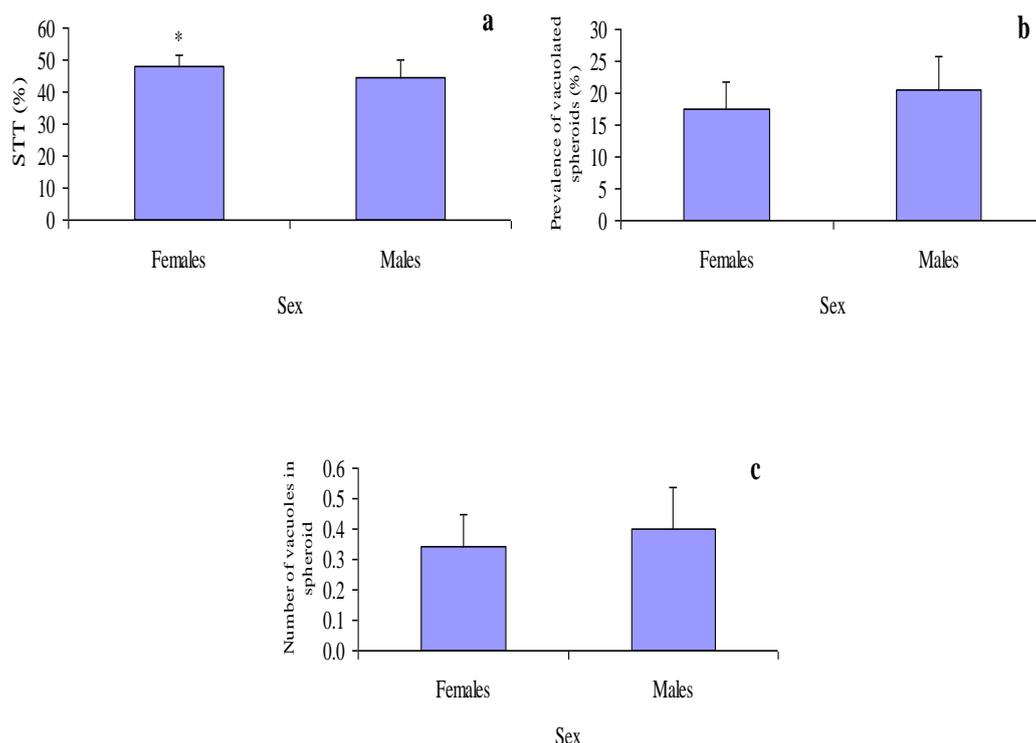


Figure 7. Mean (\pm SE) the spheroid to total tissue (STT) ratio (a), the prevalence of vacuolated spheroids (b) and the number of vacuoles in spheroids (c) of *Penaeus monodon* in both sexes. * Significant difference ($P < 0.05$).

Discussion. Initiation of non-pathognomonic signs for GAV infection as described by previous researchers (Spann et al 1995; Spann et al 1997; Spann et al 2000; Callinan & Jiang 2003; Callinan et al 2003) could also be observed during the experiment both in stock tanks and experimental aquaria. Moreover, undeveloped setae and unsynchronized development between setae and the retraction of epidermis from the setal bases suggested that these prawns might experience long moulting cycles and slow growth. Spann et al (1997) observed that prawns infected with GAV ceased moulting. However, the present study suggested that prawns had prolonged moulting activity. Therefore, the gross sign of diseases in prawns is not a good indicator of viral infection due to inconsistency of the signs, which could lead to subjectivity and other viral diseases could produce the same signs (Cowley et al 2004; de la Vega et al 2004). Furthermore, prawns infected by viral diseases sometimes do not show any gross sign of diseases (Bonami et al 1992; Nadala et al 1992; Spann et al 1995; Flegel et al 2004; Sritunyalucksana et al 2006).

Mass mortality in the stock tanks, mortality in experimental aquaria before prawns were injected either with PBS or viral free extract and the presence of LOS cells in every

single prawn indicated that these prawns have been exposed to viral infection related changes to the lymphoid organ at the farm before the experimental treatments. LPV (Owens et al 1991), LOVV (Bonami et al 1992), TSV (Hasson et al 1995), YHV (Lu et al 1995), and LOV later called GAV (Spann et al 1995; Spann et al 1997) were amongst viral diseases of penaeid prawns associated with spheroid cells within the lymphoid organ. Using RT-nPCR to screen the prevalence of GAV in *P. monodon* broodstock from different sources, Cowley et al (2000a) found that around 98% of the broodstock which supplied Australian hatcheries was infected with GAV. This promoted the vertical transmission of GAV at spawning to the larvae used in Australian farms (Cowley et al 2002b).

In addition, pathological and epidemiological studies of peripheral neuropathy and retinopathy (PNR) in farmed *P. monodon* strongly pointed out GAV as the causative agent of the disease (Callinan & Jiang 2003; Callinan et al 2003). Spann et al (2003) claimed that generally healthy cultured *P. monodon* in Australia had chronic GAV infection. A study by Munro & Owens (2005) found that the prevalence of GAV in *P. monodon* was 95%, while in *P. esculentus*, *P. merguensis*, *P. longistylus*, and *Cherax quadricarinatus* it was 0%. Furthermore, Munro (personal communication) claimed that most of cultured *P. monodon* in Australia had GAV infection without any gross signs of diseases. Furthermore, apparently *P. monodon* was the natural host and GAV was endemic in Australia (Cowley et al 2000a; Spann et al 2000). Therefore, it was most likely that these experimental prawns were also infected with GAV.

