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Integrating the *Vibrio*-resistance phenotype and gene expression data for discovery of markers used for resistance evaluation in the clam *Meretrix petechialis*

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ABSTRACT

In this study, gene expression data were used to select resistance-associated markers that indicate resistance phenotypes in the clam *Meretrix petechialis*. A total of 1772 clams from 23 full-sibling families were subjected to a *Vibrio* challenge test, and the results showed significant variation in resistance to vibriosis among different families. Three of the most resistant families and three of the most susceptible families were selected for screening of resistance-associated markers. Then, among 22 genes, 12 demonstrated a significant change in expression, and their expression levels were further analysed by *t*-test and linear regression analyses, respectively, between resistant and susceptible families. Overall, the results showed that the basal expression levels of *Big-Def*, *BIRC7*, *NFIL3*, *CTL9* and *Bax* were associated with *Vibrio*-resistant phenotypes and could be considered candidate resistance-associated markers. Further, two *M. petechialis* strains that differed in *Vibrio* resistance were used to examine the effectiveness of these five resistance-associated genes. The results further confirmed that the expression of *BIRC7* and *NFIL3* were associated with *Vibrio*-resistant phenotypes. This study integrated the *Vibrio*-resistance phenotype and gene expression data for resistance evaluation and provided potential markers for the genetic breeding of high *Vibrio*-resistance strains in the clam *M. petechialis*.

1. Introduction

The clam *Meretrix petechialis* (described previously as *M. meretrix*) is a commercially important species cultured in China (Liu et al., 2006). Outbreaks of vibriosis in molluscs often cause mass mortality and extensive economic losses in clam culture. In our previous work we found that the genetic variation of *Vibrio* resistance is ubiquitous, and moderate heritability was estimated for this trait in the clam *M. petechialis* (Liang et al., 2017). Thus, systematic breeding for genetic improvement of *Vibrio* resistance is a feasible and sustainable approach for control of this infectious disease. However, the study of disease resistance and its incorporation into breeding programmes have been hindered by the difficulty in measuring appropriate phenotypes accurately (Bishop and Woolliams, 2014).

To date, traits such as disease resistance are usually measured directly using challenge trials, and the mortality or survival rate are used as phenotypic scores. Breeding for the resistance trait is still costly and difficult as mentioned above. Therefore, marker-assisted selection (MAS) conducted directly on the selection candidates is a viable alternative approach for resistance selection and does not require exposing the clams to infection in a challenge test. However, when MAS was used in the breeding programme, markers needed to be in close linkage with the target trait or large numbers of markers needed to be genotyped for genome wide selection (GWS) (Robinson and Hayes, 2008). Although some association analyses between the molecular markers and *Vibrio* resistance have been reported in *M. petechialis* (Nie et al., 2013, 2015; Wang et al., 2011; Zou and Liu, 2016), most selected markers only account for a small fraction of the total genetic variation in survival. Thus, more effort is needed to improve the effectiveness of the markers.

Analyses of gene expression profiles as an indirect test for disease

Abbreviations: ACP, acid phosphatase; AKP, alkaline phosphatase; Bax, apoptosis regulator Bax; BIRC7, baculoviral IAP repeat-containing protein 7 isoform X3; Bcl2-l2, Bcl-2 like 2 protein; Big-Def, Big defensin; C1ql, C1q-like 23 kDa protein; Cas3/9, caspase 3/9; Cas3a, caspase 3a; Cas1, caspase-1; Cas2, caspase-2; Cas8, caspase-8; C1q, complement 1 q protein; Bf, complement factor B; CTL9, C-type lectin 9; CTLD2, C-type lectin D2; HSF, heat shock factor protein 5-like; HSP70, heat shock protein 70; HSP90, heat shock protein 90; IRF2, interferon regulatory factor 2; MyD88, myeloid differentiation primary response gene (88); NFIL3, nuclear factor interleukin-3-regulated protein

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resistance have been proposed as alternative technologies for selective breeding in Atlantic salmon (Robinson and Hayes, 2008). With the development of sequencing technologies, an increasing number of transcriptome analyses are performed in aquatic animals and the results show a direct correlation between immune gene expression level and bacterial infection, such as in differential transcriptome analyses in the fish *Epinephelus coioides* (Wang et al., 2014), the shrimp *Macrobrachium rosenbergii* (Rao et al., 2015) and the oyster *Crassostrea gigas* (Zhang et al., 2015). Our recent transcriptome study also indicated that different immune genes are involved in the response of *M. petechialis* against *Vibrio* infection (Jiang et al., 2017). Overall, these results suggest that much transcript regulation occurs in immune-related pathways, such as in antigen presentation, the complement pathway, the apoptosis pathway, and so on, when aquatic animals are challenged with bacteria. These studies have extended the list of candidate genes associated with bacterial infection; however, few works have considered the correlation between gene expression levels and resistance phenotypes, particularly in invertebrates.

In fact, the prediction of a disease-resistant phenotype from gene expression data has been widely applied in human disease studies, such as in cancer subtype discovery, cancer or normal sample discrimination, etc. (Rosenwald et al., 2002; Van De Vijver et al., 2002; Yeoh et al., 2002). The success of prediction in human disease provides a good model for use in disease-resistance evaluation in molluscs. The main objectives of our present study in the clam *M. petechialis* were 1) to evaluate resistance phenotypes using the *Vibrio* challenge test and select the extreme performing clam families, 2) to discover likely indicator genes associated with resistant traits by comparing resistant and susceptible phenotypes, and 3) to examine the effectiveness of the screened genes using two clam strains that differ in *Vibrio* resistance.

