



Quantification of Crustacean Tropomyosin, a Major Food Allergen, in Eight Species of Taiwanese Shrimp Based on Immunoassay

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Abstract

Shrimp is one of the most common food allergens in Taiwan. However, a simple method of measuring crustacean tropomyosin, the active allergen in shrimp is lacking. In this study, we aim to develop a validated enzyme-linked immunosorbent assay (ELISA) for quantifying level of tropomyosin in eight shrimp species in Taiwan. Our ELISA showed high specificity to shrimp tropomyosin, and inter-day precision and intra-day precision were 8.7% when 0.2 ng/ml were used as standard test samples. Furthermore, our ELISA had 0.030 ng/mL limited of detection (LOD) and 0.064 ng/mL limit of quantification (LOQ). We found despite the steaming process, levels of tropomyosin were still detectable which indicate active allergen in processed shrimps. Our findings in this study suggest this ELISA method can be a useful technique to study shrimp allergen for future crustacean allergen research.

Keywords Allergen · ELISA · Food allergy · Shrimp · Tropomyosin

Introduction

Food allergy is a critical aspect of food safety and has been recognized by the World Health Organization (WHO) as one of the important public health issues in the world (Kirsch et al. 2009). Our previous studies have shown that Taiwanese patients with food allergy have a higher risk of congestive heart failure, peripheral vascular disease, chronic pulmonary disease, rheumatologic disease, and renal disease (Yu et al. 2017). Currently, the mainstay of therapy is to avoid exposure to food allergens. Thus, many countries have implemented policies requiring food industry to label food packaging with allergen-containing foods, in order to prevent allergenic reactions (Koeberl et al. 2014). In the USA, food labeling requirements have identified eight food ingredients (egg, peanut, milk, tree nuts, gluten-containing cereal,

soybean, fish, and crustacean) as major allergens (Monaci and Visconti 2009). In Europe, an additional seven ingredients (mustard, celery, sesame, lupine, mollusks, sulfur oxide, and sulfites) have been required for food labeling (Monaci and Visconti 2009). In Japan, shrimp, crab, egg, milk, wheat, buckwheat, and peanut have been identified as major food allergens for food labeling (Sakai et al. 2013).

Current epidemiological studies suggested that shrimp is a major cause of food allergy both in the world and in Taiwan (Chan et al. 2014; Werner et al. 2007). Shrimp allergies frequently result in acute allergic reactions, such as atopic dermatitis and acute respiratory conditions. The major allergen in crustaceans, such as shrimp, is tropomyosin (Hajeb and Selamat 2012; Yu et al. 2011; Zhang et al. 2014), which is a heat-stable protein with α -helical coiled-coil structure (Shanti et al. 1993). This protein can be found in both muscle and non-muscle cells of animal, and has the molecular weight ranging from 34 to 38 kDa. Current research suggests only non-vertebrate tropomyosins can cause allergy (Ayuso et al. 2002). Amino acid sequence of tropomyosin is highly conserved among crustaceans (Kamath et al. 2013). Therefore, many individuals may have concurrent allergies to both shrimp and other crustaceans.

Accidental ingestion of shrimp can cause severe allergic symptoms. Furthermore, contamination in shared production lines between shrimp and processed food is an important health concern. Therefore, a reliable analytical method is

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critical for detection of food allergen. Currently, enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) have become commonly used methods for routine detection of allergens in food. PCR techniques have been utilized to detect the shrimp and crab DNA in the foods with detection limit ranging from 0.1 to 10 µg/g (Eischeid et al. 2013; Taguchi et al. 2011). However, it lacks ability to detect the protein. ELISA has the advantages of direct detection of protein, ease of use, and high sensitivity. Many commercial kits have the detection limits of 0.05–10 µg/g for allergen (Schubert-Ullrich et al. 2009). Several studies also demonstrated that ELISA techniques can accurately quantify tropomyosin levels in crustacean and its processed foods (Fuller et al. 2006; Schubert-Ullrich et al. 2009; Seiki et al. 2007; Werner et al. 2007; Zhang et al. 2014).

