



In vivo characterization of histological and immunological response of allergic protein of *Metapenaeus dobsonii* using an adjuvant free BALB/c mice model

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ABSTRACT

Shrimp allergy, a common form of food allergy is an adverse immunological response to shrimp proteins. BALB/c mice was sensitized by an adjuvant free oral administration of purified tropomyosin, from *Metapenaeus dobsonii* to characterize intestinal histological responses and immunological protein recognition pattern as it is impractical in human subjects. Sensitized mice with higher dose of tropomyosin expressed symptoms of anaphylaxis including puffiness around eyes and snout, no activity, tremor and convulsion after challenge. The responses of high level of sera IgE, tropomyosin specific IgE and histamine in the treatment groups indicated the increased allergic reaction by ELISA. Sera IgE of sensitized mice exhibited a comparable recognition pattern to tropomyosin by immunoblotting similar to human subjects. Histological changes were comparatively highly affected in the intestinal area of duodenum in the sensitized mice. Hence BALB/c mice can be used as a suitable adjuvant free shrimp allergy model for immunotherapy tools.

1. Introduction

Allergy to food is a healthcare issue of universal importance having high potential for fatality, and it has been considered as highly serious. In UK and USA the prevalence of food allergy among adults is 1–2 % and in infants it is 5 % approximately [1–26]. Both the severity and prevalence of food induced allergic reactions has increased in the past years [2]. One of the most common seafood allergies is shellfish hypersensitivity [3]. Shrimps and crabs are included in the major allergenic foods among crustaceans [4]. Although shrimp is considered as a delicacy worldwide, often shrimp allergy is associated with severe reactions such as life threatening anaphylaxis [5]. Tropomyosin is the major allergen in majority of shrimp species and the 80 % of individuals having shrimp allergy react to tropomyosin. It is a heat stable protein and its homologies are responsible for cross reactive allergy in various crustaceans and mollusks [6]. IgE mediated hypersensitive reactions are the major causative behind all allergic reactions elicited by seafood [7].

Allergic immune response and disease can be triggered by food in species like mice, rats, pigs and dogs in similar way of humans [8]. Animal models can be used to mimic the immune responses of allergic patients including production of allergen-specific IgE, release of

histamine and Th2-type cytokine responses. Successful mimicking of human allergic reactions is the most important criteria for assessing whether the model is developed or not. It is reported BALB/c mice as ideally suitable for studying shrimp allergenicity aspects as it can mimic well the responses of human allergy [9], both humoral and cellular [10]. It is well reported as a suitable model for evaluating the allergenicity of different food protein extracts [11] from peanut, cow's milk and potato. The cell-mediated immune response in mice sensitized with recombinant shrimp tropomyosin [12] was studied. A murine model using a recombinant shrimp allergen along with cholera toxin as adjuvant and its gastrointestinal and immunological changes is reported [13]. As there are few literature reports on development of adjuvant free type of shrimp allergen animal models, in the present study an adjuvant free BALB/c model was evaluated using purified shrimp tropomyosin of 37 KDa from flower tail shrimp, *Metapenaeus dobsonii* alone by intragastric administration. As the major symptoms of shrimp allergy are associated with gastrointestinal tract, detailed histological changes can throw light into shrimp hypersensitivity. The intestinal histological changes along with changes in level of shrimp tropomyosin specific immunoglobulin (IgE), serum histamine, anaphylactic symptoms and protein recognition pattern by immunoblotting was

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evaluated.

2. Materials and methods

2.1. Shrimp samples

Fresh flower tail shrimp (*Metapenaeus dobsonii*) samples in fresh condition were collected from Kalamukku landing centre of Cochin, Kerala. The samples were iced immediately at 1:1 ratio (shrimp to ice) and transferred in an insulated box to the laboratory. Collected meat was subjected to homogenization and stored at -20°C .

2.2. Purification of tropomyosin

Purified tropomyosin was prepared with slight modification to the method reported [14] at a temperature of 4°C . Homogenized meat was extracted with 10 fold volume of 20 mM Tris–HCl containing 50 mM KCl followed by centrifuging to collect pellet. The extraction process was repeated four times. Treated the final residue with absolute acetone for three times and allowed to dry overnight.

The dried acetone powder was extracted with slight modification [15] using 10 fold volume of 20 mM Tris–HCl containing 1 M KCl and 10 mM 2-mercaptoethanol, overnight. Centrifuged and the resulting supernatant was subjected to isoelectric precipitation at pH 4.5 using 1 N HCl. Dissolved the residual pellet in 20 mM Tris HCl and adjusted the final pH to 7.6 with 1 N NaOH, followed by fractionation using 30 and 50 KD Amicon (Merck Millipore) cut off filters. The final fraction was heated in boiling water bath for 10 min, immediately cooled in chilled water and collected the purified tropomyosin in solution.

