

Characterization of *Sarcoptes scabiei* Tropomyosin and Paramyosin: Immunoreactive Allergens in Scabies

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Abstract. Scabies is a human skin disease due to the burrowing ectoparasite *Sarcoptes scabiei* var. *hominis* resulting in intense itching and inflammation and manifesting as a skin allergy. Because of insufficient mite material and lack of in vitro propagation system for antigen preparation, scabies is a challenging disease to develop serological diagnostics. For allergen characterization, full-length *S. scabiei* tropomyosin (Sar s 10) was cloned, expressed in pET-15b, and assessed for reactivity with IgE antibodies from human sera. IgE binding was observed to Sar s 10 with sera collected from subjects with ordinary scabies, house dust mite (HDM)-positive and naive subjects and a diagnostic sensitivity of < 30% was observed. *S. scabiei* paramyosin (Sar s 11) was cloned, and expressed in pET-28a in three overlapping fragments designated Sspara1, Sspara2, and Sspara3. IgE and IgG binding was observed to Sspara2 and Sspara3 antigens with sera collected from ordinary scabies, and HDM-positive subjects, but no binding was observed with sera collected from naive subjects. Sspara2 displayed excellent diagnostic potential with 98% sensitivity and 90% specificity observed for IgE binding and 70% sensitivity for IgG. In contrast, the diagnostic sensitivity of Sspara3 was 84% for IgE binding and 40% for IgG binding. In combination, Sspara2 and Sspara3 provided an IgE sensitivity of 94%. This study shows that IgE binding to Sspara2 and Sspara3 is a highly sensitive method for diagnosis of scabies infestation in clinical practice. The developed enzyme-linked immunosorbent assay helps direct future development of a specific diagnostic tool for scabies.

INTRODUCTION

Sarcoptes scabiei var. *hominis* (*S. scabiei* var. *hominis*) causes a common human skin infestation known as scabies.^{1–4} Scabies occurs worldwide among people of all ages, races, genders, and social classes and has been identified as a neglected tropical infectious disease.^{5,6} It has been estimated that about 1–10% of the global population is infected with scabies with an infection rate as high as 50–80% in certain populations.⁷ Scabies ranges from common ordinary scabies (OS) with infestation of 10–15 mites per person⁸ to rare crusted scabies (CS) with infestation of up to millions of mites per person.⁹ Clinical manifestations of OS are wide ranging, depending on degree and activation of the inflammatory response, burrows, and itching. CS is a severe form of the disease and often found in patients with human immunodeficiency virus infection, human T lymphotropic virus 1 (HTLV-1) infection, organ transplant patients, the mentally impaired, and physically incapacitated. It has also been recognised in overtly immunocompetent patients.¹⁰ These patients have been recognized as core transmitters of scabies,¹¹ due to hyperproliferation of mites.

Scabies is a challenging disease to diagnose due to difficulties in isolation of *Sarcoptes* mite on the human host in OS⁴ and physical signs confounding with other skin diseases. There is no in vitro propagation system of *S. scabiei* var. *hominis* for antigen preparation. Routinely used diagnostic methods are direct observation of clinical signs including the typical history of pruritus, presence of inflammatory papules, and visible lesions on the host body. The microscopic identification of scabies mites or eggs in infected skin scrapings is rarely carried out but shows less than 50% sensitivity.^{12,13}

Clinical diagnosis is often complicated because visible lesions on scabies patients are sometimes masked by eczema or impetigo or are atypical.⁴ Alternative diagnostic options include polymerase chain reaction (PCR)¹⁴ and dermatoscopy,¹⁵ both of which have been demonstrated as informative in patients where mites or mite DNA can be located. This practice is uncommon worldwide and not easily applied to clinical health settings.

Because of the lack of sensitivity in the present diagnostic methods for human scabies, there is a great need for the development of a reliable serodiagnostic test such as enzyme-linked immunosorbent assay (ELISA) to control scabies at both the individual and community level. There is no commercially available ELISA for the diagnosis of scabies in humans but an ELISA has been developed for detection of mange in animals utilizing whole mite antigen (WMA) extracts from *S. scabiei* var. *suis*.^{16,17} However, the reported specificity and sensitivity of these tests are variable.^{18,19}

Expressed sequence tag (EST) libraries from *S. scabiei* var. *hominis* and var. *vulpus* are available, and include homologues of multiple house dust mite (HDM) allergens, opening new possibilities in scabies research.^{20–23} As a result, several *S. scabiei* homologues to multiple HDM allergens have been sequenced, cloned, and expressed including *S. scabiei* serine proteases, cysteine proteases, glutathione S-transferase, and apolipoprotein.^{21,22,24,25} The *S. scabiei* var. *hominis* cysteine protease and apolipoprotein have been shown to bind scabies specific antibodies in IgE ELISA tests.²⁵ The *S. scabiei* apolipoprotein is considered a potentially valuable serodiagnostic agent as it has been proved to be highly specific for scabies and does not show cross reactivity with HDM allergens.^{12,26} Recombinant *S. scabiei* cofilin also showed potential value as a diagnostic antigen and could be used to develop an ELISA-based serological test for the diagnosis of scabies in animals.²⁷

Tropomyosin, a muscle protein, is classified as a group 10 allergen.²⁸ It is reported as a major allergen of shellfish that is

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cross-reactive with *Der p 10*, the HDM tropomyosin²⁹ and an important component of immune and allergic reactions.³⁰ Tropomyosin has a conserved sequence and is a pan-allergen in invertebrates including shrimps, molluscs, mites, and cockroaches^{31,32} with 80% amino acid sequence homology between different species, that is, *S. scabiei*, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Blomia tropicalis*, and *Psoroptes ovis*. Several isoforms of the protein exist within a species.³³ *Sarcoptes scabiei* recombinant tropomyosin was evaluated as vaccinating agent, but showed no significant protection against sarcoptic mange in rabbits.³⁴

