1. Background

In recent years, the incidence of allergic diseases has shown an upward trend, resulting in a global public health problem (1-4). As an inhalant allergen, the dust mite attracted great attention in allergological research (5-8). It was confirmed that dust mites *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* are the major indoor allergens that can cause bronchial asthma, allergic rhinitis, atopic dermatitis, and other allergic diseases (7-9).

The study of dust mite allergens is the basis for understanding the allergic diseases caused by dust mites. Each day, more and more new dust mite allergens are being identified (10, 11), greatly increasing our knowledge of dust mite allergens, promoting the levels of diagnosis, prevention, and treatment of the dust mite allergic diseases. On the basis of our previous study (12), 84 allergen proteins of *D. farinae* were identified using LC-MS/MS methods. These allergen proteins include some previously reported house dust mite allergens.

**Keywords:** *Dermatophagoides farinae*; Expression; Hsp60
mite allergens, many kinds of enzymes, translation elongation factor, ribosomal protein, outer membrane (lipo-) protein, keratin, glycine cleavage system protein, binding protein, heat shock protein, regulation protein, hypothetical protein, pol polyprotein, pol protein, and etc. As an inhalant allergen, the heat shock protein 60 (Hsp60) from D. farinae was further investigated to identify undetected D. farinae allergens.

2. Objective
In this study, to identify the new allergen the partial cDNA sequence of the D. farinae Hsp60 was determined. Moreover, the cDNA fragment was cloned and transformed into E. coli for the prokaryotic expression of the protein. The immunological characteristics of the expressed product were analyzed by ELISA and Western blot methods to offer a foundation for future investigation of the allergic diseases caused by Dermatophagoides farinae.

3. Materials and Methods

3.1. Dust mite D. Farinae
We used a vacuum cleaner (PHILPS FC6048, Qingdao co., LTD.) to collect samples of dust mites from the room and pillow of some students at the Guangzhou Medical University. A surgical mask was used to cover the vacuum tube. The surgical mask was replaced each time. The retrieved samples were placed on a plastic plate (KA061, 9 cm), and the mites were detected with a zero brush under the body vision microscope (smz-168, Motic company). Dust mite D. farinae was cultured at a constant temperature (27 ± 0.5 ºC) and humidity (relative humidity of 75 % ± 1 %) in our laboratory and afterwards stored at -80 ºC.

3.2. Plasmid
Prokaryotic expression vector pET-32a (+) was kindly provided by Professor Peng Lifen of Guangdong Medical College. P^MD18-T vector, an effective vector for Cloning of PCR products (TA Cloning), was purchased from TaKaRa (Biotechnology, Dalian, China).

3.3. Serum
The hypersensitivity of patients to D. farina was determined with the Uni-CAP 100-automatic allergen detection system (Pharmacia, Sweden), and their sera were collected by the Guangzhou Institute of Respiratory Diseases and Kingmed Diagnostics Center. Serum samples from 50 patients, with levels of specific IgE (S IgE) against D. farina at 3 or more, were mixed and used in this study. The negative control sera lacking the S IgE were collected from the people with negative skin prick test results and without a history of allergy to house-dust mites.

3.4. RNA Extraction and cDNA Synthesis
The homogenate of 300 dust mites D. farinae was mixed in 1ml Trizol (Invitrogen, USA) and RNA was extracted according to the Trizol Reagent’s instructions. Then the RNA was reverse-transcribed into cDNA with PrimeScript RT-PCR Kit (TaKaRa, Dalian, China).

3.5. PCR Amplification
On the basis of the characteristic and conservative sequences of the Hsp60 homologues of other species, the degenerate primers were designed. The upstream primer was 5’-GATGAACCNA(T/G)TTYNTAARMGNGG-3’, and the downstream primer was 5’-AGTC ACCAAATCCNGGNCYTNTNAC-3’.

PCR amplifications were performed using the prepared cDNA as a template, and the following cycling parameters were used in the PCR reactions (BIO-RAD T100, USA): 94 ºC for 5 min, 30 cycles of 94 ºC for 45 s, 60 ºC for 45 s, and 72 ºC for 90 s, and 72 ºC for 10 min.

3.6. Sequencing Analysis
The PCR products were sequenced by Takara Biotechnology Companies (Dalian, China). To investigate homology, the acquired nucleotide sequence and the corresponding amino acid sequence were analyzed with BLAST (http://www.ncbi.nlm.nih.gov/).