2.3. Animals used

Male and female BALB/c mice of 8 weeks old reared in the animal house facility of ICAR-Central Institute of Fisheries Technology, Cochin were used for the experiment. The experiment was conducted after the approval of institute animal ethics committee. The animals were hygienically reared in well ventilated cages. The animals were maintained with feed free of shrimp ingredient.

2.4. Sensitization and challenge protocol

The experimental animals were divided into three groups with six animals in each group. The groups included one control and two treatments. The experimental protocol was designed with some modifications to the method reported previously [16] and has been repeated three times (Fig.1). T1 group and T2 group was sensitized by

intragastric gavage of 2.5 mg/Kg and 1.25 mg/Kg purified tropomyosin respectively at an interval of seven days. The course of sensitization includes 0th, 8th, 15th and 22nd day. After 7 days of last sensitization, the animals were subjected to overnight fasting followed by challenge on 29th day with 14 and 7 mg/Kg purified tropomyosin for T1 and T2 respectively. While equal volume of phosphate buffered saline (PBS) was given to control group. Animals were sacrificed on 30th day followed by collection of blood and preparation of sera. The sera were stored at -80°C until analysis.

2.5. Assessment of systemic anaphylaxis

The evaluation of responses of systemic anaphylaxis was carried out by close observation of treated mice for 30–40 min after challenge. The scoring was carried out as per the reported method [17] in presence of trained personnel. Score 0 is for absence of symptoms; score 1 is for scratching and rubbing around the nose and head; score 2 is for puffiness around eyes, pilar erecti, reduced activity, and or decreased activity with increased respiratory rate; score 3 is for wheezing, labored respiration, cyanosis around the mouth and tail; score 4 is for no activity after prodding or tremor and convulsion; score 5 is for death.

2.6. Analysis of mouse sera IgE level

The assay was carried out by using a 96 wells ELISA kit for mouse IgE for sera (Catalogue number RAB0799 from Sigma Aldrich, USA) as per manufactures instruction.

2.7. Analysis of tropomyosin specific IgE level

Tropomyosin specific IgE level in mice sera was analyzed by indirect ELISA technique as per the method reported [18]. Wells of 96 well ELISA plate (Thermo scientific) were coated overnight at 4°C with 100 μL of purified tropomyosin (2 mg/ml) in carbonate buffer (pH 9.6). The plate was washed five times using 0.05 % Tween 20 in phosphate buffered saline (PBS-T). Blocking was carried out using 3 % bovine serum albumin (BSA) in PBS at 37°C for two hours. Diluted mice sera (1:10) samples in 3 % BSA in PBS were added to the plate and incubated at 37°C for two hours. Incubated with horse radish peroxidase conjugated anti mouse IgE (Biorad) at 1:100 dilution for one hour at 37°C . After washing the plates were incubated with substrate solution of 0.1 % OPD (O-phenylene diamene) and 0.03 % H_2O_2 in 0.5 M phosphate citrate buffer (pH 5) for 30 min at room temperature. The reaction was stopped using 2 N H_2SO_4 and measurement of absorbance values were done at 490 nm using a microplate reader (Robonik, Readwell touch ELISA plate analyser). The data was taken after subtracting the

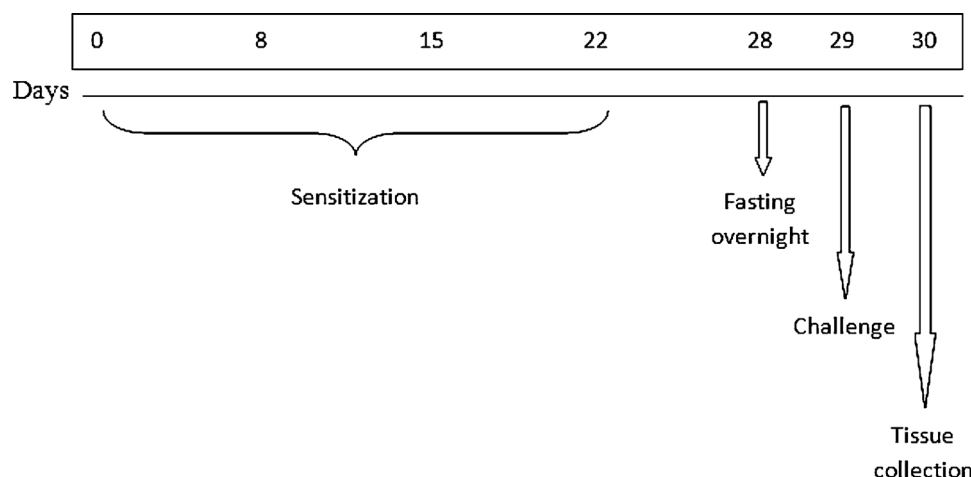


Fig. 1. Schematic diagram of tropomyosin induced sensitization and challenge protocol of BALB/c mice.

background value. The method is specific and sensitive to measure tropomyosin specific IgE level. All the analyses were performed in triplicate and the results were given as mean $\pm$ standard error.