In this study, we aimed to develop a rapid, validated ELISA with improved sensitivity and precision to detect tropomyosin in shrimp and processed food. Specifically, we will quantify the tropomyosin levels in both raw and cooked shrimps consumed in Taiwan, as well as in commercially processed foods.

Materials and Methods

Samples

Fresh samples of whiteleg shrimp (*Litopenaeus vannamei*), giant river prawn (*Macrobrachium rosenbergii*), spear shrimp (*Parapenaeopsis hardwickii*), giant tiger prawn (*Penaeus monodon*), green tiger prawn (*Penaeus semisulcatus*), red shrimp (*Solenocera melanthero*), sakura shrimp (*Sergia lucens*), chicken breast, pork tenderloin, and tilapia fillet were obtained from local markets in Pingtung, Taiwan. Southern rough shrimp (*Trachypenaeus curvirostris*) was purchased from a local market in Tainan, Taiwan (Fig. 1). Commercial crustacean processed foods were obtained from supermarkets in Pingtung, Taiwan.

Sample Preparation

Extraction methods by Liang et al. were used with minor modifications (Liang et al. 2008). Two grams of sample was mixed with 40 mL of 20 mM Tris-HCl buffer (pH 7.5, containing 3% NaCl), and homogenized for 1 min. Then, the sample was centrifuged at 10,000×g for 30 min at 4 °C, and the supernatant was taken out as protein extracts. Protein extracts were stored at −20 °C. Cooked shrimps were steamed in laboratory for 3 min using raw shrimps, following similar protein extraction techniques.

Sandwich ELISA Procedure

The standard curve recommended by manufacturer's instructions was not in linear shape and used a large quantity of

reagents, so our research group modified the protocols to improve the analytical quality of ELISA. Briefly, 96-well were coated with monoclonal antibody 1A6 (Indoor Biotechnologies, USA) and blocked with PBS-T buffer. Tropomyosin controls (Indoor Biotechnologies, USA) ranged from 0.125 to 2 ng/mL were used as linear standard curve standards, and sample protein extracts were performed in triplicates. Subsequently, polyclonal rabbit anti-shrimp antibody antibodies (Indoor Biotechnologies, USA) were added and incubated with secondary peroxidase-conjugated goat anti-rabbit IgG (Jackson Laboratories, USA). Absorbance was read at 450 nm by Model Epoch Microplate Spectrophotometer Epoch-A451 (BioTek, USA). Quantifications of tropomyosin performed using serial dilution to fall in the OD of the standard curve.

Validation of ELISA

The precision of the method was evaluated by intra-day assay and inter-day assay. The former quantified the same standard sample using the same procedure three times within 1 day by ELISA, and the latter quantified the same standard sample once a day in three continuous days. Concentration of standard samples were 0.2, 0.8, and 1.6 ng/mL, and the results of each assay was expressed as the coefficient of variation (CV%). The limit of detection (LOD) of the method was calculated as the mean of the nine blank samples plus three times of the standard deviation. The limit of quantification (LOQ) of the method was calculated as the mean of the nine blank samples plus ten times of the standard deviation (Werner et al. 2007). The specificity of the method was evaluated through cross-reactivity test using chicken breast, pork tenderloin, and tilapia fillet as samples for ELISA analysis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