Paramyosin is a major HDM allergen and designated as group 11 allergen.³⁵ It has a molecular weight of approximately 98 kDa and is conserved among a variety of species.³⁶ In general, invertebrate paramyosin is cross-reactive in many invertebrates.^{31,37} The sheep scab mite *P. ovis* paramyosin has been identified as homologous to *Der p 11*^{38,39} and helminth paramyosin recognized as a target for protective immunity.⁴⁰ The paramyosin protein of *S. scabiei* is reported as a monomeric and dimeric protein that is highly abundant⁴¹ and comparable to paramyosin from other species.⁴²

Because of the potential significance of tropomyosin and paramyosin in host allergic immune response and their potential usefulness as serodiagnostic markers for human scabies, we investigated the IgE specificity of *S. scabiei* tropomyosin (Sar s 10) and paramyosin (Sar s 11). The primary aim of this study was to determine whether *S. scabiei* var. *hominis* recombinant tropomyosin (Sar s 10) and paramyosin fragments (Sspara1, Sspara2, and Sspara3) could be used in a sensitive and specific diagnostic assay for scabies infestation in humans by measuring IgE and IgG level in serum from subjects with scabies compared with those with allergy to HDM and naive individuals.

MATERIALS AND METHODS

Ethical approval and sample collection. Ethical approval for collection of mites and blood samples was granted by the Dean, Faculty of Sciences of Pir Mehr Ali Shah Arid Agriculture University Rawalpindi Pakistan and Executive Director of hospital following guidelines of National Bioethics Committee of the Pakistan Medical Research Council (Permit or letter No. PMAS-AAUR/ZOOL 362 approved on March 22, 2012). The serum samples were collected after written informed consent was received from three groups of 96 participating individuals: OS subjects ("ordinary," $N = 48$), subjects with allergy to HDM based on clinical history and positive skin prick test to a panel of common aeroallergens ("HDM," $N = 24$), and subjects naive to scabies and allergy with no known exposure to scabies or allergy ("naive," $N = 24$). The cases of OS were confirmed by clinical observation and positive identification of mites and mite parts under the microscope. Samples were stored at -80°C until assessed. For the isolation of RNA, stored mites originally collected from CS patients were used, with ethical approval from the Menzies School of Health Research Ethics committee (Approval 01/15). The adult mites were collected in 100 μL of cold Trizol (Invitrogen) and stored at -80°C until RNA extraction.

RNA extraction and amplification of cDNA encoding Sar s 10 and Sar s 11 fragments. Mites were homogenized in Trizol using a Minilys homogeniser (Bertin Technologies, Bretonneux, France). After phase separation, the aqueous

phase was column purified by Purelink RNA mini Kit (Life Technologies, Mulgrave, Victoria, Australia) following manufacturers protocol. The concentration was checked on the NanoDrop 2000 Spectrophotometer (Nanodrop Technologies, Scoresby, Victoria, Australia). One microgram of total RNA was transcribed to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Chadstone, Victoria, Australia) as per manufacturer protocol. For the amplification of Sar s 10 cDNA, primers were designed based on *Der p 10* sequence using the Primer3 software (<http://frodo.wi.mit.edu/>) (Table 1). Cycling conditions for Sar s 10 primer were initial denaturation 95°C 2 minutes, followed by 35 cycles of 95°C , 30 seconds; 50°C , 30 seconds; 72°C , 2 minutes; with final extension at 72°C , 8 minutes.

For the amplification of Sar s 11 cDNA, three sets of primers were designed to amplify these three overlapping paramyosin fragments (Sspara1, Sspara2, and Sspara3) based on *S. scabiei* var. *vulpus* sequence⁴³ (Table 1). The nucleotide positions of these overlapping Sar s 11 fragments were Sspara1 = 1–1,612 bp, Sspara2 = 1,300–2,100 bp, and Sspara3 = 1,750–2,652 bp. Cycling conditions for Sspara1, Sspara2, and Sspara3 were initial denaturation 95°C , 2 minutes, followed by 45 cycles of 95°C , 30 seconds; 58°C , 30 seconds; 72°C , 2.5 minutes; with final extension at 72°C , 8 minutes.

PCR products were ligated into pGEM-T-easy cloning vectors (Promega, Alexandria, New South Wales, Australia), transformed into α -select competent cells (Bioline, Alexandria, New South Wales, Australia), and sequenced (Big Dye 3.1, Applied Biosystems, Alexandria, New South Wales, Australia). Sar s 11 fragment sequences assembled into contigs. The identified nucleotide sequence was translated and analyzed by BLASTx (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) for homology in GenBank protein database of Sar s 10 and Sar s 11. The resulting top hit amino acid sequences of Sar s 10 and Sar s 11 were aligned using ClustalW (<http://www.genome.jp/tools-bin/clustalw>) and aligned sequences were further used for phylogenetic tree construction using the software Molecular Evolutionary Genetics Analysis (MEGA 6).⁴⁴

Expression and purification of recombinant Sar s 10 and Sspara1, Sspara2, and Sspara3. The cDNAs encoding Sar s 10 and Sspara1, Sspara2, and Sspara3 were subcloned into pET-15b and pET-28a expression vectors, respectively, in frame with the 6-His tag and then expressed in the *Escherichia coli* strain BL21 (DE3) (Bioline). Recombinant protein expression was induced by addition of 0.4 mM isopropyl- β -D-thiogalacto-pyranoside to the culture at 37°C for 4 hours. For purification of the recombinant Sar s 10 and Sar s 11 protein fragments, the pellet was resuspended in native lysis buffer (50 mM NaH_2PO_4 , 300 mM NaCl, pH 8.0), treated with lysozyme (1 mg/mL) and one complete ethylenediaminetetraacetic acid-free protease inhibitor cocktail tablet (Roche, Castlehill, New South Wales, Australia), incubated for 30 min following sonication. The lysate was centrifuged for 30 minutes ($10,000 \times g$ at 4°C) and the supernatant collected for purification. The recombinant proteins were purified using Nickel-nitroloacetic acid (Ni-NTA) agarose column as per the manufacturer's standard protocol (QIAGEN) under native conditions. The purified proteins (Sar s 10, Sspara1, Sspara2, and Sspara3) were quantified using the QubitTM Protein Assay Kits provided with the Qubit[®] 2.0 Fluorometer following the manufactures' protocol (Life Technologies). Proteins were

