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# Differential gene expression in black tiger shrimp, *Penaeus monodon*, following administration of oxytetracycline and oxolinic acid

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# ABSTRACT

The intensification of shrimp farming systems has led to the spreading of a variety of bacterial and viral diseases that continue to plague the shrimp industry worldwide. Efforts to combat these pathogenic organisms include the use of immunostimulants, probiotics, vaccines and antibiotics. Although a few studies have already reported on the effects of various stimuli on shrimp, the effect of antibiotics, particularly on the changes in the shrimp transcriptomic profile have yet to be reported. Here we show that injecting shrimp with oxytetracycline and oxolinic acid alters the expression of genes in the black tiger shrimp, *Penaeus monodon*, lymphoid organ. These antibiotics, especially oxylinic acid, down-regulated the expression of a few immune-related genes, most notably penaeidin, proPO, clotting protein, profilin and whey acidic protein.

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# 1. Introduction

Pathogenic microorganisms are responsible for the heavy losses suffered by shrimp farms worldwide [1,2]. Diseases caused by *Vibrio* species and bacilliform virus, such as the White Spot Syndrome Virus (WSSV) for instance, can rapidly spread resulting in low survival rates in both hatchery and grow-out ponds [3]. Cumulative mortalities from these pathogens can reach 100% within days after the onset of infection [4]. Since shrimps are thought to have only their innate immune mechanisms to combat diseases, measures have been developed not only to prevent the spread of diseasecausing pathogens but also to enhance the shrimp's immune system. Such interventions include the use of immunostimulants [5,6], probiotics [7], vaccines [8] and antibiotics [9].

Antibiotics are used in shrimp culture for both therapeutic and prophylactic purposes [10]. Two of the most common antibiotics used in shrimp are oxytetracycline and oxolinic acid. Oxytetracycline (OTC) is active against a wide variety of bacterial species including Gram-negative and Gram-positive aerobic and anaerobic bacteria. Its popularity is due to its effectiveness, relative safety, low rate of accumulation in edible tissue and short tissue elimination time [11]. OTC is also one of the only four antimicrobial agents approved by the US Food and Drug Administration for use in food fish and only one of the few drugs approved for use in invertebrates [12]. It is used against many bacterial diseases including vibriosis and necrotizing hepatopancreatitis infections [13]. Oxolinic acid (OA), a quinolone, on the other hand is an antibiotic effective against a variety of Gram-negative bacteria and works primarily by inhibiting the synthesis of bacterial DNA [14]. It is used mainly in shrimp farms in Asia to treat vibriosis [9,10,15].

The effects of antibiotics on innate immunity, specifically on the expression of shrimp genes, have yet to be reported. There are, however, certain chemicals which were reported to suppress the shrimp immune system making shrimp vulnerable to pathogens [16].

Microarray technology has been used to analyze the changes in gene expression after DNA vaccination [17], stimulation [18], exposure to a chemical pollutant [19], and during larval development [20] of various fish species. In shrimp, previous reports have highlighted the use of microarray in identifying the genes that

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were differentially expressed after WSSV infection[21] and immunostimulation with peptidoglycan [22].

We previously identified a large number of genes by expressed sequence tag (EST) analysis of normal and WSSV-infected haemocytes of kuruma shrimp, *Marsupenaeus japonicus* [23] and haemocytes of black tiger shrimp [24]. In this study we used a microarray covering 2036 of these genes to examine the transcriptomic profile, particularly of immune-related genes, of black tiger shrimp lymphoid organ after administration of oxytetracycline and oxolinic acid.

# 2. Materials and methods

# 2.1. Shrimp

Apparently healthy black tiger shrimp, weighing 12–15 g were used in this experiment. They were checked for bacterial and viral infection prior to the administration of antibiotics and were kept in optimal rearing condition throughout the experiment.