The possibility of bacterial infection as secondary pathogen in this experiment was indicated by the presence of granulomatous reaction in the lymphoid organ, hepatopancreas and gills. This inflammatory type reaction resembled melanized nodule formation as described by Egusa et al (1988) in *P. japonicus* infected *Vibrio* sp. These melanized nodules were formed as a protective response to bacterial infection, to confine the infected tubules and to prevent bacterial spreading (Egusa et al 1988; Jiravanichpaisal & Miyazaki 1994). It was apparent that the lymphoid organ as well as hepatopancreas and gills were highly vulnerable to bacterial infection. Furthermore, this inflammatory reaction, once again confirmed that the spheroid cells were not produced due to bacterial infection, contrary to the published work of Van de Braak et al (2002) and Alday-Sanz et al (2002) but as a major mechanical defense against viral infection (Anggraeni & Owens 2000; Rusaini & Owens 2010a,b). It was not surprising that a perfect combination between GAV and bacterial infection might overwhelm the protective responses that led to the death of the infected animals.

Stress during transportation, a decrease of salinity from 40 ppt at the farm to 35 ppt and crowded stock tanks in the laboratory created stress and might have suppressed the immune capability of the prawns. As a result, virus might easily proliferate and bacteria as an opportunistic pathogen could also make a contribution to mortalities. Owens et al (1998) found that rapid change of salinity and low level of oxygen were associated with mortalities in prawns infected with SMV. Furthermore, de la Vega et al (2004) revealed that stress related to captivity, handling, invasive clinical procedures, and transportation could enhance viral propagation and mortalities. In the tanks, moulting and moribund infected prawns might be cannibalized by other healthy prawns that in turn infected themselves (Callinan & Jiang 2003).

As previously described (Rusaini & Owens 2010a), this present study also confirmed that the spheroid cells had no association with body weight and total length of animals. Consistent with the study of Anggraeni & Owens (2000) where the size of the prawns infected with MCMS which did not correlate with the spheroid area. Furthermore, the present investigation clearly demonstrated that the spheroids to total tissue ratio, the prevalence of vacuolated spheroids and the number of vacuoles in the spheroid had no association with moult stages. This emphasized that the fluctuation of the abundance of the spheroid cells in the lymphoid organ was not driven by moulting as proposed by Anggraeni & Owens (2000). This indicated that another factor or mechanism worked throughout the life of the prawns causing fluctuation of the spheroid cells and it appeared it was linked to the lunar cycle (Rusaini & Owens 2010a).

The ratio of STT influenced by the moon phase once again appeared in the interaction between moon phase and treatments. It was found that in the control group the STT ratio which was lowest at new moon as observed in previous studies (Rusaini & Owens 2010a), rose for the next phase, reached a peak at full moon and then decreased at last quarter moon. However in the GAV infected group, the ratio of STT was lowest at first quarter moon, increased at the next phase and had a maximum at last quarter moon and reduced at full moon. It seemed that the STT ratio in the infected group developed and eliminated a week later than in the control group. This might indicate that in prawns highly infected with GAV or other viral diseases changes in the lymphoid organ react slowly in eliminating the LOS cells compared to less infected animals.

As expected, the STT ratio of the GAV injected group was significantly higher than the control group. This indicated that even though the experimental prawns already had infections (bacteria and virus); the additional injection of cell-free extract of GAV amplified the production of the spheroid cells within the lymphoid organ. This finding was supported by the work of Anggraeni (1998) who found that the increase of spheroid cells was only associated with viral infection and not correlated to bacterial infections as stated by Alday-Sanz et al (2002) and Van de Braak et al (2002).

Sexes related differences on the abundance of the spheroid cells in penaeid prawns were not consistent with the experiment of Rusaini & Owens (2010a), which showed that there was no significant difference on STT ratio between males and females. However, this current study clearly demonstrated a female bias on the ratio of STT. Some studies revealed the inconsistency of relationship between sexes and prawns' immunodefence. On one hand, Sequeira et al (1995) reported the presence of sex related DHC in association within the cycle of the moult. Owens & O'Neill (1997) found female bias on haematological components of penaeid.

On the other hand, it was found that haematological variation was not significantly different between female and male prawns (Chen & Cheng 1993; Cheng & Chen 2001; Cheng et al 2002). Sheridan et al (2000) who investigated sex related variation in arthropods infected with parasites showed that, in general, there was no sex bias among invertebrate hosts but it could exist in any specific host-parasite system. Furthermore, these authors suggested that the interaction between the immune and endocrine system in vertebrates might not exist in invertebrates. The negative feedback system between immunodefence and the expression of sexual features and behavior provided by testosterone as observed in vertebrates was absent in invertebrates. Therefore, it was not surprising if higher parasitic infection in males was common in vertebrates but not in invertebrates. In short, it is suggested that sex-related changes in immunological components of penaeid prawns is still unclear. These discrepancies might be due to the differences in health status, pathogens, the maturation of animals, or species differences.

Conclusions. The present study clearly showed that the abundance of spheroid (LOS) cells in the lymphoid organ of *P. monodon* had no association with days post-injection, moult stages, lunar phase, and prawns size. However, a sex bias appeared on the variation of spheroid to total tissue ratio. Furthermore, presumptive GAV infection enhanced the formation of these cellular changes and this immune reactivity was only associated with viral infection but not with bacterial infection.

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