Cloning and characterisation of the SpToll gene from green mud crab, *Scylla paramamosain*

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Toll/Toll-like receptors (TLRs), one of the most important pattern recognition receptors (PRRs), play a crucial role in innate immune responses in both invertebrates and vertebrates. In this study, we cloned and characterised a Toll gene from *Scylla paramamosain* (SpToll). Bioinformatic analysis predicted that SpToll contained one open reading frame of 3018 bp and encoded a single-pass transmembrane domain protein of 1005 amino acids. Further, SpToll could be clustered into one branch along with other arthropod Tolls in a phylogenetic tree. SpToll transcripts could be detected by RT-PCR from all tissues examined including the heart, gill, hepatopancreas, stomach, intestine, muscle, eyestalk and hemocytes. Infection by *Vibrio parahaemolyticus* up-regulated SpToll mRNA expression in hemocytes after 48 h. The profile of single nucleotide polymorphisms (SNPs) in the leucine-rich-repeats (LRRs) domain of SpToll in three healthy crabs was then evaluated. Two hundred and twenty SNPs with a frequency of about 1.0–4.0% were identified in hemocyte DNA/cDNA. Surprisingly, the adenine to guanine transition at position 1372 (c.1372A > G) had a frequency of about 50%. Finally, the results showed that challenge with *V. parahaemolyticus* stimulated the appearance of two sets of SNPs in crabs. More importantly, the c.1372A > G mutation could contribute to a low mortality after *V. parahaemolyticus* infection and introduce variation of charge and secondary structure into the SpToll polypeptide. In summary, these studies suggested a novel Toll homologue in crab and identified a SNP with potential pathogen-resistant activities. The result will be important for the investigation of crab immune defense mechanisms.

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1. Introduction

The green mud crab *Scylla paramamosain* is a commercially important crustacean distributed widely throughout the Indo-Pacific region. This species has a long history of being cultured along the southeast coasts of China. Recently, several outbreaks of diseases caused by viruses, bacteria and parasites had been reported to produce mass mortalities (Weng et al., 2007; Li et al., 2008). Since the invertebrates lack a true adaptive immune system, research on the innate immune system in *S. paramamosain* will be important for the control of infectious diseases and therefore the development of a sustainable crab farming industry.

Several immune factors including antimicrobial peptides (scygonadin and crustin) (Wang et al., 2007; Imjongjirak et al., 2009), prophenoloxidase (proPO) (Ko et al., 2007), hemocyanin (Yan et al., 2011), serine proteinases (Vaseeharan et al., 2006), antioxidant enzymes (Liu et al., 2010), and anti-lipopolysaccharide factor (ALF) (Imjongjirak et al., 2007) have been identified and characterised in crab species. Toll/TLRs play a crucial role in both the eradication of invading pathogens and the bridging the innate and adaptive immunity (Akira et al., 2001; Goldstein, 2004; Pasare and Medzhitov, 2004). Typically, members of the TLR family share a characteristic structure of several extracellular LRRs involved in pathogen recognition, a transmembrane segment and an intracellular Toll/Interleukin-1 receptor (TIR) domain for signaling (Xu et al., 2000). Up to now, at least ten TLRs in human, thirteen TLRs in mouse, nine Tolls in *Drosophila* and three Tolls in other arthropods have been cloned and characterised (Tautz et al., 2000; Beutler, 2004; West et al., 2006; Mekata et al., 2008; Yang et al., 2007, 2008). However, a Toll homologue in *S. paramamosain* has not yet been reported.

In this study, a SpToll gene was cloned and characterised from the green mud crab *S. paramamosain*. Results also showed that SpToll mRNA expression in hemocytes was up-regulated after bacterial infection, and that SNP mutations in the LRRs domain might be associated with enhanced resistance of crab to pathogens.
2. Materials and methods

2.1. Experimental animals

Live healthy green mud crabs (*S. paramamosain*) (average weight: 150 g) were obtained from Shantou Huaxun Aquatic Product Corporation (Shantou, Guangdong, China). They were acclimatized at room temperature for one week before the experiments were carried out.

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted from *S. paramamosain* tissues using RNAiso Plus (Takara, Japan) according to manufacturer instructions. The extracted RNA was treated with RNase-Free DNase (Takara, Japan) to remove contaminating DNA, and cDNA was synthesized using the M-MLV RTase cDNA Synthesis Kit (Takara, Japan) or the SMART RACE cDNA Amplification Kit (for RACE) following manufacturer instructions.