2.8. Analysis of histamine level in sera

The assay for serum histamine level was performed using a 96 wells ELISA kit for mouse histamine (Catalogue number EL-1634 from Origin) as per the manufactures instruction.

2.9. SDS PAGE and immunoblotting with mice sera

Performed Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified tropomyosin as reported [19] using Mini Protean 3 Apparatus (Bio-Rad, USA). It was carried out with a 12 % polyacrylamide separating gel and a stacking gel of 4 % at 150 V for 50–60 min. The shrimp extracts were mixed with Lamelli buffer in the ratio of 1:4, heated at 95 °C for 4 min, cooled and loaded for running along with a wide range protein marker (Sigma) as a reference. After running the gels, it was stained with Coomassie blue R-250 and destained with acetic acid solution. In case of immunoblotting, prestained dual colour protein marker (Biorad) was used.

For immunoblotting the separated proteins (purified tropomyosin) by SDS PAGE were transferred electrophoretically to a 0.45 μ m nitrocellulose membrane using Mini Trans-Blot System (Bio-Rad, USA) at 80 V for 120 min. Then the membrane was blocked using 3 % BSA (w/v) in TBS (Tris buffered saline) and washed using 0.05 % Tween 20 in TBS. Then it was subjected to incubation with mice sera collected from groups of control, T1 and T2 at dilution of 1:25 in blocking buffer for 2 h at room temperature. Washed the membrane and incubated in horse radish peroxidase conjugated anti mouse IgE (Biorad), diluted 1:500 in blocking buffer for 1 h at room temperature. The blots developed were subsequently subjected to visualization by staining using Opti-4CN substrate kit from Biorad.

2.10. Histology

After 24 h of challenge animals were sacrificed and collected liver, duodenum, jejunum and ileum. The collected tissues were washed with 0.9 % saline and fixed in buffered formalin (1:20) for 48 h and later embedded with paraffin. Paraffin sections of 5 μ m thickness and stained with eosin. Observed for allergic responses in various tissues.

2.11. Statistical analysis

The results were given as mean together with standard error. Statistical significance of data was determined using one way analysis of variance by SPSS.16 (SPSS Inc., Chicago, IL, USA). Pearson's correlation test was used to determine the relationship between IgE, tropomyosin specific IgE, histamine and systemic anaphylaxis score.

3. Results and discussion

As shrimp allergy can be lifelong and can result anaphylactic reactions of life threatening nature even during exposure to allergen in minute level, studies on hypersensitive responses using animal models can support the future development of immunotherapeutic drugs in an eminent way.

3.1. Tropomyosin purification and SDS PAGE

Protein components of raw and cooked extracts in PBS (Phosphate buffered saline) were analysed by SDS PAGE along with purified tropomyosin and given in Fig.2. The molecular weight of purified tropomyosin is 37 KDa. The content of purified tropomyosin was determined by Biuret method and used for sensitizing and challenging of BALB/c

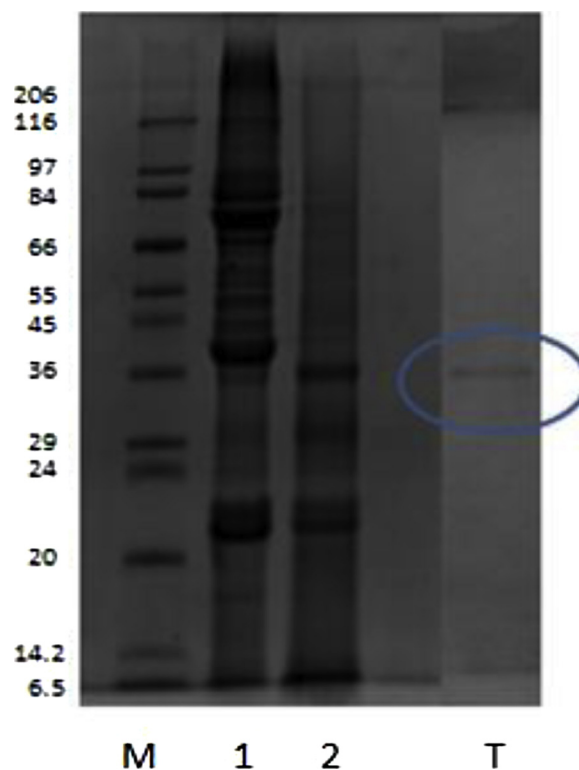


Fig. 2. SDS PAGE of purified protein Tropomyosin. M: Protein marker; 1: Raw extract; 2: Cooked extract; T: Purified tropomyosin.

mice as per the protocol described.