TABLE 1
Tropomyosin and paramyosin primers with restriction sites

Protein	Primer name	Primer sequences 5' to 3'	Primer restriction sites	Vector	Size (bp)	Molecular mass (kDa)
Tropomyosin	Sar s 10_F	GGCAGCCATATGTATGAGGCC ATCAAG	NdeI, BamHI	pET15b	855	31
	Sar s 10_R	GCAGCCGGATCCTCA CTAGTGATTTATAGC				
Paramyosin 5'-fragment (Sspara1)	Sspara1_F	ATCTGGATCCATGTCTG CTAGATCAGCTAAATTC	BamHI, HindIII	pET28a	1600	55
	Sspara1_R	CTCGAAGCTTGTG CGATCT CGGTTTTCAAT				
Paramyosin middle fragment (Sspara2)	Sspara2_F	ATCTGGATCCGATGATTTG GCTGAATGCAA	BamHI, HindIII	pET28a	800	29
	Sspara2_R	CTCGAAGCTTTAATCG TTCCTGTTCTCCAC				
Paramyosin 3'-fragment (Sspara3)	Sspara3_F	ATCTGGATCCCTTCAAGCAC ACTATGATGAG	BamHI, HindIII	pET28a	900	33
	Sspara3_R	CTCGAAGCTTGT TTGTTTTGTTGAGATTTATC				

NdeI, BamHI, and HindIII restriction sites are underlined.

analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and immunoblotting. Recombinant proteins (2 µg/lane) were separated by 12% SDS-PAGE and stained with Coomassie Brilliant Blue R-250, or by immunoblotting. For immunoblotting, purified proteins (2 µg), Sar s 14.3²⁵ (2 µg) as a positive control, and bovine serum albumin (0.5 µg) as a negative control were run on SDS-PAGE and proteins transferred to nitrocellulose membranes using the Biorad Trans-Blot Turbo Transfer System (Biorad, Gladesville, New South Wales, Australia). Membranes were blocked with 5% skim-milk powder (SMP) in phosphate-buffered saline with 0.5% Tween 20, overnight at 4°C. Membranes were incubated with 1:50 dilutions of pooled sera from 1) three OS subjects; 2) three HDM subjects; and 3) three scabies naive subjects, followed by anti-human IgE alkaline phosphatase (AP)-conjugated H&L (Abcam ab7426, Sapphire Bioscience, Melbourne, Victoria, Australia). Between each step, membranes were washed three times with 0.05% PBS-T. Membranes were developed and visualized with AP substrate (BCIP/NBT) (Sigma-Aldrich, Castle Hill, New South Wales, Australia).

Enzyme-linked immunosorbent assay. A three-stage ELISA was performed for both tropomyosin and for fragments of paramyosin (individually and then cocktail of Sspara2 and Sspara3 fragments). Briefly, 2 µg/well Sar s 10 and Sspara2 and Sspara3 fragments was bound to High Protein 96 well plates (Corning) (Sigma-Aldrich) in carbonate coating buffer (15 mM/L Na₂CO₃, 35 mM/L NaHCO₃, 3 mM/L NaN₃, pH 9.6) overnight at 4°C. Plates were blocked with 1% SMP-PBS-T (0.01%) at 37°C for 2 hours, then serum applied at a 1:10 (IgE assay) or 1:200 (IgG assay) diluted in 1% SMP-PBS-T (0.01%) at 37°C for 2 hours. Between each step, plates were washed three times with 0.05% PBS-T. Secondary antibody, either 1/1,000 anti-human IgE (Abcam ab91559, Sapphire Bioscience) (IgE assay) or AP-conjugated anti-human IgG dilution (Sigma-Aldrich, A9544) diluted 1/2,500 in 1% SMP-PBS-T at 37°C for 2 hours. After washing, AP-conjugated polyclonal rabbit anti-goat IgG H&L (Abcam ab6742, Sapphire Bioscience) (1/1,000 dilution) (IgE assay) was incubated at 37°C for 2 hours. Color was developed using phosphatase substrate (Sigma-Aldrich) dissolved in glycine buffer (0.1 M/L glycine, 1 mM/L MgCl₂, 1 mM/L ZnCl₂, pH 10.4) and developed for 30 minutes at 37°C and absorbance was measured at 405 nm with the Perkin-Elmer Enspire 2300 Multimode Reader and Enspire software 4.13 (Perkin-Elmer, Waltham, MA).

Statistical analysis. Prism v. 5.01 (GraphPad Software, Inc., San Diego, CA) software was used to carry out statistical analysis. Differences between groups were assessed by nonparametric Kruskal–Wallis analysis of variance. To check the distribution of two unmatched groups, nonparametric Mann–Whitney *U* tests were used. Comparisons were considered to be significant at *P* values of < 0.05. The ELISA cutoff was calculated by taking the average of the negative control group (naive) + 2 standard deviations. To calculate sensitivity percentages following formula was used: true positives/(true positives + false negatives) × 100% whereas specificity percentages were calculated by: (true negatives/[true negatives + false positives]) × 100%.²⁶ The receiver operator characteristic (ROC) curves and the area under the curve were calculated using Prism for subjects with a current scabies

infestation (OS) against subjects with HDM allergy, or no infestation (naive).

RESULTS

Amplification of cDNA-encoding Sar s 10 and Sar s 11.

The PCR product obtained by amplification with Sar s 10 primers (F/R) contained an open reading frame (ORF) of 855 bp and encoded a predicted protein of 284 amino acids. The Sar s 10 sequence was deposited in the European Nucleotide Archive database under accession number LT669813. The top hits when the nucleotide sequence for Sar s 10 was searched against GenBank protein database using Blastx, which showed 99% identity with *S. scabiei* var. *suis*, *D. farinae* and 97% with *D. pteronyssinus* and *P. ovis* to Sar s 10, respectively. The next two species with closest homologues were *Chortoglyphus arcuatus* and *Lepidoglyphus destructor* with 95% identity. With Sar s 10 *Glycyphagus domesticus* and *B. tropicalis*, tropomyosin showed 91% and 94% identification (Figure 1).