2.3. Degenerate primer design and SpToll fragment cloning

A pair of degenerate primers was designed in the conserved region of the LRRs domain from shrimp Toll protein sequences and crab ESTs (GenBank Accession Nos. ABKS8729.1, BAF99007.1, BACG8890.1, DY308063.1 and FG984065.1). PCR reactions were performed using primers CT-F and CT-R (Table 1) with 1 cycle of denaturation at 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s, followed by a 10 min extension at 72 °C. The PCR products were extracted and inserted into pMD-19T (Promega, Madison, WI). Recombinant clones grown on MacConkey agar (Sigma, St. Louis, MO) were identified using blue–white screening. Positive clones were cloned and sequenced. Positive clones were picked and confirmed by sequencing.

2.4. Rapid amplification of cDNA ends (RACE)

cDNA ends of SpToll were obtained using the SMART RACE cDNA Amplification Kit (Clontech, USA). The universal primer mix (UPM) plus Toll-GSP5 and Toll-GSP3 provided in the kit (Table 1) were used for amplification of the 5’-end and 3’-end, respectively, of the SpToll cDNA. The PCR reactions were performed with an initial denaturation at 94 °C for 3 min, then 5 cycles of 94 °C for 30 s and 72 °C for 2 min, 5 cycles of 94 °C for 30 s and 70 °C for 2 min, then 27 cycles of 94 °C for 30 s and 68 °C for 30 s and 72 °C for 2 min, and a final 72 °C for 10 min. All amplified products were cloned and sequenced as described in Section 2.3. Finally, the SpToll complete cDNA was cloned by RT-PCR with primers WSpToll-F and WSpToll-R (Table 1).

2.5. Bioinformatic analysis

The nucleotide sequence and deduced amino acid sequence of the SpToll cDNA were analyzed using the BLAST algorithm (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/). Structures within SpToll were predicted by the Simple Modular Architecture Research Tool (SMART) program (http://smart.embl-heidelberg.de/). Potential N-linked glycosylation sites were predicted by NetNGlyc 1.0 Serve (http://www.cbs.dtu.dk/services/NetNGlyc/).

2.6. mRNA expression of SpToll

Total RNA was extracted from the *S. paramamosain* heart, gill, hepatopancreas, stomach, intestine, muscle, eyestalk and hemocytes. Two microgram of the total RNA was then used for the synthesis of cDNA. The SpToll cDNA fragment was amplified using primers SpToll-F and SpToll-R (Table 1) under the following conditions: pre-denaturation at 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, followed by a final elongation at 72 °C for 10 min. As an internal loading control, the crab beta-actin cDNA fragment was amplified with primers beta-actin-F and beta-actin-R (Table 1) using the same PCR amplification conditions.

2.7. Immune challenge tests

For the immune challenge test, an experimental group and a control group were set up with each group having three crabs. Each individual was injected with 0.2 ml of a poly I: C (polynosinic-polycytidylic acid, SIGMA, 1 mg/ml), *V. parahemolyticus* (10⁷ CFU/ml) or 0.8% NaCl solution. Hemocytes were collected from each crab at 0, 3, 6, 12, 24, and 48 h for RNA extraction. After cDNA synthesis, a quantitative real-time RT-PCR assay was performed using an ABI PRISM-7300 Sequence Detection System (Applied Biosystems, Foster City, CA). The primers of the target gene (SpToll-F and SpToll-R) and internal control gene (beta-actin-F and beta-actin-R) were used for amplification of a segment of 143 and 88 bp, respectively. Data from the quantitative real-time RT-PCR analysis were subjected to the one-way analysis (one-way ANOVA) followed by an unpaired, two-tailed t-test.