3.2. Systemic anaphylactic responses

Most of the oral food induced anaphylaxis in humans is done by IgE antibody, mast cell or basophil pathway [20] while in mice systemic anaphylaxis it can be mediated by IgE or IgG1 antibodies [21]. Observation of the systemic anaphylactic responses were carried out after challenge on day 29th and shown in Fig. 3. All the tropomyosin sensitized mice were exhibited responses in comparison to control mice. All the mice of treatment groups had symptoms of scratching and rubbing around nose and head. The mice coming under T1 group exhibited symptoms of puffiness around eyes and snout (score 2, Fig. 3A) and no activity, tremor and convulsion (score 4, Fig. 3B). Sensitized mice exhibited obvious symptoms after 30–40 min after challenge. In the case of mice under group T2, the symptom of scratching and rubbing round eyes and nose (score 1) and puffiness around eyes and snout (score 2) has seen mainly in two cases. There was no death of animal after challenge. The control group didn't show any symptoms during sensitization and challenge. There was no symptom of diarrhea after challenge in both treatment groups. Diarrhea in 41.7 % of tropomyosin sensitized mice along with cholera toxin as adjuvant is reported [13]. Hypersensitive responses to food items like peanut [17], ovaalbumin [22] and rice protein [23] have also been reported previously.

3.3. Mouse IgE level

The sera IgE level on the day after challenge (30th day) was measured and given in Fig. 4. There observed an increase in the level of sera IgE in the treatments in comparison to control value of 7.87 ng/ml. In case of treatments the IgE levels were 33.69 and 21.23 ng/ml respectively. The continuous sensitization and challenge protocol has resulted a significant increase in the IgE level of sera ($p < 0.005$). This can be due to the increased mast cell degranulation in presence of shrimp allergen tropomyosin. Increase in mast cell degranulation and IgE level