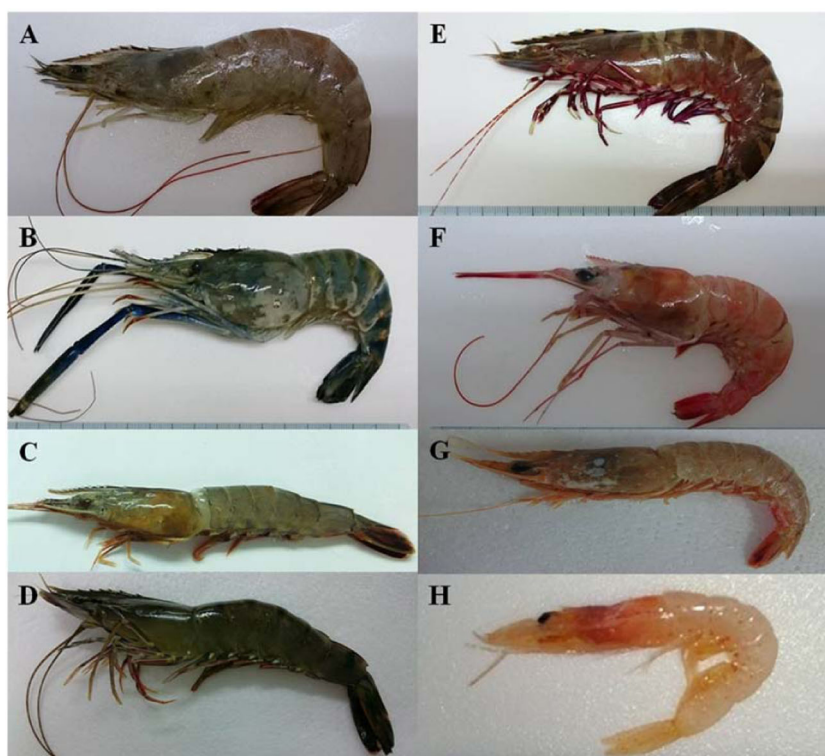
The sample protein extracts were heated to 100 °C for 5 min, and 10 µL were loaded into each well. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by Mini-PROTEAN® Electrophoresis System (Bio-Rad, USA) with 60 V for 20 min, then 80 V for 120 min at room temperature. Precision Plus Protein™ Dual Color Standards (Bio-Rad, USA) was used as protein marker. The separated proteins were stained with Bio-Safe™ Coomassie G-250 Stain (Bio-Rad, USA).

Results

Standard Curve of ELISA

The concentrations of five tropomyosin standards were 0.125, 0.25, 0.5, 1, and 2 ng/mL. As Fig. 2 showed, the working

Fig. 1 Eight shrimp species in Taiwan. **a** Whiteleg shrimp (*Litopenaeus vannamei*). **b** Giant river prawn (*Macrobrachium rosenbergii*). **c** Spear shrimp (*Parapenaeopsis hardwickii*). **d** Giant tiger prawn (*Penaeus monodon*). **e** Green tiger prawn (*Penaeus semisulcatus*). **f** Red shrimp (*Solenocera melantho*). **g** Southern rough shrimp (*Trachypenaeus curvirostris*). **h** Sakura shrimp (*Sergia lucens*)



range of the standard curve was displayed as linear curve ranged from 0.125 to 2 ng/mL with correlation coefficient (R^2) of 0.9991.

Levels of Tropomyosin in Shrimps

Table 1 shows the levels of tropomyosin in raw and cooked shrimps. Giant tiger prawn, whiteleg shrimp, and green tiger prawn have the three highest contents of tropomyosin among raw samples, and the tropomyosin levels in them were more than 6 mg per gram of raw shrimp meat. In contrast, red shrimp, green tiger prawn, and whiteleg shrimp have the three highest contents of tropomyosin among cooked samples, and the tropomyosin levels in all cooked shrimp were ranged from

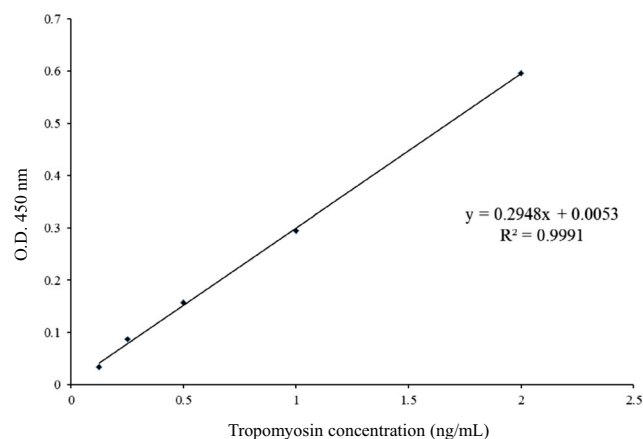


Fig. 2 Standard curve of ELISA for tropomyosin measurement

0 to 3 mg/g, except for red shrimp. This result clearly demonstrates that tropomyosin was partially disrupted after being steamed for 3 min, but decreasing levels varied by shrimp species.