# 2.2. Construction of the cDNA microarray Chip

A total of 2036 unique genes, most of which were prepared from previous EST analysis results [23,24] make up the array chip. The selection of genes spotted onto the microarray slide was carried out carefully to eliminate duplication. Fragments of these genes were then amplified, purified and concentrated before they were sent to the company (NGK Insulators, Limited, Nagoya, Japan) for spotting.

Each gene was spotted twice while housekeeping genes, like  $\beta$ actin and imoti, and lambda DNA were also spotted onto each slide to serve as controls. The same array was used in our previous works on shrimp [25,26].

# 2.3. Administration of antibiotics

Shrimp were administered 330  $\mu$ g/g body weight of oxytetracycline or 330  $\mu$ g/g body weight of oxolinic acid suspended in PBS. Control samples were injected with equal volume of PBS. Samplings were done at 1, 3, and 7 days after administration of antibiotics. Ten (10) shrimp per group were taken at each sampling period. The lymphoid organs were removed, stored in RNA later (TAKARA, Japan) and kept at -80 °C until use.

#### 2.4. Microarray analysis

# 2.4.1. Preparation of lymphoid organ cDNA for microarray analysis

Total RNA from lymphoid organs was isolated using TRIzol (Invitrogen Life Technologies, USA) following the manufacturer's instructions. cDNA was prepared from total RNA, labeled indirectly with aminoallyl-dUTP (LabelStar Array Kit, cDNA Labeling Module, Qiagen, USA) and purified using QIAquick Spin columns (QIAquick PCR Purification Kit, Qiagen, USA) following manufacturer's protocol.

The purified cDNAs were then precipitated with 0.2 M sodium bicarbonate, labeled with monofunctional dyes, Cy5 (Amersham, USA) for the antibiotics-injected samples and Cy3 (Amersham, USA) for control samples and were then purified using MinElute Spin columns (LabelStar Array Kit, cDNA Cleanup Module, Qiagen, USA) prior to hybridization.

# 2.4.2. Hybridization of labeled cDNA to the microarray chip

Cy3 and Cy5 labeled cDNAs were mixed with the hybridization buffer with formamide and hybridized to the microarrays for 16– 18 h at 42 °C. The subsequent washing procedures were carried out following previous protocols with modification [27,28].

#### 2.4.3. Scanning and visualization

The microarray slide glass was scanned with GenePix 4000B array scanner (Axon Instruments, Inc) and the resulting images were then analyzed using GenePix Pro 4.0 array analysis software (Axon Instruments, Inc.). Signal intensities were normalized and calculated following Kurobe et al. [27]. The median signal intensity and the ratio of medians were used as appropriate measures of gene expression. The ratio was calculated from the signal intensity of test or stimulated genes divided by the signal intensity of the control. Genes with a ratio of at least 2.0 were considered to be up-regulated while those with ratios of 0.5 or lower were considered to be down-regulated. Both expressed and differentially expressed genes were classified accordingly based on the similarity of their deduced functions.

#### 2.4.4. Clustal and TreeView analysis

The microarray data was further analyzed by Cluster Program 3.0 (Clustering library version 1.27) using the average linkage hierarchical clustering algorithm with Euclidian distance as the similarity metric [29]. The cluster analysis results were visualized with TreeView program (version 1.60) [30]. Genes belonging to specific clusters were grouped and classified further according to their deduced functional categories.

#### 3. Results

# 3.1. Differential expression

The percentage of genes expressed in every sampling period for both antibiotics did not vary significantly (Table 1). The number of differentially expressed genes (i.e., genes that were either upregulated or down-regulated by a factor of two after antibiotic administration) and the percentage of differentially expressed genes after OA administration, however, was more than those after OTC administration (Table 1). The number of differentially expressed genes is highest at day 3 for antibiotics, 55% for OA and 46.2% for OTC. It is interesting to note that the number of upregulated genes is increased after day 1 p.a. for both antibiotics with the increase most noticeable between days 1 and 3. In contrast down-regulated genes in OA decreased in every sampling period while down-regulated genes for OTC decreased after day 3.