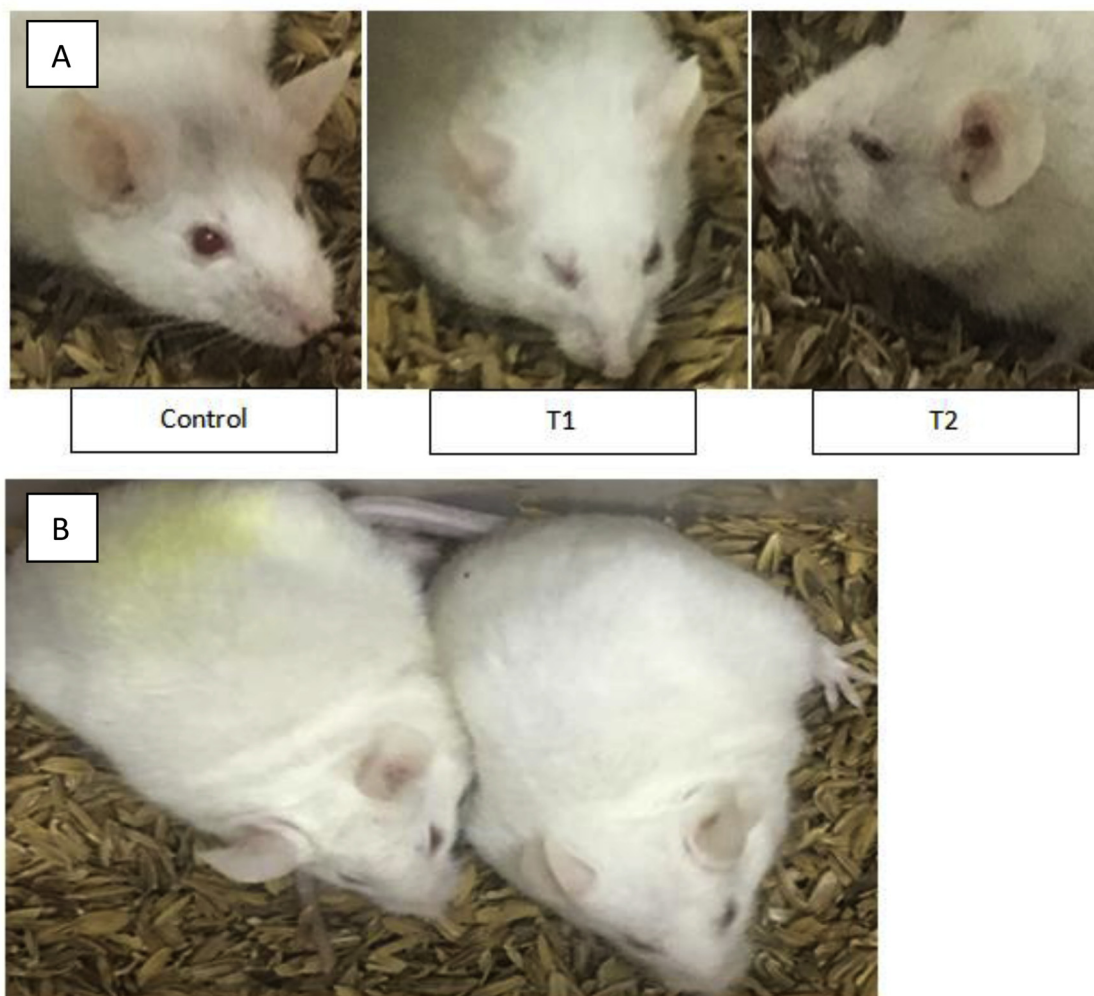
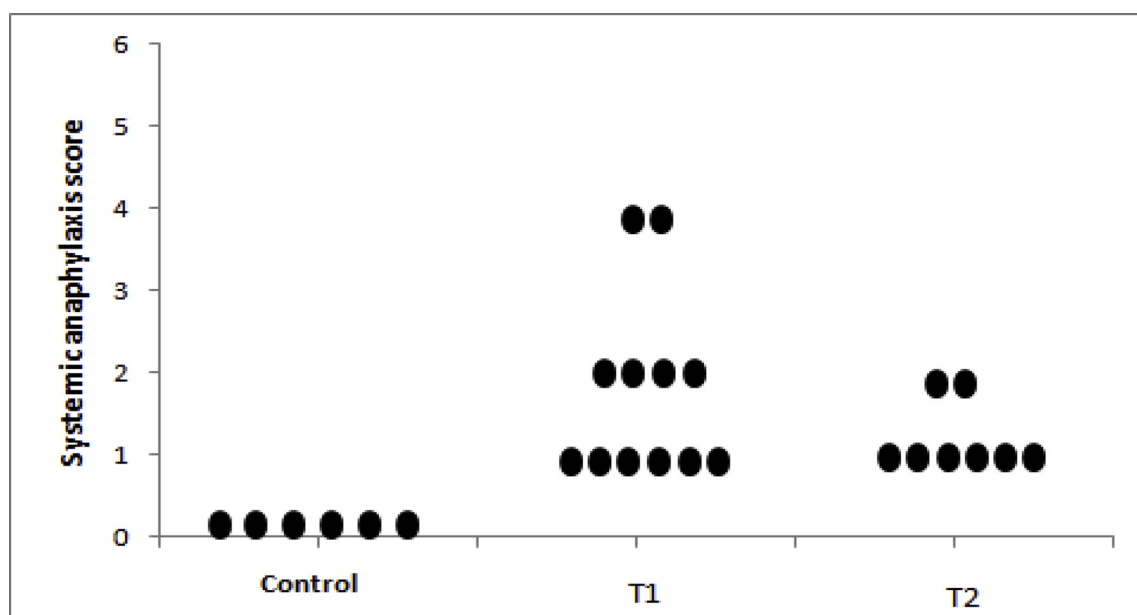


Fig. 3. Systemic anaphylaxis score after challenge. Control, T1 (higher dose level) and T2 (lower dose level). A & B – Images of BALB/c mice after challenge.

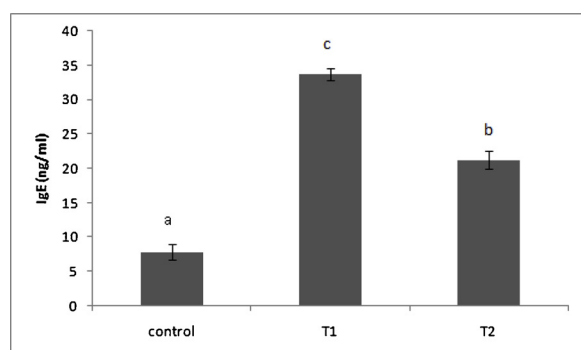


Fig. 4. Serum IgE levels in mice by ELISA; Control, T1 (higher dose level) and T2 (lower dose level). Data is shown as mean $\pm$ standard error, $n = 6$. Bars with different letters are significantly different at $p < 0.005$.

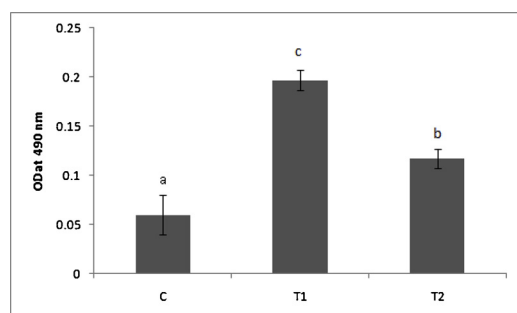


Fig. 5. Serum tropomyosin specific IgE levels in mice by ELISA; Control, T1 (higher dose level) and T2 (lower dose level). Data is shown as mean $\pm$ standard error, $n = 6$. Bars with different letters are significantly different at $p < 0.005$.

subsequent to shrimp allergen sensitization in a mouse model through intraperitoneal injection is previously reported [9].