The sequence of the three partial overlapping fragments of paramyosin Sar s 11 (Sspara1, Sspara2, and Sspara3) was assembled into contigs using DNA Dragon-DNA Sequence Contig Assembler Software (<http://www.dna-dragon.com/download.php>). The complete Sar s 11 ORF was 2,652 bp in length and encoded 884 amino acids and is available in the European Nucleotide Archive under accession number LT669814. The sequences showed highest homology to Sar s 11 were from *S. scabiei* var. *suis*, var. *canis*, var. *cuniculi* with amino acid identity of 99%. *Choriotopes panda* and *P. ovis* had amino acid identity of 83% and amino acid identity of 82% was showed by *Choriotopes texanus*, *D. farinae*, and *D. pteronyssinus*, whereas the amino acid identities of Sar s 11 was lower at 79% for *B. tropicalis* (Figure 2).

Expression and purification of recombinant Sar s 10 and Sar s 11 fragments. The full coding sequence of the *S. scabiei* Sar s 10 and Sar s 11 fragments was cloned into the vectors pET-15b and pET-28a, respectively. The analysis of expressed

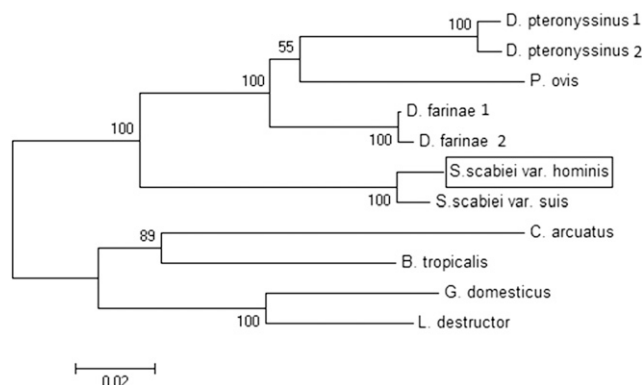


FIGURE 1. Relationships of identified Sar s 10 sequence with selected tropomyosin sequences. The amino acid sequences of the identified molecules were aligned using ClustalW with phylogenetic tree was constructed using MEGA 6. Abbreviations and accession numbers are as follows: *D. pteronyssinus* 1 (AAB69424.1), *D. pteronyssinus* 2 (ACI32128.1), *P. ovis* (CAJ38272.1), *D. farinae* 1 (BAA04557.1), *D. farinae* 2 (AIO08865.1), *S. scabiei* var. *hominis* (LT669813), *S. scabiei* var. *suis* (AFH08744.1), *C. arcuatus* (AEX31649.1), *B. tropicalis* (ABU97466.1), *G. domesticus* (AAQ54614.1), *L. destructor* (Q9NFZ4.1).

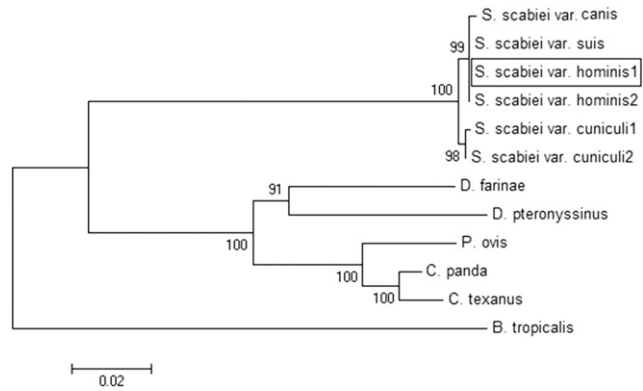


FIGURE 2. Relationships of identified Sar s 11 sequence with selected paramyosin sequences. The amino acid sequences of the identified molecules were aligned using ClustalW with phylogenetic tree was constructed using MEGA 6. Abbreviations and accession numbers are as follows: *S. scabiei* var. *canis* (AF317670.1), *S. scabiei* var. *suis* (EU606031.1), *S. scabiei* var. *hominis* 1 (LT669813), *S. scabiei* var. *hominis* 2 (AF462195.1), *S. scabiei* var. *cuniculi* 1 (EU116341.1), *S. scabiei* var. *cuniculi* 2 (DQ131648.1), *D. farinae* (KM010008.1), *D. pteronyssinus* (AY189697.1), *P. ovis* (AM114275.1), *C. panda* (EU543652.1), *C. texanus* (EF053124.1), *B. tropicalis* (AF525465.1).

and purified recombinant Sar s 10 protein using SDS-PAGE showed a single band of approximately 31 kDa. Immunoblotting showed that recombinant Sar s 10 was recognized by sera from subjects with OS. IgE binding was also observed with HDM and naive sera (Figure 3A).

Analysis of the expressed and purified overlapping recombinant fragments Sspara1, Sspara2, and Sspara3 on SDS-PAGE showed 55-, 29-, and 33-kDa bands. Immunoblotting analysis demonstrated no IgE binding to Sspara1 by sera from subjects in the OS, HDM, and naive groups. Recombinant Sspara2 and Sspara3 fragments were recognized by sera from the OS group, with strong IgE binding to both Sspara2 and Sspara3 fragments. The IgE binding was not observed with HDM and naive sera (Figure 3B).

Specific IgE binding to *S. scabiei* var. *hominis* recombinant Sar s 10 and Sar s 11 fragments. IgE binding to Sar s 10. For Sar s 10, no significant mean difference ($P = 0.249$) between specific IgE binding in the sera from the OS, HDM and the naive groups were observed (Figure 4).