2.8. Analysis of SNP profile in LRRs domain

For determination of the SNP sites in the SpToll LRRs domain (LRR11–15, 1155–1564 bp) at the individual level, PCR-based DNA/cDNA libraries were constructed as described previously with modifications (Belyavsky et al., 1989). Briefly, DNA was obtained from hemocytes of three healthy crabs using the Genome DNA Extraction Kit (Dongsheng Biotech Company, China). RNA isolation and cDNA synthesis were carried out as described in Section 2.2. Total DNA and cDNA were used to perform PCRs using SpToll primers LSpToll-F and LSpToll-R (Table 1). The products were cloned as described in Section 2.3. Positives colonies were selected on LB agar plates containing 40 mg/ml 5-bromo-4-chloro-3-indoly-L-D-galactoside and 100 μg/ml ampicillin (Sangon, Shanghai, China).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Nucleotide sequences of primers.</th>
</tr>
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<tbody>
<tr>
<td>Primer</td>
<td>Sequence (5’–3’).</td>
</tr>
<tr>
<td>Degenerate PCR</td>
<td></td>
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<tr>
<td>CT-F</td>
<td>TCCCTACCCATATSCNARTTY</td>
</tr>
<tr>
<td>CT-R</td>
<td>GTCACAAATCAGTGGGRTTCNCKT</td>
</tr>
<tr>
<td>RACE PCR</td>
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<tr>
<td>Toll-GSP5</td>
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</tr>
<tr>
<td>Toll-GSP3</td>
<td>CGGGAGACCATGTTGAGCAGTTT</td>
</tr>
<tr>
<td>UPM (long)</td>
<td>CTAATAGCTACATATAGGCGAACGAGTATC</td>
</tr>
<tr>
<td>UPM (short)</td>
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</tr>
<tr>
<td>WSpToll-F</td>
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<tr>
<td>WSpToll-R</td>
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<tr>
<td>SpToll-R</td>
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<td>Beta-actin-F</td>
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<tr>
<td>Beta-actin-R</td>
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<td>LSpToll-F</td>
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<td>LSpToll-R</td>
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<td>DToll-F</td>
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<tr>
<td>DToll-R</td>
<td>TGGTACCCACAGCCGTAA</td>
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Fig. 1. Cloning and sequence analysis of SpToll. (A) Nucleotide and deduced amino acid sequence of SpToll. The amino acid sequence was shown under the cDNA sequence. The initiation and stop codons were shown in bold. The polyadenylation signal AATAAAs were enclosed in solid lines and the poly A tail was underlined with dotted line at the C-terminal part. The predicted signal peptide was underlined. The 14 potential N-linked glycosylation sites were shaded in gray. The transmembrane domain and TIR domain were underlined with curved and double lines, respectively. (B) Alignment of SpToll ectodomain to the consensus sequence of leucine-rich repeats (LRRs) of TLRs. Conserved residues were shaded in gray. An insertion with 14 amino acid residues in LRR13 was indicated. (C) Schematic diagram of the SpToll protein. SP: the predicted signal peptide, gray square; LRR: leucine-rich repeat, blocks; LRR CT: leucine-rich repeat C-terminal domain; TM: transmembrane domain, black bar; TIR: Toll-IL-1R domain.
Six libraries each containing $10^6$ clones were established. Ninety-six positive colonies from each library were picked into a 96-well plate randomly and sequenced by Shenzhen Genomics Institute (Shenzhen, China). All SNP sites were listed and compared by statistical analysis.

2.9. PCR-DGGE

Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis was performed as previously described (Sheffield et al., 1989). In brief, a region of the SpToll cDNA was
amplified using specific primers: DToll-F and DToll-R (Table 1). A GC clamp was added to primer DToll-F to improve band separation. Concurrently, a total of three *V. parahemolyticus* treated-crabs were acquired as described in Section 2.7. Hemocytes were collected from each crab at 0, 12, 24, and 48 h for RNA extraction and cDNA synthesis. PCR amplification reactions were performed in a volume of 50 µl containing 1.25 U of high fidelity Taq (TaKaRa LATaq™ Hot Start Version; TaKaRa Bio Inc.), 10 µl of LATaq Buffer (2 mM MgCl2), 200 mM of each dNTP, 0.5 mM of each primer and 100 ng of template cDNA. The cycling conditions were as follows: denaturing at 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. The products were purified and loaded into an 8% (wt/vol) polyacrylamide gel with a urea-formamide denaturing gradient ranging from 30% to 65%. DGGE was performed in 1× TAE buffer using a DGGE-2001 system (CBS Scientific, Del Mar, CA) at 150 V and 60 °C for 8 h. The gels were stained for 30 min in 1× TAE buffer containing ethidium bromide and photographed immediately under UV light using a Gel Doc XR system and analyzed using the Quantity One software (Bio-Rad, Hercules, CA). Further, two extra bands (S1 and S2) in a DGGE profile were excised with a sterile razor blade and resuspended in 50 µl TE buffer. After elution at 4 °C overnight, the bands were re-amplified by PCR with DToll-F/R primers. The PCR products were then cloned and sequenced as described in Sections 2.3 and 2.5 for subsequent sequence analysis.