3.4. Tropomyosin specific IgE level

A generally accepted standard in development of mouse models is the induction of specific IgE response to allergenic component and quantification by optimized ELISA technique [1]. Tropomyosin specific IgE level in mice sera was measured and shown in Fig. 5. The analysis demonstrated a significant ($p < 0.005$) increase in absorbance both in the case of T1 and T2 in comparison to control sera. Due to tropomyosin sensitization and challenge, IgE of both treatments showed an elevated level of absorbance towards purified tropomyosin at 0.197 and 0.117. Mice sensitized with higher dose expressed higher IgE response than lower dose administered one. Similar trend was reported in case of rice protein by BALB/c mice model [23]. Increase of shrimp allergen specific IgE during time course level to peak, followed by a decrease and attainment of stable condition was reported [24]. The specific nature of IgE was again confirmed by immunoblotting. The specific IgE antibody measurement in humans was reported to be used as a diagnosis tool to understand the severity in egg and peanut allergies [25].

3.5. Mouse histamine level

The histamine levels in sera can vary depending upon sensitization by allergens. The variation in serum histamine level on the day after challenge (30th day) was evaluated and compared with control mice sera and depicted in Fig. 6. The histamine level was varying between the individual mice and it was significantly higher ($p < 0.005$) in both treatments of T1 (266.86 ng/ml) and T2 (208.45 ng/ml) in comparison to control (125.98 ng/ml). The increased level of histamine in the treated mice is an indication of increased mast cell degranulation after

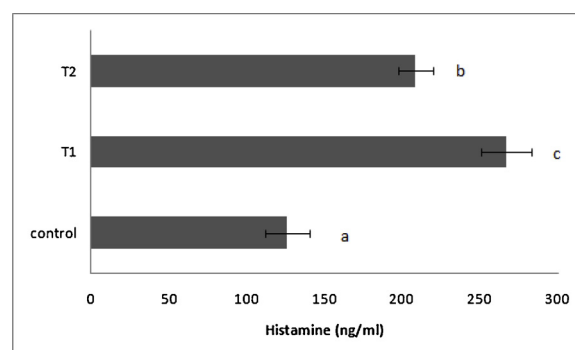


Fig. 6. Serum histamine levels in mice by ELISA; Control, T1 (higher dose level) and T2 (lower dose level). Data is shown as mean $\pm$ standard error, $n = 6$. Bars with different letters are significantly different at $p < 0.005$.

challenging with purified tropomyosin. Due to the cross linking of antigen to IgE bounded receptors of mast cells or basophils, degranulation process can be induced and biochemical mediators like prostaglandins, cytokines, leukotrienes, histamine etc will be secreted. The serum histamine level cannot reflect mice allergy for a long period of time as the half life period of histamine in vivo is transient [9,26].

3.6. Immunoblotting with mice sera

In order to further validate this BALB/c mice model for shrimp allergen, tropomyosin the allergic protein was subjected to analysis of recognition using sera collected from sensitized mice (T1 and T2) under this study as primary antibody through immunoblotting technique. Fig. 7 shows the binding of anti mouse IgE antibody to tropomyosin protein on immunoblots after incubation with sera of tropomyosin sensitized BALB/c mice as primary antibody. It was demonstrated that all the individual mice sera of both sensitized groups (T1 and T2) contained tropomyosin specific IgE antibodies except in case of control mice. Even though the mice IgE blot observed to be not strong as that of human IgE blot as per our previous study report [2,7,27–32,35], it is clear that all the mice IgE blot are directed against tropomyosin, the major shrimp allergen of 37 KDa purified from *M. dobsonii*. Hence the

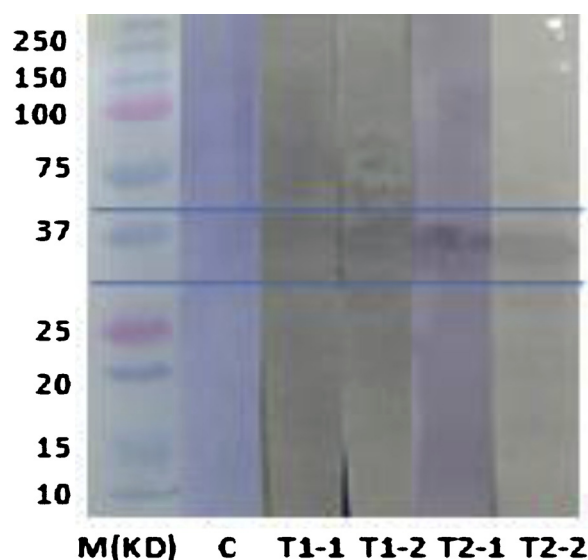


Fig. 7. Immunoblotting analysis of IgE antibodies present in sensitized mice against purified tropomyosin from shrimp. Lane M (marker), c (control), T1-1(Pooled sera 1 of higher treatment level), T1-2(Pooled sera 2 of higher treatment level), T2-1(Pooled sera 1 of lower treatment level) and T2-2(Pooled sera 2 of lower treatment level).