Grouping together the genes according to their deduced functions, we found that the number of up-regulated genes having immune-related functions increased at day 3 after administration of both antibiotics (Table 2). Conversely, down-regulated immunerelated genes decreased at day 3 after administration of both antibiotics. The same trend was also found for genes involved in metabolism and catabolism, genes having various cellular activities, ribosomal RNA genes and genes with unknown functions, whose number of up-regulated genes is increased at day 3 after administration of both antibiotics.

## 3.2. Effect of antibiotics on immune-related genes

Administration of antibiotics appeared to down-regulate a number of immune-related genes. Prophenoloxidase (proPO), clottable protein and  $\beta$ -thymosin were down-regulated by both antibiotics at day 3 and/or 7, while penaeidin, haemocyanin, whey acidic protein, and profilin were down-regulated by both antibiotics at day 1 (Table 3). The expression of other immune-related genes, meanwhile remained unchanged or even up-regulated after day 1 p.a. including  $\alpha$ -2 macroglobulin and TNF-induced protein.

## 3.3. Clustering and functional analysis

Three major clusters were identified. One cluster included genes that were consistently up-regulated by OTC but is down-

# Table 1

Overview of the black tiger shrimp transcriptomic profile following OTC and OA injection.

	Sampling period								
	Day 1		Day 3		Day 7				
	OA	OTC	OA	OTC	OA	OTC			
Total number of spotted genes	2036	2036	2036	2036	2036	2036			
Total number of genes expressed	925	1113	900	807	932	741			
Percentage of expressed genes	45.43	54.67	44.20	39.64	45.78	36.39			
Up-regulated genes ( $\geq$ 2-fold increase in expression)	7	17	145	110	156	104			
Known genes	3	7	44	35	50	34			
Unknown genes	4	10	101	75	106	70			
Down-regulated genes ( $\leq$ 0.5-fold decrease in expression)	401	234	350	263	229	162			
Known genes	149	86	158	119	120	79			
Unknown genes	252	148	192	144	109	83			
Total number of differentially expressed genes	408	251	495	373	385	266			
Percentage of differentially expressed genes	44.11	22.55	55.00	46.22	41.31	35.90			
Total number of genes expressed (by both antibiotic treatments)	738		607		576				
Up-regulated genes (by both antibiotic treatments)	0		53		67				
Known genes	0		6		20				
Unknown genes	0		47		47				
Down-regulated genes (by both antibiotic treatments)	128		190		114				
Known genes	39		92		58				
Unknown genes	89		98		56				
Total number of differentially expressed genes	128		243		181				
Percentage of differentially expressed genes	17.34		40.03		31.42				

#### Table 2

Overview of the expressed and differentially expressed genes of black tiger shrimp following OTC and OA injection grouped according to their deduced function. Group 1 (immune-related genes); Group 2 (metabolism and catalytic activity); Group 3 (various cellular functions); Group 4 (ribosomal RNA); Group 5 (unknown function).

Group	Day 1						Day 3					Day 7						
	Oxolinic acid			Oxytetracycline		Oxolinic acid		Oxytetracycline		Oxolinic acid			Oxytetracycline					
	Expressed	Up	Down	Expressed	Up	Down	Expressed	Up	Down	Expressed	Up	Down	Expressed	Up	Down	Expressed	Up	Down
1	45	1	19	57	3	12	44	5	17	45	4	11	48	11	12	40	4	11
2	53	0	24	60	1	14	46	5	24	46	6	16	51	8	21	44	5	14
3	156	1	67	188	1	50	159	26	63	138	16	56	166	24	45	127	18	32
4	103	1	39	101	2	10	107	8	54	88	9	36	110	7	42	91	7	22
5	568	4	252	707	10	148	544	101	192	490	75	144	557	106	109	439	70	83
Total	925	7	401	1113	17	234	900	145	350	807	110	263	932	156	229	741	104	162

regulated by OA from day 1 to day 3. This cluster included 37 genes, 26 of which have unknown functions (Supp Fig 1a). Another cluster included genes up-regulated in day 1 after administration of both antibiotics but is down-regulated at days 3 and 7 by OA. This

cluster included 28 genes, 15 of which have unknown functions (Supp Fig 1b). The third cluster included genes down-regulated at day 1 by both antibiotics. This cluster included 78 genes, 44 of which have unknown function. This cluster had the highest