IgE binding of human sera to Sspara2 and Sspara3. For Sspara2 and Sspara3, a clear distinction between specific IgE binding in the sera from OS, HDM, and naive subjects was observed. The Mann-Whitney test was used to analyze differences between group means. The IgE binding to Sspara2 for the OS individuals was significantly higher ($P < 0.0001$) than the HDM and naive individuals. The HDM group mean was significantly different to the mean response for subjects naive to scabies ($P = 0.0081$) (Figure 5, left panel).

The mean IgE binding to Sspara3 for the OS group compared to other groups was also significantly greater ($P < 0.0001$). The HDM subjects mean IgE response to Sspara3 was significantly different than the mean response for subjects naive to scabies ($P = 0.0379$) (Figure 5, right panel).

IgG binding of human sera to Sspara2 and Sspara3. The ordinary HDM allergy and naive subjects sera were also checked by indirect ELISA for binding of IgG to Sspara2 and Sspara3.

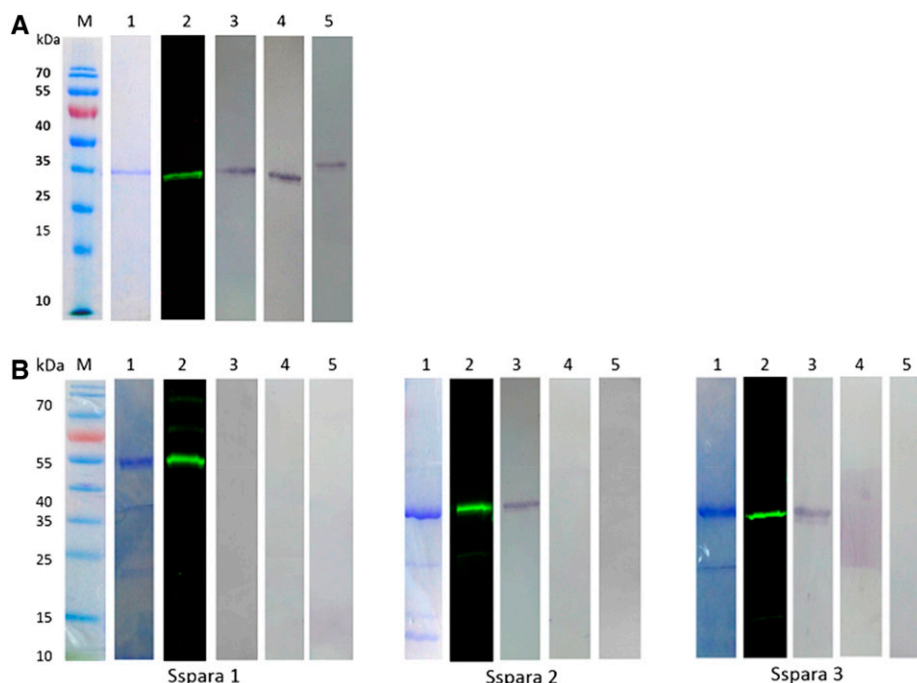


FIGURE 3. SDS-PAGE and IgE Immunoblot of recombinant Sar s 10 and Sar s 11 fragments. (A) For Sar s 10 immunoblot: Lanes: M, protein molecular weight markers; 1, purified recombinant Sar s 10; 2, anti-His immunoblot for detection of Sar s 10. IgE immunoblot probed with sera from ordinary scabies subjects (3), house dust mite (HDM) subjects (4), and naive subjects (5). (B) For Sspara1, Sspara2, and Sspara3 fragments immunoblot: M, protein molecular weight markers; 1, purified recombinant Sspara1, Sspara2, and Sspara3; 2, anti-His immunoblot for detection of Sar s 11 fragments. IgE immunoblot probed with sera from ordinary scabies subjects (3), HDM subjects (4), and naive subjects (5). This figure appears in color at www.ajtmh.org.

There was a significant difference in mean IgG binding to Sspara2 between the OS group and for the subjects naive to scabies group ($P < 0.0001$). Interestingly, however, there is no significant difference in mean IgG binding between OS and HDM allergy subjects ($P = 0.3907$) (Figure 6, left panel).

The IgG binding to Sspara3 was significantly different between the OS group and for the subjects naive to scabies group ($P = 0.0130$), as determined by Mann-Whitney U test. There was no significant difference between OS and HDM subjects ($P = 0.7969$) (Figure 6, right panel).

IgE and IgG binding of human sera to the antigen cocktail. IgE binding to the antigen cocktail (mixture of Sspara2 and Sspara3) of individual OS sera ($N = 48$) was high. The mean value of the OS group was significantly higher compared with HDM and naive groups ($P < 0.0001$). The HDM allergy subjects mean IgE response to the cocktail antigen was significantly different than the mean observed for subjects in the naive to scabies group ($P = 0.018$) (Figure 7, left panel).

The mean IgG binding to the antigen cocktail for the OS group was significantly greater compared with HDM ($P = 0.0007$) and naive groups ($P < 0.0001$). There was also significant difference in IgG binding between HDM and naive subjects ($P = 0.0024$) (Figure 7, right panel).

Comparison of diagnostic sensitivity and specificity of recombinant Sar s 10 and Sar s 11 fragments. The diagnostic sensitivity of the Sar s 10 for detection of active scabies infestation was very low. The assay had 30% and 67% sensitivity and specificity, respectively, with a positive likelihood ratio of 0.95 and a negative likelihood ratio of 1.11. For Sspara2, 98% sensitivity and 90% specificity were observed with a positive likelihood ratio of 5.48 and a negative

likelihood ratio of 0.03. The area under curve was 0.9706. In regard to IgG binding, Sspara2 has 70% sensitivity and 96% specificity and area under curve was 0.82. The diagnostic sensitivity of IgG was low compared with IgE with this antigenic fragment. For Sspara3, 84% sensitivity and 100% specificity were observed, with a positive likelihood ratio of 4.00 and a negative likelihood ratio of 0 and area under curve was 0.9600. With regard to IgG binding, Sspara3 has 40% sensitivity and 87.5% specificity and area under curve was 0.70. The diagnostic competence of the antigen cocktail was calculated at 94% sensitivity and 100% specificity for IgE binding and 62% sensitivity and 92% specificity for IgG binding. In comparing both antigens, that is, Sspara2 and Sspara3, Sspara2 gave excellent diagnostic specificity and sensitivity (Table 2).