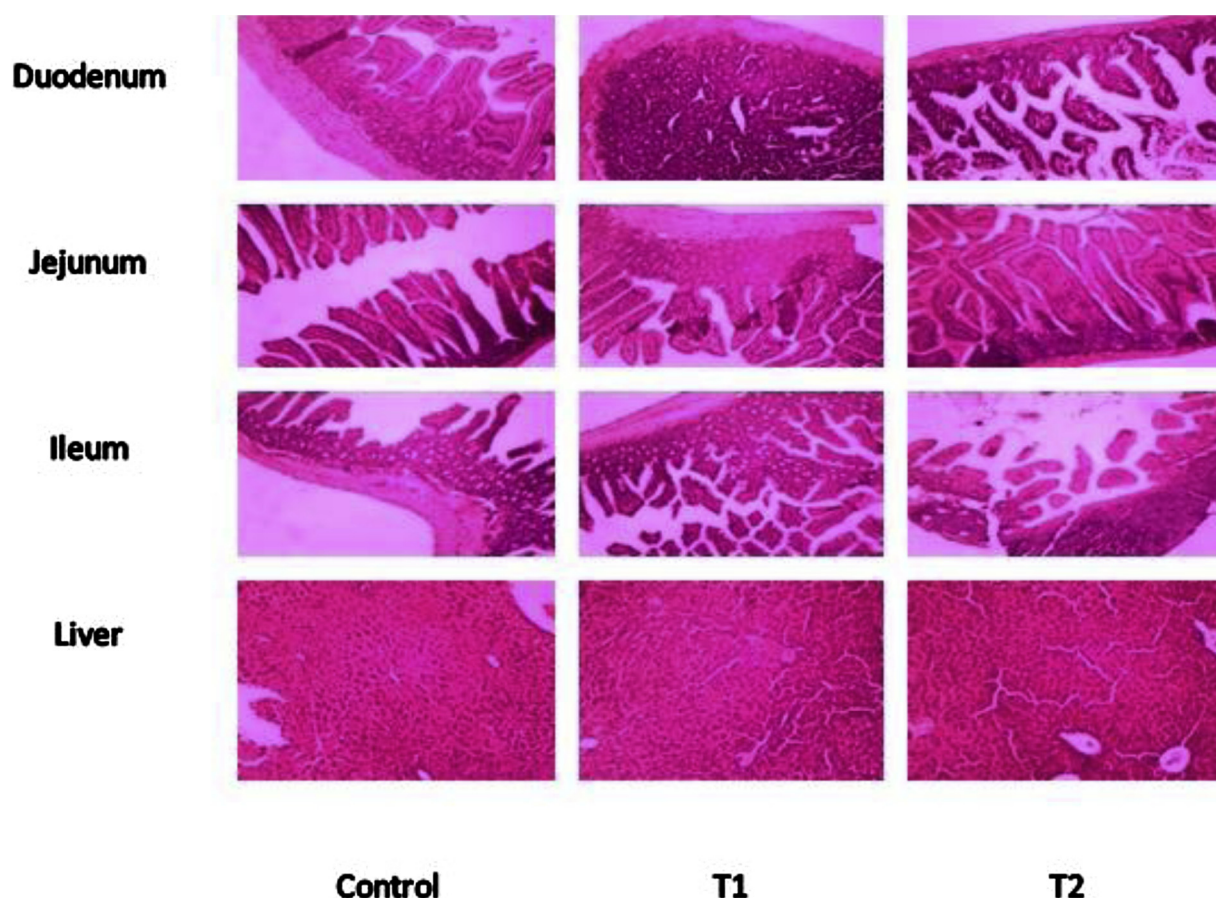


Fig. 8. Histopathology images of duodenum, jejunum, ileum and liver of control and treatment groups (T1-higher level) and (T2-lower level).

allergen profile recognized by sensitized mice sera was in agreement with that of shrimp allergic patient sera and the results further validate the development of BALB/c mice model for shrimp allergen, tropomyosin.