#### Table 3

Effect of antibiotics administration on some immune-related genes in black tiger shrimp. Up-regulated and down-regulated genes are defined as genes whose expression differed by a factor of two or more.

Name	ID	OTC d1	OTC d3	OTC d7	OA d1	OA d3	OA d7
Penaeidin-3c precursor	sh834	0.413	0.984	0.683	0.328	0.650	0.763
Whey acidic protein	sh889	0.417	0.520	0.889	0.236	0.122	0.483
C-type lectin	HpaN0076	0.426	0.531	0.673	0.358	0.994	0.410
Protein-kinase c inhibitor	sh771	0.468	0.486	0.572	0.513	0.565	0.739
Profilin	N133	0.511	0.503	0.475	0.289	0.314	0.442
Thymosin beta-9	sh1028	0.519	0.510	0.585	0.297	0.280	0.363
Hemocyanin	HpaN0002	0.523	0.734	0.72	0.455	1.155	0.794
Proteinase inhibitor-signal crayfish	sh610	0.659	1.209	0.98	0.739	2.335	0.928
Alpha2-macroglobulin homolog	KS-2-10_A04	0.793	3.185	2.739	1.207	2.118	2.936
26S proteasome regulatory subunit	sh622	0.831	2.171	N.D.	0.977	1.505	1.553
Thymosin beta-11	sh960	0.907	0.456	0.514	0.425	0.480	0.529
Clottable protein	sh971	0.908	0.334	0.579	0.482	0.236	0.41
TNF-induced protein	LPN0015	0.959	2.155	1.683	0.651	1.635	2.666
Serine protease	LPN0449	1.047	0.985	1.23	0.322	1.17	1.413
Prophenoloxidase	sh685	1.114	0.351	0.401	0.994	0.242	0.205
Cathepsin L	LPN0255	1.121	1.262	1.899	0.5	1.133	3.083
RING-box protein 2 (Rbx2)	MJ_H_1_190	1.141	1.511	1.187	N.D.	1.127	2.011
Penaeidin-2 precursor	sh1018	1.276	N.D.	N.D.	0.448	N.D.	N.D.
11.5 kDa antibacterial protein	sh970	1.394	1.987	1.577	1.474	1.953	2.108
ferritin	N407	1.503	2.413	2.287	1.37	3.442	5.957
Kazal type 2	PJV413f	4.879	N.D.	N.D.	0.928	2.136	2.102
β-thymosin	N318	N.D.	0.243	0.275	N.D.	0.057	0.17

number of immune-related genes (5) from among the three clusters (Supp Fig 1c). The complete list of genes included in the three clusters as well as their deduced functions is described in supplementary Table 1.

Other major functions in cluster 1 were ribosomal (8%) and various cellular functions (14%). In cluster 2, the major functions were metabolism and catalytic activity, ribosomal (8%) and various cellular functions, which made up 42% of the total genes in the cluster. In cluster 3, the major functions were ribosomal (12%) and various cellular functions (19%). The percentages of genes with immune-related functions in the three clusters were 5%, 4%, and 7%, respectively.