3.7. TA Cloning
According to the sequencing result, specific PCR primers were designed. (Upstream primer was 5’-GGGATCCCAATGGATTGATTACACGC-3’ and downstream primer was 5’-CGCTGAGTCTGGTGGCTTCACAG-3’, underlines show the Bam HI and Xho I restriction sites, respectively). Then PCR amplification of the D. farinae Hsp60 gene fragment was carried out, and the reaction conditions were 94 ºC for 5 min, 30 cycles of 94 ºC for 45 s, 65v for 45 s, 72 ºC for 45 s, and 72 ºC for 10 min. PCR products were purified by an agarose gel DNA extraction kit (Tiangen Biotech, Beijing, China) and subsequently were inserted into the P^MD18-T vector to construct P^MD18-T-Hsp60. The recombinant plasmid was identified with PCR amplification and restriction enzymes digestion.

3.8. Subcloning
The plasmid P^MD18-T-Hsp60 was digested with Bam HI and Xho I (TaKaRa, Dalian, China).
3.12. Western Blot Analysis
The purified recombinant Hsp60 was transferred onto PVDF membrane according to the BIO-RAD Trans-Blot SD operations guide. The sera from the patients hypersensitive to *D. farina* were diluted 25-fold and were used to react with the recombinant protein at 4 °C overnight. Goat anti-human IgE-HRP (KPL Inc. USA) was diluted at a ratio of 1:2500, then reacted with the membrane for 1h, and finally the results were visualized using the ECL chemiluminescence method.

4. Results

4.1. PCR Amplification and cDNA Sequencing
Using the degenerate primers, a product about 500 bp was successfully amplified by PCR. As shown in Figure 1, the PCR product was sequenced. BLAST indicated that the cDNA sequence was 90% similar to the Hsp60 of bacterium *Variovorax*, and the potential amino acid sequence showed 94% similarity to the Hsp60 of bacterium *Acidovorax avenae*.

4.2. Construction of a Plasmid Expressing pET-32a(+) -Hsp60 Recombinant Protein
Specific PCR primers with restriction sites BamH I and Xho I were used to amplify the Hsp60 gene fragment of *D. farinae*, and the result (Fig. 2A) showed that the size (about 520 bp) of the amplified product is consistent with our expectation. The results of PCR amplification and the BamH I and the XhoI restriction enzymes digestion of pMD18-T-Hsp60 are shown in Figure 2B, suggesting that the target gene was successfully cloned into the vector. Subsequently, the Hsp60 gene fragment in recombinant plasmid pMD18-T-Hsp60 was subcloned into plasmid pET-32a (+), and Figure 2C shows that the recombinant plasmid pET-32a(+) -Hsp60 was successfully constructed.

4.3. Expression and Solubility Analysis of the Recombinant Protein
As shown in Figure 3A, in comparison with the control, pET-32a (+)-Hsp60 could express a protein about 37KD, which is consistent with putative molecular mass of the fusion protein. In addition, the expression of the recombinant protein could be found in the supernatant and pellet of culture medium, and the expression of the recombinant protein in the supernatant demonstrates higher water-solubility (Fig. 3B).

4.4. Purification of the Recombinant Protein
The result for the purification of the soluble Hsp60

[This continues with detailed descriptions and results of the purification process, including the use of Ni²⁺-NTA agarose resin, SDS-PAGE analysis, and ELISA results for detecting the recombinant protein.]

Iran J Biotech. 2018;16(3):e1697
fusion protein is shown in Figure 4, which implies the recombinant protein was purified effectively.

4.5. ELISA
The average OD values of the patient sera, the negative control sera, and the blank controls were 0.383, 0.126, and 0.079, respectively. The OD values of the patient sera is >2.1-fold of the negative control sera, which proves that the recombinant protein can react with SIgE in the patient sera.

4.6. Western Blot Analysis
The results for the Western blot analysis of the recombinant Hsp60 are shown in Figure 5, which shows that the recombinant protein could be specifically recognized by SIgE in the patient sera.