Precision and Specificity of ELISA

Intra-day assay and inter-day assay were performed in precision test. As shown in Table 2, the CV% of intra-day precision were 8.70, 3.33, and 1.24 when 0.2, 0.8, and 1.6 ng/mL were used as standard test samples, respectively. When same standard test samples were used, the CV% of inter-day precision were 8.70, 10.00, and 6.90%, respectively. In addition, the relative error were 15% or less when 0.2, 0.8, and 1.6 ng/mL were used as standard test samples, representing the measured concentration approximated standard concentration.

Levels of tropomyosin in chicken breast, pork tenderloin, and tilapia fillet were 0.31 ± 0.03 $\mu\text{g/g}$, 0.04 ± 0.00 $\mu\text{g/g}$, and 0.03 ± 0.00 $\mu\text{g/g}$, respectively (Table 3).

Protein Profile of Shrimp Extracts

Figure 3a shows the protein profile of raw shrimp extracts by SDS-PAGE, and the band with 36 kDa in each line was tropomyosin. After steaming shrimps for 3 min, the tropomyosin band was still observed in the gel (Fig. 3b). The result was consistent with our previous data that tropomyosin was retained after heat treatment. In contrast, most of the other

Table 1 Levels of tropomyosin in eight shrimps

Common name	Scientific name	Tropomyosin (mean $\pm$ SD) (mg/g)		Decrease of tropomyosin (%)
		Raw	Cooked	
Whiteleg shrimp	<i>Litopenaeus vannamei</i>	7.17 $\pm$ 0.52	1.66 $\pm$ 0.21	76.8
Giant river prawn	<i>Macrobrachium rosenbergii</i>	4.73 $\pm$ 0.21	1.55 $\pm$ 0.41	67.2
Spear shrimp	<i>Parapenaeopsis hardwickii</i>	5.83 $\pm$ 0.60	0.58 $\pm$ 0.08	90.1
Giant tiger prawn	<i>Penaeus monodon</i>	7.59 $\pm$ 0.45	1.14 $\pm$ 0.08	85.0
Green tiger prawn	<i>Penaeus semisulcatus</i>	6.44 $\pm$ 1.10	2.93 $\pm$ 0.35	54.5
Red shrimp	<i>Solenocera melantho</i>	4.50 $\pm$ 0.28	4.93 $\pm$ 0.02	–
Southern rough shrimp	<i>Trachypenaeus curvirostris</i>	4.11 $\pm$ 0.65	1.42 $\pm$ 0.05	65.5
Sakura shrimp	<i>Sergia lucens</i>	5.83 $\pm$ 0.10	0.64 $\pm$ 0.05	89.0

Each sample was tested in triplicate ($n = 3$)

SD, standard deviation

protein in shrimps were degraded without observable bands in the gel.

Levels of Tropomyosin in Processed Foods

Table 4 shows the tropomyosin levels in processed foods. Tropomyosin was detected in all crustacean-containing foods. Among them, shrimp dumpling had the highest tropomyosin levels at 67.25 μ g/g. In contrast, products without crustacean ingredients showed only trace amount (lower than 0.02 μ g/g) of tropomyosin.

Discussion

Tropomyosin is the major allergen in fresh shrimp, which accounts for 80% of shrimp and crab allergies (Jeoung et al. 1997). The levels of tropomyosin or the existence of allergic proteins in shrimps consumed in Taiwan play an important role in understanding the food allergy epidemic in Taiwan. This is the first study to analyze the levels of tropomyosin in eight species of Taiwanese shrimps. Immune reactions from allergen exposure are quite variable among individuals. Some may have severe reactions with trace exposure while others may have little reaction with large quantity of exposure. This

study aims to investigate the levels of tropomyosin in various species to provide consumers more information for allergy exposure.

As the ELISA protocol used in this study was modified from original instructions, we recheck the validation of this analytical method. LOD and LOQ of ELISA were 0.030 and 0.064 ng/mL, respectively, showing the good sensitivity of this method. As a previous study reported only non-vertebrate tropomyosins may cause allergy (Ayuso et al. 2002), we used pork, chicken, and fish as test samples to assess the specificity of ELISA in our study. The results showed low concentration of tropomyosin in pork, chicken, and fish using this method, and the measured concentration was 1/10000 of that in shrimps, suggesting the good specificity of ELISA. Jenkins et al. reported that the homology of crustacean tropomyosin with pig, chicken, and fish tropomyosin was 55% (Jenkins et al. 2007). Our result was consistent with the previous study that shows the low cross-reaction with crustacean tropomyosin to pork, chicken, and fish.