DISCUSSION

Scabies and Sar s 10. The tropomyosin (Sar s 10) and paramyosin (Sar s 11) encoding genes of *S. scabiei* var. *hominis* were characterized and inferred as immuno-reactive allergens in scabies. Sar s 10 from *S. scabiei* var. *hominis* has an open reading frame of 855 bp and 285 amino acids, coding for an allergenic 31 kDa protein. Tropomyosin is considered a pan-allergen in invertebrates³¹ and reported as a cross reactive allergen among invertebrates⁴² because of the high degree of amino acid sequence identity.³⁴ Sar s 10 amino acid sequence revealed a 99% sequence homology with *S. scabiei* var. *suus*.³³ This protein also revealed 97% homology to the group 10 allergens of *D. pteronyssinus*, *D. farinae*, and *P. ovis*.⁴⁵ The *S. scabiei* tropomyosin also displayed 90–96%

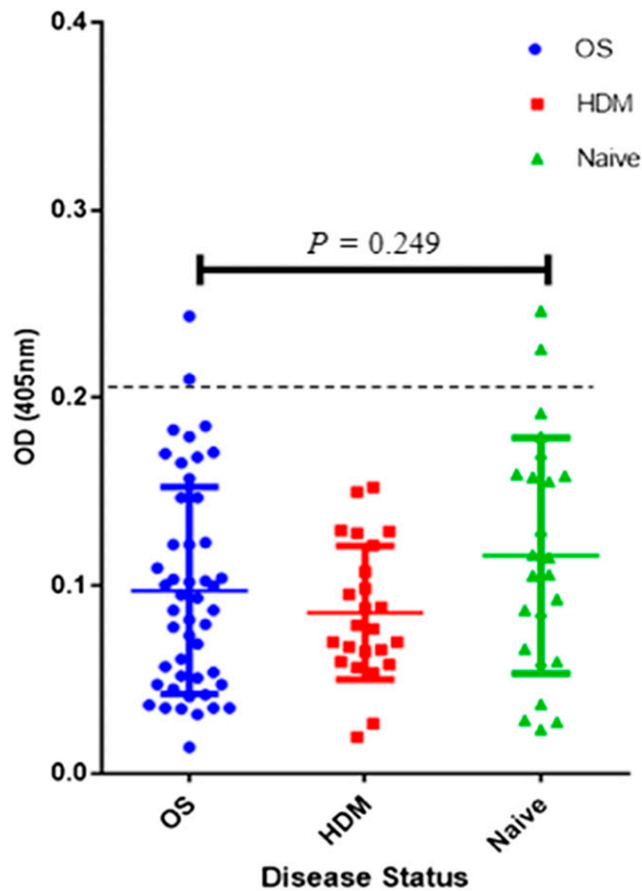


FIGURE 4. IgE binding to Sar s 10 in subjects with ordinary scabies (OS), house dust mite allergy (HDM), and naive to scabies (Naive). The cutoff value of the average of the naive +2 standard deviations was calculated to be 0.223. This figure appears in color at www.ajtmh.org.

homology with storage mites, that is, *L. destructor*, *G. domesticus*, *B. tropicalis*, *C. arcuatus*. This result was not unexpected and in support of previous data reporting the group 10 HDM allergen as highly conserved³¹ and cross-reactive with tropomyosin of species such as shellfish and other arthropods.⁴⁶

Among insects, *S. scabiei* tropomyosin had 80% amino acid identity with *Periplaneta americana*, *Blattella germanica*. It has been demonstrated that tropomyosin protein from cockroaches shares 80% homology with other arthropod tropomyosin.^{47,48} Tropomyosin was also found to be a major allergenic component accounting for the cross-reactivity between cockroaches and dust mites.⁴⁷ In several studies tropomyosin is reported as major shellfish allergen and commonly used as a biomarker for detection of shellfish allergens.⁴⁹ The tropomyosin protein from cockroaches also shares homology of 58%, 64%, 74% with other invertebrates, that is, *Ascaris lumbricoides*, *Sinonovacula constricta*, *Haliotis diversicolor*, respectively.⁵⁰ Because of the high sequence identity of Sar s 10 with other mite and invertebrate tropomyosins, it is unlikely to be a specific allergen for Sarcoptic mites and may have high cross-reactivity with tropomyosin of other mites.³²

Immunoanalysis of Sar s 10. Western blot analysis showed IgE binding by pooled OS patient sera and additionally with pooled HDM positive and naive sera. Previous studies reported that naive and HDM patient sera exhibited strong binding to variety of *S. scabiei* antigens.⁴⁶ Western blot and dissociation-enhanced lanthanide fluorescence immunoassay (DELFI) has also demonstrated that individuals with scabies and HDM allergy showed strong IgE binding to both scabies mites and HDM antigens.^{51,52}

The sera from OS subjects, HDM-positive subjects, and naive subjects showed no clear difference in specific IgE