2. Materials and methods

2.1. Experimental clams

Twenty-three full-sibling families were produced in the hatchery laboratory of Zhejiang Mariculture Research Institute (Wenzhou, China). Briefly, 11 sires and 11 dams were chosen randomly from the clam population to produce 23 full-sibling families according to the Berg and Henryon design (Berg and Henryon, 1999) with some modification. The details of full-sibling families breeding design and rearing conditions were described in Liang et al. (2017). The clams that were approximately 15 months old with a mean weight of 7.62 ± 1.54 g were collected randomly from each family.

Moreover, a clam *M. petechialis* strain exhibiting a high *Vibrio* resistance level (strain R), which was developed by 10-year successive selection, and a control stain (strain C) were used in the subsequent application research. The clams about one-year old were collected randomly from the clam strains. All the clams were acclimated at 26 °C in continuous aerated seawater and fed with *Isochrysis galbana* for one week before the *Vibrio* challenge.

2.2. Vibrio challenge and sample collection

To determine if families were resistant or susceptible, the *Vibrio* challenge test was executed according to Liang et al. (2017) in October 2015. Briefly, approximately 100 clams from each of the 23 families were randomly collected and divided into three replicates. Each replicate was placed in a single cage, and all 69 cages were placed into a water tank and challenged by immersion with a dose of 1×10^7 CFU ml^{-1} MM21 of *Vibrio parahaemolyticus* strain (Yue et al., 2010). The fresh seawater and *Vibrio* were renewed once per day. A consistent immersion *Vibrio* challenge was applied until a total mortality of ~5% was observed for all the clams, the bacteria-containing seawater was removed, and then the clams were reared only in fresh seawater. By this way, the average cumulative mortality of clams would approach 50–60% at the termination of the experiment (Liang et al., 2017). The daily mortality was calculated based on the total number of clams used in the challenge test, and the mortality rate was recorded daily. Five hepatopancreas samples from each family were taken before the challenge test (uninfected group) and at 6 days post-infection (infected group), respectively. These samples were dissected, immediately frozen in liquid nitrogen and stored at − 80 °C until use.

An independent *Vibrio* challenge test was carried out in strain R and strain C, and approximately 180 clams from each strain were randomly collected and divided into three replicates. The experimental conditions and the statistical method of mortality were consistent with the above-mentioned challenge test except that the bacteria-containing seawater was removed on day 10. Twenty hepatopancreas samples from each strain were taken before the challenge test, dissected, immediately frozen in liquid nitrogen and stored at − 80 °C until use.

2.3. Comparison of resistant and susceptible phenotypes

To evaluate the resistance and susceptibility of different families to *Vibrio* infection, a multivariate survival statistical analysis was performed using a frailty model with a baseline hazard function according to the Cox model (Clayton, 1978; Cox, 1972) in all the clam families. The families with an estimated hazard ratio (HR) > 1 ($P < 0.05$) were classified as susceptible families, while the families with an HR < 1 ($P < 0.05$) were classified as resistant families. Estimates were obtained with 95% confidence intervals. The multivariate survival statistical analysis was performed in SAS 9.4 software (SAS Institute Inc.). Compared to the challenge test performed in July 2015 (Liang et al., 2017), the common resistant and susceptible families were obtained and exhibited in a Venn diagram. Then, the mortality data of common resistant and susceptible families were represented using Kaplan-Meier mortality curves (Kaplan and Meier, 1958). A survival statistical analysis between the two strains was also conducted using the above method.

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted from hepatopancreas using an EZNA® Total RNA Kit II (Omega Bio-Teck, USA) according to the manufacturer's instruction. RNA degradation and contamination was monitored on 1% agarose gels and then quantified using a NanoDrop-1000 spectrophotometer (Thermo Scientific, USA). Single-strand cDNA was synthesized from Total RNA (1 μg) using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Japan) according to the manufacturer's protocol.

2.5. Gene expression analysis

Based on the *M. petechialis* transcriptome database (GenBank accession number: SRR4241880, SRR4241881, SRR4241882 and SRR4241884) (Jiang et al., 2017), 22 immune-related genes were selected for detecting the regulation of gene expression levels by quantitative real-time PCR (qRT-PCR) between the uninfected (n = 30) and infected (n = 30) groups using the clams randomly selected from the 23 families. These selected genes included eight apoptosis-related genes, three complement system-related genes, three heat shock response-related genes, two lectin genes and six other immune-related genes. The *β-actin* and *EF1α* genes were used as internal references to normalize the relative expression levels between samples (Jiang et al., 2017). The sequences of specific primers for qRT-PCR are listed in Supplemental Table S1. The efficiency of each primer pair was analysed with serial two fold dilutions of cDNA (1, 1/2, 1/4, 1/8 and 1/16) to determine that the primers of reference genes and each censored gene had a similar efficiency. qRT-PCR was performed in QuantStudio 6 Flex (Applied Biosystems, USA) using a QuantiNova SYBR Green PCR Kit (Qiagen, Germany). Each reaction was performed in triplicate, and the following conditions were used: 95 °C for 2 min, 40 cycles of 95 °C for