Generally speaking, the lower of the CV%, the better of the precision, and the acceptable level of CV% was below 20% (Kamath et al. 2013). Our result shows the ELISA used in this study has good precision. According to the validation data from manufacturer, the ELISA developed in our study showed better precision and sensitivity in low tropomyosin concentration. In

Table 2 Inter-day and intra-day precision of ELISA in this study

Standard concentration (ng/ml)	Inter-day precision		Relative error	Intra-day precision		Relative error
	Result (mean $\pm$ SD) (ng/ml)	CV (%)		Result (mean $\pm$ SD) (ng/ml)	CV (%)	
0.20	0.23 $\pm$ 0.02	8.70	15.00%	0.23 $\pm$ 0.02	8.70	15.00%
0.80	0.90 $\pm$ 0.09	10.00	12.50%	0.90 $\pm$ 0.03	3.33	12.50%
1.60	1.74 $\pm$ 0.12	6.90	8.75%	1.61 $\pm$ 0.02	1.24	0.63%

Each sample was tested in triplicate ($n = 3$) and each experiment included a standard curve

SD, standard deviation; CV, coefficient of variation

Table 3 Levels of tropomyosin from chicken, pork, and fish samples

Sample	Tropomyosin (mean $\pm$ SD) ($\mu\text{g/g}$)
Chicken breast	0.31 ± 0.03
Pork tenderloin	0.04 ± 0.00
Tilapia fillet	0.03 ± 0.00

Each sample was tested in triplicate ($n = 3$)

SD, standard deviation

addition, we change the original standard curve into linear shape ranged from 0.125 to 2 ng/mL with correlation coefficient of 0.9991. We further verified that the relative errors of measured concentration were low when 0.2, 0.8, and 1.6 ng/mL were used as standard test samples, suggesting good validity in the linear range.

Shrimps are often cooked prior to consumption, so we determined tropomyosin levels in cooked shrimps in this study. Our results showed tropomyosin levels decreased in most of the species after steaming. We also demonstrated only trace tropomyosin levels measured in canned crab in this study. Previous studies suggested the decrease levels may be the result of masking of tropomyosin epitope from heating,

Table 4 The levels of tropomyosin in commercial processed foods

Name	Tropomyosin ($\mu\text{g/g}$)
Canned crab ^a	34.13
Crab crackers ^a	10.74
Shrimp meat ball ^a	22.16
Shrimp dumpling ^a	67.25
Cuttlefish-flavored crackers ^b	0.01
Instant curry ^b	0.02

^a Showing “Contain shrimp and crab ingredients” on the label of the package

^b Showing “No shrimp and crab ingredients but produced through the same production line for crustacean-containing products” on the label of the package