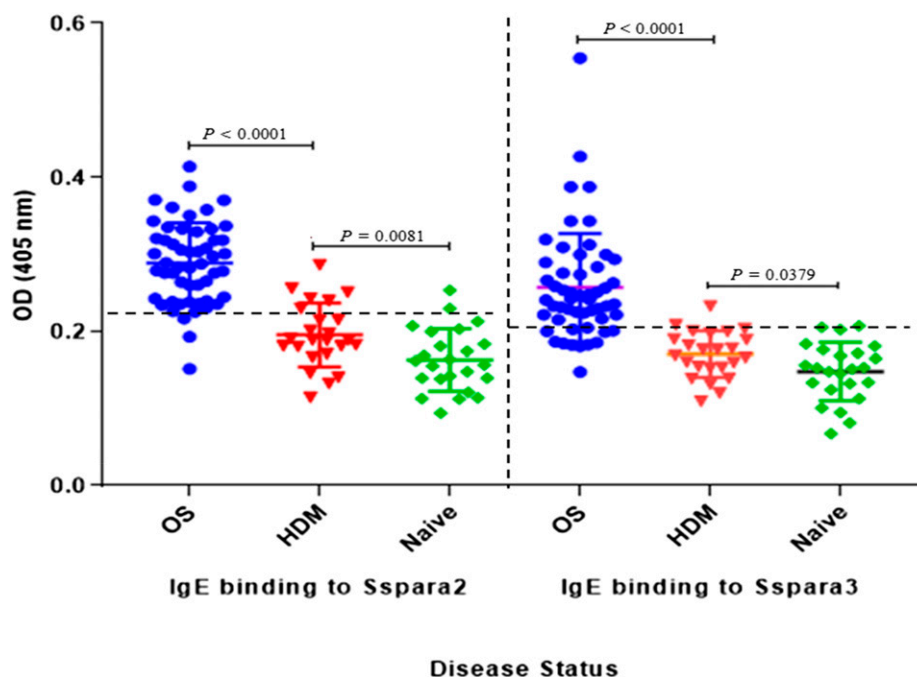


FIGURE 5. IgE binding to Sspara2 and Sspara3 in subjects with ordinary scabies (OS), house dust mite allergy (HDM), and naive to scabies (Naive). Left panel: The cutoff value of the average of the naive +2 standard deviation was calculated to be 0.24. Right panel: The cutoff value of the average of the naive +2 standard deviations was calculated to be 0.22. This figure appears in color at www.ajtmh.org.

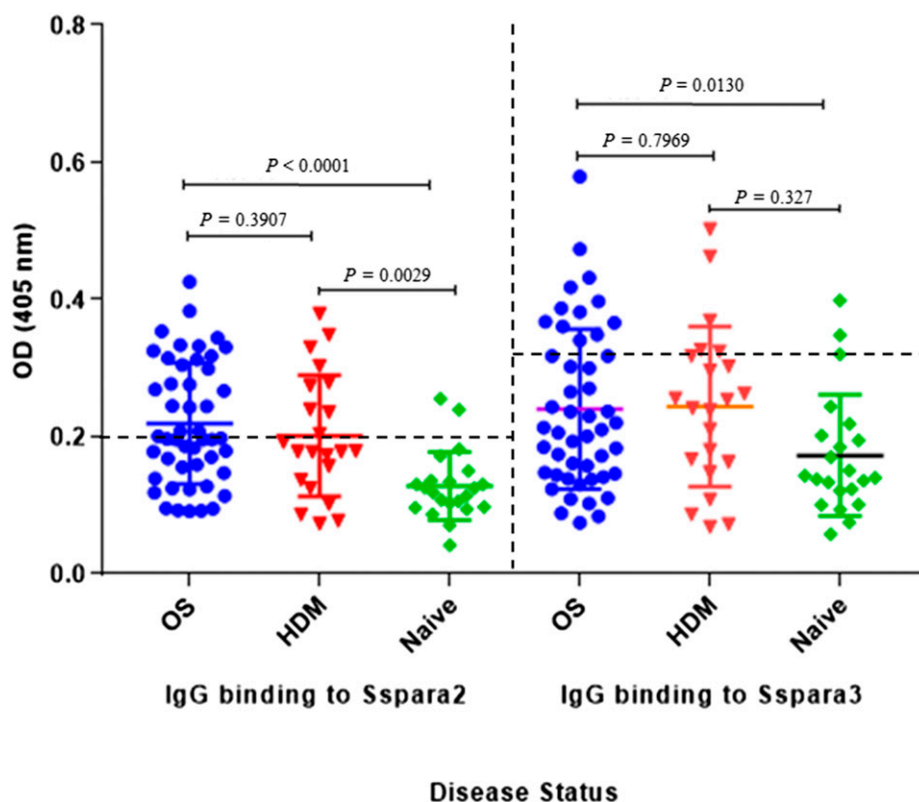


FIGURE 6. IgG binding to Sspara2 and Sspara3 in subjects with ordinary scabies (OS), house dust mite allergy (HDM), and naive to scabies (Naive). Left panel: The cutoff value of the average of the naive +2 standard deviation was calculated to be 0.2. Right panel: The cutoff value of the average of the naive +2 standard deviations was calculated to be 0.34. This figure appears in color at www.ajtmh.org.

binding to Sar s 10. Strong IgE antibody binding to tropomyosin from *A. lumbricoides* and *P. americana* has been reported in sera from patients with cockroach allergy.⁵⁰ From this study, high homology of tropomyosin within invertebrates has been established and studies have shown that tropomyosin from phylogenetically related organisms have IgE reactivity from allergenic individuals.^{33,47} Thus, the observed homology in the multiple sequence alignment and subsequent binding of IgE antibodies in all groups to Sar s 10 are consistent with other studies.

Scabies and Sar s 11. The cDNA for *S. scabiei* var. *hominis* Sar s 11 is 2,652-bp long encoding for 884-kDa protein.⁴³ The three oligonucleotide fragments of Sar s 11 from *S. scabiei* var. *hominis* have open reading frames of 1,612-, 800-, and 900-bp nucleotides. These three overlapping fragments have a deduced amino acid sequence of 537, 267, and 300 amino acids encoding for 55-, 29-, and 33-kDa allergenic protein fragments designated as Sspara1, Sspara2, and Sspara3 respectively. Paramyosin is found only in invertebrates³⁵ and the molecule is evolutionary somewhat conserved among a variety of species.⁵³ Paramyosin has been identified as a major immunogen in parasites and has shown potential as a vaccine candidate for parasitic diseases such as filariasis and schistosomiasis.⁵⁴ It is a major allergen associated with HDM allergy.³⁴ The BLAST analysis of Sar s 11 amino acid sequences revealed an 99% sequence homology with *S. scabiei* var. *canis*,⁴³ *S. scabiei* var. *hominis*,⁵⁵ *S. scabiei* var. *cuniculi*.⁵⁶ A sequence homology of 100% was shown to a partial paramyosin amino acid sequence of *S. scabiei* var. *hominis*.²⁰