### 2.10. Evaluation of SNPs on crab’s resistance to pathogens

A total of 75 crabs were treated with *V. parahemolyticus* (2.5 × 10⁴ CFU/g of crab) as described in Section 2.7. Total DNA isolated from the hemocytes of each crab was used for PCR analysis as described in Section 2.8. PCR products were sequenced directly as described previously (Zakeri et al., 1998) to identify SNP mutations. SpToll A/A, G/G, or A/G genotypes were designated to crabs that were homozygous or heterozygous, respectively, for adenine and/or guanine at position 1372. The genotype was determined by sequencing of SpToll, e.g. the homozygote (A/A or G/G) at position 1372 would show only one peak, while heterozygote (A/G) would present two peaks in the chromatogram. The cumulative mortality of the crabs was recorded every 12 h post infection. A Kaplan–Meier plot (log-rank χ² test) was then used to investigate the correlation of SNPs with pathogen resistance in crabs. Discovery Studio 2.5 (Accelrys, San Diego, CA) was used to predict the effect of SNPs on the structure variation of LRRs domain in SpToll.

### 3. Results

#### 3.1. Cloning of the SpToll gene from *S. paramamosain*

A full length cDNA of Toll in *S. paramamosain* (SpToll) was obtained by RT-PCR amplification after obtaining the sequences of the 5’ and 3’ end of SpToll using RACE (See Sections 2.3 and 2.4...
for detail). Sequence analysis predicted that the full length cDNA of 3843 bp contained a 395 bp 5′ untranslated region (UTR), a 3018 bp open reading frame (ORF) and a 430 bp 3′ UTR, which included tandem polyadenylation signal sequences (AATAAA) and a partial poly A-tail of 27 bp (Fig. 1A). Alignment of the deduced 1005 amino acid sequence with the TLR consensus sequence identified 15 LRRs (residues 145–520) within the extracellular domain (Fig. 1B), a single-pass transmembrane segment (residues...
765–787), and an intracellular TIR domain (residues 816–954) (Fig. 1C). In addition, an insertion of 14 residues in LRR-13 (Fig. 1B), a signal peptide of 27 amino acids and 14 potential N-linked glycosylation sites were also identified (Fig. 1A).

3.2. Phylogenetic analysis of SpToll

A Toll/TLR phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) software (version 4.0).
Except the DmToll9 from Drosophila melanogaster, the result showed that SpToll and members in the arthropod branch, including PmToll (Penaeus monodon), FcToll (Fenneropenaeus chinensis), LvToll (Litopenaeus vannamei) and MJToll2/MJToll (Marsupenaeus japonicus), could be clustered into a subgroup (Fig. 2A). In addition, an amino acid sequence alignment between these Toll members is shown in Fig. 2B. The identity rates ranged from 39.0% to 43.0%.

3.3. Expression of SpToll mRNA and response to pathogen or poly I: C stimulation

Expression of SpToll mRNA was determined using RT-PCR. As shown in Fig. 3, a predominant band with an expected size of 143 bp was observed in all examined tissues including the heart, gill, hepatopancreas, stomach, intestine, muscle, eyestalk and hemocytes. Furthermore, a quantitative real-time RT-PCR assay was carried out to determine the transcript levels of SpToll in hemocytes of S. paramamosain treated with V. parahaemolyticus or poly I: C. After an initial decrease at 3 h, SpToll mRNA expression increased about 2.5-fold in the 48 h post V. parahaemolyticus treatment in comparison to that of the control (Fig. 4A). In contrast, there was not a significant difference in the transcript levels of hemocyte SpToll between poly I: C- and 0.8% NaCl solution-treated crabs during the entire 48 h period (Fig. 4B).