Figure 1. Sequencing result, nucleic acid sequence alignments, AA sequence alignments and evolutionary relationships of the D. farinae Hsp60 with its reference species. (A) Sequencing result; (B) Multiple nucleic acid sequence alignments; (C) Multiple aa sequence alignments; (D) Evolutionary relationships. The alignments and phylogenetic tree were generated using the Megalign program in LASERGENE (DNAStar 7.0) with the Clustal V method, and sequence distance was calculated using weight matrix PAM250. Gaps were introduced by the alignment program to maximize the homology.

Figure 2. Cloning and verification of plasmid pET-32a(+) -Hsp60. (A) PCR amplification of D. farinae Hsp60. Lane M: DNA Marker; Lane 1: PCR product. (B) Identification of recombinant plasmid PMD18-T-Hsp60. Lane M: DNA Marker; Lane 1: PCR product of the recombinant plasmids PMD18-T-Hsp60; Lane 2: Recombinant plasmid PMD18-T-Hsp60 digested with BamHІ and XhoІ. (C) Identification of recombinant plasmids pET-32a(+) -Hsp60. Lane M: DNA Marker; Lane 1: Recombinant plasmid pET-32a(+) -Hsp60 digested with BamHІ and XhoІ; Lane 2: PCR product of recombinant plasmid pET-32a(+) -Hsp60.
5. Discussion
The heat shock proteins (HSPs) are important for the growth and the development of prokaryotes and eukaryotes. It is well known when organisms are stimulated by physical, chemical, and pathological factors, the expression levels of the HSPs increase. It has been reported that the HSPs can be recognized by the immune system, and is involved in the antigen processing and presenting (14). Moreover, evidently the HSPs of some parasites could induce immunological response in the host, indicating their function as antigen (15, 16). In addition, it has been confirmed that the Hsp70 of *D. farinae* may function as an allergen (17). Based on the results of our previous study (12), we suspect that *D. farinae* Hsp60 is also an allergen. In combination with another research conducted by our group (18), we have demonstrated the existence of a Hsp60 family of *D. farinae*.

In this study, the partial cDNA sequence of the *D. farinae* Hsp60 was successfully identified and it was efficiently expressed in the prokaryotic expression system. Moreover, ELISA and western blot analysis revealed that the expressed recombinant protein could be specifically recognized by SIgE in sera of the *D. farinae* allergic patients. The results suggest that the Hsp60 may be an undetected *D. farinae* allergen.

Currently, using the patients’ sera as a probe, the immunological screening of the cDNA library is a routine method to identify and discover new dust mite allergens (19). However, with high-throughput proteomics analysis technology, our group screened out eighty-four proteins (included Hsp60) of *D. farinae*, and we identified some of them as possible potential allergens (12). Moreover, the Hsp60 from *D. farinae* was successfully identified in this study. Our finding suggests a selectable way for screening dust-mite allergens, which also provides a new approach to identify new dust-mite allergens.

6. Conclusions
Taken together, the results in this study provide a solid foundation for further analyzing the allergenicity of *D. farinae* Hsp60 and its possible clinical application.

Figure 3. Expression and solubility analysis of the recombinant protein. (A) SDS-PAGE analysis of the expression of pET-32a(+) -Hsp60. Lane M: molecular mass standards; Lane 1: Expression product of pET-32a(+) (control); Lane 2: Expression product of pET-32a(+) -Hsp60. (B) Solubility analysis of pET-32a(+) -Hsp60 expression product. Lane M: molecular mass standards; Lane 1: Expression product of pET-32a(+) (pellet); Lane 2: Insoluble expression product of pET-32a(+) -Hsp60 (pellet); Lane 3: Expression product of pET-32a(+) (supernatant); Lane 4: Soluble expression product of pET-32a(+) -Hsp60 (supernatant).

Figure 4. Purification of the recombinant protein Lane M: molecular mass standards; Lane 1: Expression product of pET-32a (+); Lane 2,3: unpurified and purified expression products of pET-32a (+)-Hsp60, respectively.

Figure 5. Western blot analysis of the recombinant Hsp60. Lane M: molecular mass standards; Lane 1: Expression product of pET-32a (+); Lane 2: Expression product of pET-32a (+)-Hsp60.
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Authors’ contributions
ZML and HJL carried out most of the experiments and wrote the manuscript. DXC has extensively revised the manuscript and the experimental design. JL, MSC, TC, QH, XDL, XML, XD, and HXS helped with the experiments. All the authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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