# 4. Discussion

The shrimp lymphoid organ is a primary site of bacterial accumulation and bacteriostasis [31,32] and plays a role in the elimination of viral particles and other infectious agents [33,34]. Here we showed that the expression of several genes in the shrimp lymphoid organ including those that are involved in immune response was altered after administering antibiotics. The effects of the two antibiotics, however, are slightly different from each other. An earlier study on the pharmacokinetics of OA and OTC showed that both antibiotics were rapidly and widely distributed to tissues outside of the haemolymph [15]. OA, however, had a larger volume of distribution which suggested that OA is more extensively distributed than OTC [15]. OA was also found to be eliminated more slowly than OTC [15]. This may explain why there were far more differentially expressed genes in the lymphoid organ of OAtreated samples, particularly down-regulated genes. This may also account for the higher number of down-regulated immune-related genes in OA than in OTC. Although dopamine have been shown to suppress the immune system of shrimp [16], to our knowledge this is the first report on the immunosuppressive effect of antibiotics on shrimp genes.

Immune-related genes, most notably penaeidin, proPO, clotting protein, profilin and whey acidic protein, were significantly downregulated especially by OA. Penaedins were first isolated in pacific white shrimp, *Litopenaeus vannamei*, and display antimicrobial activity against fungi and bacteria with a predominant activity against Gram-positive bacteria [35] and have been proposed to be linked with the surviving capacity of shrimp [36]. ProPO, meanwhile is an essential component in melanin synthesis and in crustaceans was shown to be important against bacterial pathogens [37,38]. Most importantly, both the penaeidins and ProPO are mainly produced in the haemocytes [39,40].

The presence of these two genes could be because (1) shrimp lymphoid organ was found to contain many haemocytes [32], (2) lymphoid organ cells also share similar characteristics to semigranular and, in particular, large granular haemocytes and (3) had phenoloxidase activity [41]. The clotting protein is a gene involved in haemolymph coagulation in shrimp and its absence renders shrimp susceptible to viral and bacterial pathogens [42]. The lymphoid organ was reported to be one of the major tissues producing this gene [43]. Profilin is a protein important for regulating actin polymerization essential for many cellular processes and in shrimp it was found to be up-regulated after Vibrio challenge [44]. On the other hand, genes having whey acidic protein domains shown to possess antimicrobial properties [45] are expressed at a higher level in virus-resistant shrimp and is highly up-regulated during the early phase of WSSV infection [46].

In addition,  $\alpha$ 2-macroglobulin, Rbx2 and 11.5 kDa antibacterial protein genes were up-regulated in both the antibiotics administration and our previous PG stimulation study. In contrast, four (4) genes; thymosin beta-9, clottable protein, ProPO, and thymosin

beta-11, were up-regulated in PG stimulated shrimp [22], but these 4 genes were down-regulated by the antibiotics (OA or OTC) administration. This inverse expression patterns of the 4 immunerelated genes provided further support that the administration of antibiotics is associated with suppression of immune function in shrimp.

The down-regulation of genes involved in actin microfilament formation such as profilin and thymosin may also affect the capacity of lymphoid organ cells to phagocytose bacteria. Further work, however, is needed to verify this assumption.

The down-regulation of genes involved in the immune response in shrimp by OA and OTC has also been observed in other cultured species, such as fish. OA has been reported to suppress mitogenic response of rainbow trout (*Oncorhynchus mykiss*) head kidney cells [47] although at the recommended treatment doses, it did not suppress the nonspecific and specific immune responses of salmonids [48]. Likewise, OTC has been shown to down-regulate rainbow trout head kidney cells response to mitogens [47], delay mitogenic response and decrease serum immunoglobulin levels in carp [49,50], and reduce both nonspecific and specific immune response activities in salmonids [48]. The apparent similarity in immunosuppressive actions of OA and OTC in two very different immune systems (invertebrates and vertebrates) suggests that the effect of the antibiotics is wide-ranging and thus warrants further investigation.

In summary, we showed that antibiotics used to treat and prevent microbial infections in shrimp affect the shrimp transcriptomic profile and down-regulate the expression of a few immune-related genes.

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# Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2009.05.010.

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