3.4. Detection of SNPs in the SpToll LRRs domain

It has been reported recently that polymorphism in TLRs is associated with an altered host response to pathogens (Bell et al., 2003). We therefore decided to measure SNPs in SpToll first and then investigate its potential relationship to pathogen resistance. To this end, six plasmid libraries of the SpToll LRRs region were constructed using genomic DNA and cDNA from hemocytes of S. paramamosain (see Section 2.8 for details). The SNP profile was then evaluated based on sequences of approximately six hundred clones. Two hundred and twenty SNPs were identified by multiple nucleotide sequence alignments. Most of the mutations had a frequency of about 1.0–4.0% except the frequency of an adenine to guanine transition at position 1372 which was about 50% (Fig. 5). It was also noteworthy that several SNPs, (11, 5 and 9 SNP sites in crab 1, 2 and 3, respectively), were identical in both the genomic DNA and cDNA, while only 2 SNPs were found in all of the crabs.

3.5. Association of SNPs with pathogen resistance in crab

Based on the above analysis, we tested using a PCR-DGGE analysis whether the SNP profile in crabs infected with V. parahaemolyticus was modulated. Compared to the control, a band named S1 appeared at all time points examined while a second band (named S2) appeared 24 and 48 h post infection (Fig. 6A). After cloning and sequencing, a total of 6 SNPs at positions 1235, 1263, 1282, 1372, 1479 and 1541, were found in the two extra bands by an alignment analysis (Fig. 6B). A challenge test was then carried out to determine whether there is an association between the SNPs observed and crab resistance to V. parahaemolyticus. DNA was isolated from hemocytes of each crab that survived the 96 h treatment. SNPs were then identified using PCR followed by sequencing. The analysis indicated that the cumulative mortality of crabs with a homozygous G/G genotype at position 1372 was significantly less than those of crabs with a homozygous A/A or heterozygous A/G genotype 24 h after injection of V. parahaemolyticus (Fig. 7A).

Therefore, a bioinformatic analysis was performed to predict the effect of SNP at position 1372. The A to G transition would result in a positive charge lysine (K) to negative charge glutamic acid (E) substitution at amino acid position 458. This could potentially cause perturbation of a predicted alpha helical structure within the insertion residues of 446–459 in LRR13 (Fig. 7B).

4. Discussion

The Toll protein was discovered in 1980 as an essential factor for dorsal ventral patterning in Drosophila (Nüsslein-Volhard et al., 1980). Later studies found that Toll protein was also involved in
innate immune responses (Lemaitre et al., 1996, 1997). An analysis showed that selective activation of the Toll pathway could control the expression of an antifungal peptide gene. Subsequently, it was demonstrated that Toll/TLR proteins exist not only in insects, but also in other invertebrates and in vertebrates (Medzhitov et al., 1997; Beutler, 2004; West et al., 2006; Tauszig et al., 2000). In this study, a Toll homologue, SpToll in the crab *S. paramamosain*, was cloned and characterised (Fig. 1A). SpToll displayed a structure characteristic of the Toll/TLR family (Fig. 1C), and clustered into one branch along with other arthropod Tolls in a phylogenetic tree.

Fig. 5. Analysis of SNP site and mutation frequency of SpToll LRR domain from 3 individual crabs. (A), (C) and (E) represent analysis of crab DNA (crab 1, 2 and 3). (B), (D) and (F) represent analysis of cDNA (crab 1, 2 and 3).
(Fig. 2A). Consistent with the expression profiles of TtToll and LvToll in \textit{Tachypleus tridentatus} and \textit{L. vannamei}, respectively (Inamori et al., 2004; Yang et al., 2007), the transcripts of SpToll was found in multiple tissues of \textit{S. paramamosain} (Fig. 3). Therefore, these results strongly suggest that SpToll is a novel Toll homologue in crab.

Toll/TLRs belong to an ancient family of pattern recognition receptors (PRRs) that play a crucial role in the innate immune responses (Akira et al., 2001, 2006; Lemaitre et al., 1996; Medzhitov et al., 1997). TLRs bind to different pathogen-associated molecular patterns (PAMPs) and lead to distinctive anti-pathogen responses (Medzhitov and Janeway, 1997, 2002; Akira et al., 2006). For instance, TLR1, TLR2 and TLR6 recognize lipids, whereas TLR7, TLR8 and TLR9 bind to nucleic acids (Akira et al., 2006). The crystal structure of the LRRs region in human TLR3 resembles a horseshoe and this region is involved in the interaction between TLRs and PAMPs (Bell et al., 2003, 2005). The length and position of an insertion in the LRRs domain has been thought to be strongly associated with binding to specific PAMPs (Jin and Lee, 2008).