All the mice IgE blots were showing strong resemblance in tropomyosin binding pattern even though the mice were individually sensitized and challenged by oral administration. The specific recognition of hen's egg and cow's milk proteins by sensitized BN rat immune system is reported previously [28]. The present study is the first time report of comparable shrimp allergic protein recognition pattern in BALB/c mice immune system. This result also demonstrates that immunoblotting is highly sensitive in determining the specific IgE antibody, tropomyosin. The comparable shrimp allergen profile also confirmed the suitability of BALB/c model for in vivo characterization of shrimp allergen.

3.7. Histopathology of small intestinal segments and liver

Induction of food intolerance and intestinal inflammation due to food allergens is mainly linked with gut associated mucosal defense in lymphoid tissues [29,30]. The mucous layer of gastro intestinal tract is important in innate host defense. Few studies have reported the intestinal effect of hypersensitivity due to tropomyosin. The histological examination of liver and small intestinal segments such as duodenum, jejunum and ileum were carried out and given in Fig. 8.

In case of liver, both control and treatments were showing normal structure. Portal triads and hepatic veins were normal. Both hepatocytes and kuffer cells were showing normal morphology. Histologic examination of duodenum segments of higher dose treatment (T1) revealed the core of papillary structures and lamina propria showing diffuse infiltrate of lymphocytes, plasma cells and histocytes. While in lower dose treatment (T1), lamina propria and core of papillary

structures showed collections of lymphocytes, plasma cells and histocytes only. There was no observation of swelling. Both in the control and treatments the glands and villi appeared normal. Histology of jejunum didn't show much variation between the treatments and control. Core of the villi and lamina propria showed collections of lymphocytes, plasma cells and histocytes. In the histology of ileum of higher dose treatment (T1), stroma showed diffused infiltrate of lymphocytes, plasma cells and histocytes. While in the case of T2 it was absent.

The number of studies reported on intestinal regional difference upon allergen sensitization is limited. Comparatively higher affected region of intestine in the present study is duodenum. Both jejunum and ileum were lesser affected in comparison to duodenum. It is also reported the occurrence of severe effect in the duodenum region of gut in their shrimp tropomyosin animal hypersensitivity model [13]. The food allergens can induce intestinal vascular permeability in a significant manner in the duodenum and proximal jejunum, not the regions of distal jejunum and ileum [31]. The gut regional variation in food allergy can be partly due to the variation in the nutrient adsorption capacity and the microbiota associated with [32,33].

3.8. Pearson's correlation analysis of allergenicity

The values of sera levels of IgE, tropomyosin specific IgE, histamine and score of systemic anaphylaxis score of individual mice of control and treatments were used to calculate the Pearson's correlation analysis and given in Table 1. A highly significant correlation at $p < 0.001$ was observed between immunoglobulins present in the sera and responses of individual animal. Correlations of systemic anaphylaxis score and measurements of sera level of IgE, tropomyosin specific IgE and histamine was in the range of 0.94–0.96 and were highly significant indicating the elevation of both total and shrimp allergen specific IgE

Table 1

Correlation between total IgE, tropomyosin specific IgE, histamine level and systemic anaphylaxis score.

	IgE	Tropomyosin specific IgE	Histamine	Systemic anaphylaxis score
IgE	1			
Tropomyosin specific IgE	0.96**	1		
Histamine	0.95**	0.92**	1	
Systemic anaphylaxis score	0.94**	0.96**	0.95**	1

** Correlation is significant at 0.01 levels (two tailed).

along with histamine in the treatment groups after challenge. Even though the systemic response score was carried out in presence of trained personnel, it can be subjective to some extent. The quantifiable measurements of allergic response based on sera levels of immunoglobulins and histamine indicated a strong correlation with the anaphylaxis scoring. So the given model simulated the human situation closely in an adjuvant free oral administration manner.

Shrimp is a common cause of adverse food allergic reactions in individuals of hypersensitive nature. In the present study, an adjuvant free mice model for shrimp allergen, tropomyosin was developed and the mice subjected to sensitization was showing puffiness around eyes and snout, no activity, tremor and convulsion after challenge at higher dose level administration. Significantly high levels of tropomyosin specific IgE and histamine were present in the sera of sensitized group after the day of challenge. Histological analysis revealed the alterations in the highly affected duodenum region of intestine. The developed animal model is further validated by the specific tropomyosin recognition pattern of sensitized mice sera which is similar to the tropomyosin recognition pattern by sensitive human immune system. The sera levels of IgE, specific IgE and histamine showed strong correlation with systemic anaphylaxis score. The results support the usage of an adjuvant free BALB/c mice model for shrimp allergy research, immunotherapy development and for evaluating hypoallergen formulations. Also the demonstration of histological and immunological response obtained in this adjuvant free model can be further enhanced with future studies focusing on evaluation of inflammatory response by immunohistochemistry or transcription factor/ cytokine mRNA expression in the affected duodenal region, spleen cells and also detection of type 2 cytokines in the serum along with anti-TM non-IgE isotypes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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