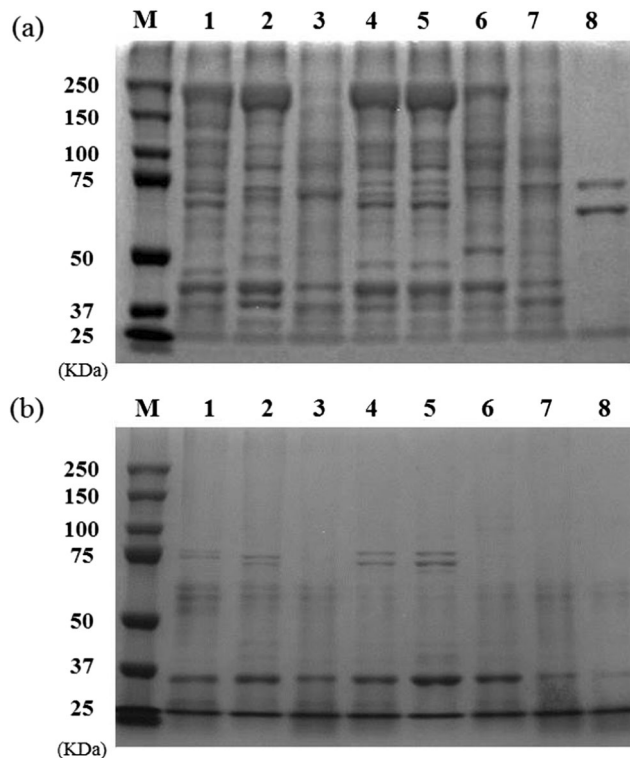


Fig. 3 Protein profiles of eight shrimps in this study. **a** Raw samples. **b** Cooked samples. M, protein marker; 1, whiteleg shrimp (*Litopenaeus vannamei*); 2, giant river prawn (*Macrobrachium rosenbergii*); 3, spear shrimp (*Parapenaeopsis hardwickii*); 4, giant tiger prawn (*Penaeus monodon*); 5, green tiger prawn (*Penaeus semisulcatus*); 6, red shrimp (*Solenocera melanthero*); 7, southern rough shrimp (*Trachypenaeus curvirostris*); 8, sakura shrimp (*Sergia lucens*)

leading to low binding affinity with specific-IgE. Our findings were consistent with the previous study showing that the intensive heat processing was possible to reduce the allergenicity of the tropomyosin. On the other hand, the literature reported that protein denaturation resulted in formation of insoluble complex (Downs and Taylor 2010; Lasekan and Nayak 2016), which may explain why we had lower tropomyosin extraction and levels. Interestingly, levels of tropomyosin in red shrimp were not decreased after steam treatment. As amino acid sequence of crustacean allergen was relatively well preserved among species (Chen et al. 2013; Shiomi et al. 2008), a possible explanation could be variable tropomyosin structure for red shrimp, which increased its heat stability compared with other species. Future study should elucidate the relationship between shrimp species and structure of tropomyosin.

Although over-heating treatment leads to tropomyosin disruption, other methods of reducing allergenicity of the food should be considered. Studies showed that enzyme, such as protease and horseradish peroxidase, may reduce the allergenicity of tropomyosin via degrading tropomyosin into small fragments (Liu et al. 2017; Shimakura et al. 2005). Shrimp and crab processed food reduce the allergenicity of tropomyosin after Maillard reaction because of the reaction with monosaccharide (Nakamura et al. 2006; Nakamura et al. 2005). Other processing methods, including r-radiation and ultrasound, also disrupt the structure of the epitope and result in lower allergenicity (Li et al. 2007; Li et al. 2006). Besides heat processing, we will further conduct the study to elucidate the effect of various processing methods on tropomyosin in the future.

Shrimps have high-quality protein, nutritional value, and savory taste, making them popular with consumers worldwide. We found that low levels of tropomyosin levels of cooked shrimps used in this study triggered allergic responses in patient sera (data not shown). This is consistent with previous studies showing trace amount and heated shrimps are still

capable of inducing allergic reactions (Abdel Rahman et al. 2013; Kamath et al. 2014; Vang et al. 2013).

In this study, we also demonstrated the tropomyosin levels in sakura shrimp, which is a valuable shrimp in Pingtung, Taiwan. They were captured in specific place and season, and were served as local food. Moreover, the specific shrimps were usually added into processed food leading to accidental ingestions of allergen. Consequently, it is important to develop a reliable analytical method for allergen measurement and detection. According to our results, the ELISA used in this study was also a useful technique to detect tropomyosin in shrimps and crustacean-containing processed foods.

Conclusion

An ELISA was successfully developed in this study, and the sensitivity, precision, and specificity were validated. This could be used in allergen inspection and quality control during food processing. Importantly, this is the first study that investigated the levels of tropomyosin in raw and cooked shrimps consumed in Taiwan, and the tropomyosin contents decreased after heat treatment, but varied depending on species. These findings will improve the study of shrimp allergy in the future.

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Compliance with Ethical Standards

Conflict of Interest Shin-You Lin declares that he has no conflict of interest. Chih-Hung Lee declares that he has no conflict of interest. Edward S. Huang declares that he has no conflict of interest. Shyang-Chwen Sheu declares that she has no conflict of interest. Hsu-Sheng Yu declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

Abbreviations ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WHO, World Health Organization

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