Here, we found an insertion of 14 amino acid residues located in LRR13 of SpToll (Fig. 1B). Similarly, an insertion of 7, 7 and 11 residues has been identified in the LRR10 of MjToll, LvToll and FcToll, respectively, in shrimps (Mekata et al., 2008; Yang et al., 2007, 2008). In addition, it has been documented that glycosylation in TLRs may influence receptor surface presentation, trafficking, and pattern recognition (Weber et al., 2004). Our analysis indicated the presence of 14 potential N-linked glycosylation sites located in the extracellular domain of SpToll. However, direct evidence for the binding of SpToll to PAMPs is still missing.

SpToll transcript levels were increased significantly after \textit{V. parahemolyticus} treatment (Fig. 4A). This is similar to the findings that LvToll and FcToll mRNA transcription is strongly up-regulated after infection \textit{Vibrio harveyi} or \textit{Vibrio anguillarum} (Wang et al., 2010; Yang et al., 2008). In contrast, the expression levels of SpToll in crabs treated with poly I: C showed no significant difference when compared to the control. This result is similar to those found for MjToll and PmToll expression after exposure to poly I: C and WSSV (White Spot Syndrome Virus) treatment, respectively (Mekata et al., 2008; Arts et al., 2007). Although SpToll may not be directly involved in the immune defense against viruses, its similarity to other TLRs suggests that it may bind extracellular products from pathogens thereby functioning as a PRR.

Evidence suggests that mutations in TLRs can alter the ability of the host to respond to pathogens (Bell et al., 2003). Omueti et al. found that a variant of TLR1 (SNP P315L located in LRR11) was greatly impaired in mediating responses to bacterial lipopeptides (Omuetu et al., 2007). Arbour et al. reported that mutations in TLR4 (Asp299Gly and Thr399Ile) were associated with differences in LPS responsiveness in humans (Arbour et al., 2000). Lorenz et al. suggested that a mutation in TLR2 (Arg753Gln) might predispose individuals to life-threatening bacterial infections (Lorenz et al., 2000).

To investigate whether SNPs exist in the LRRs domain of SpToll, six plasmid libraries were constructed using genomic DNA and cDNA isolated from hemocytes of three healthy crabs. Two hundred and twenty SNPs in the LRRs region were identified by clone sequencing (Fig. 5). Interestingly, only a few SNP sites were identical between the genomic DNA and cDNA. It is possible that there is RNA editing in crabs, but we have not examined this possibility. Two groups of SNPs presented in the two extra DGGE bands (S1 and S2) with a total of 6 SNP sites (1235, 1263, 1282, 1372, 1479, Band S2) (Fig. 6).
and 1541) (Fig. 6), suggesting an enrichment of haplotype SNPs in pathogens-treated crabs. It was worthy to note that SNP c.1372A > G was found in both S1 and S2, and that crabs with this particular SNP displayed resistance to *V. parahemolyticus* (Fig. 7A). The result was similar to the report by Ciobanu et al. that showed SNPs might impact shrimp resistance to Taura syndrome virus (Ciobanu et al., 2009).

In conclusion, a novel Toll homologue in *S. paramamosain* (SpToll) was cloned and characterised. To our knowledge, this is the first report showing that SNPs in a Toll homologue could be involved in crab resistance to pathogenic infection. Further investigation will be required to explore the SpToll signaling pathway. This research will be helpful in establishing strategies for disease control and the development of a sustainable crab aquaculture.

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**Fig. 7.** Effects of SNP c.1372A > G on crab’s susceptibility to bacterial infection and LRRs’ structure variation. (A) Comparison of cumulative mortalities among *V. parahemolyticus*-infected crabs with a homozygous or heterozygous A/A, G/G or A/G genotype. A total of 75 crabs (25 crabs in each genotype) were treated with *V. parahemolyticus*. Mortality was measured in each treatment groups and was recorded every 12 h after injection. (B) Comparison of deduced amino acid sequences (residues 385–520) and predicted secondary structure of LRRs (LRR 11–15) between A/A and G/G genotype. LRRs amino acid sequences were aligned based on their structures. Conserved leucines were shadowed in gray. The K458E SNP (c.1372A > G) was shown in bold. The positions of α helices and β strands were indicated by coils and arrows above the sequences, respectively.
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References