Immunoanalysis of Sar s 11. Immunoanalysis of Sspara1, Sspara2, and Sspara3 fragments of *S. scabiei* var. *hominis* was performed via Western blot and a three-stage ELISA. Western blot analysis showed strong IgE binding by pooled OS sera to Sspara2 and Sspara3 as compared with Sspara1 that showed no IgE binding. This suggests the 5'-end of the protein is non-immunogenic compared with middle fragment and 3'-end and that paramyosin is indeed recognized by sera from individuals that have generated an immune response to scabies mites. It has previously been reported that C- and N-terminal of paramyosin are much less conserved when compared with paramyosin from other species.⁴¹

Paramyosin appears to be an important immunogen in scabies. ELISA detection indicated that the IgE binding of sera from the OS group was significantly higher to both Sspara2 and Sspara3 as compared with HDM and naive sera. There is also a clear distinction between specific IgE binding in the OS sera compared with naive sera when the antigen cocktail ELISA was performed. This is a similar result to that observed for other scabies antigens, namely, Sar s 3 (serine protease),⁵⁷ Sar s 8 (glutathione S-transferases [GST]),²⁴ and Sar s 14 (apolipoprotein).¹²

Both Sspara2 and Sspara3 also bound IgG from OS subjects and additionally from subjects with HDM allergy. Both fragments do not seem to be strongly recognized by sera from individuals in the naive to scabies group as reported in previous studies.²⁴ *Sarcoptes scabiei* has been demonstrated to be antigenically cross-reactive with HDMs, that is, *D. pteronyssinus* and *D. farinae*.^{45,58,59} The IgG and collagen binding activities in paramyosins of worms and

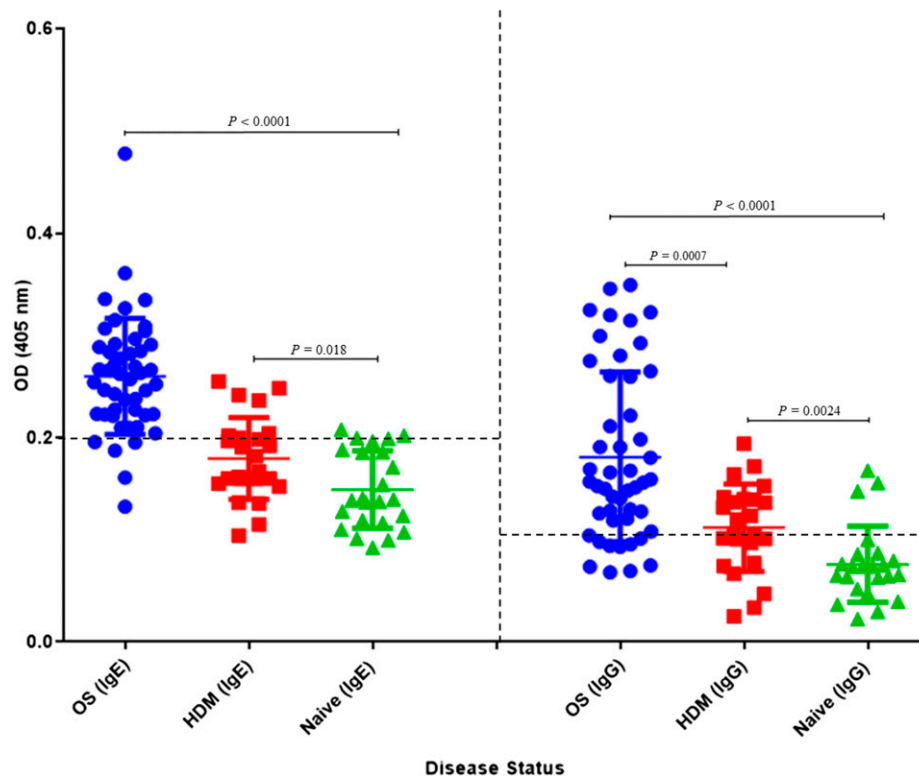


FIGURE 7. Comparison of antigen cocktail-specific IgE and IgG binding to in subjects with ordinary scabies (OS), house dust mite allergy (HDM), and naive to scabies (Naive). Left panel: The cutoff value of the average of the naive +2 standard deviation was calculated to be 0.2. Right panel: The cutoff value of the average of the naive +2 standard deviations was calculated to be 0.15. This figure appears in color at www.ajtmh.org.

molluscs are ultimate targets of immunological attacks.⁶⁰ Recombinant paramyosin *Boophilus microplus* (Bm PRM) is reported to be functionally related to host immune system evasion and considered as a new concealed antigen.⁶¹ Immunization with the selected *S. scabiei* recombinant proteins as a vaccine candidates induced high levels of humoral responses (IgGs and IgE) but had no protective efficacy against *S. scabiei* infestation.⁶²

The sensitivity and specificity are important in the performance of diagnostic tests.⁶³ A previous study using DELFIA reported that rSar s 14 has excellent diagnostic capability, with 100% sensitivity and 93.75% specificity and proved to be a highly sensitive method for diagnosis of scabies infestation in clinical practice.¹² ELISA-based diagnosis using Sar s 11 fragments is also highly sensitive and specific for diagnosis of patients with OS. The Sspara2/Sspara3 cocktail ELISA showed IgE sensitivity at 94%. The Sspara2 ELISA with area under the ROC curve of 0.9706 provides a highly discriminatory assay for OS infestation. Several human studies also reported increase in total and antigen-specific IgE in scabies,^{10,25,64}

and the development of ELISA for human scabies has focused on detection of IgE, rather than the more commonly used IgG, which was considered a less specific marker of infection.^{12,25}

In conclusion, our results demonstrate that the applicability of tropomyosin (Sar s 10) to immunodiagnosis of scabies infestation is limited likely due to the high sequence similarity between Sar s 10 and the tropomyosin of other invertebrate species, in agreement with previous studies.^{30,42} Results suggest that *S. scabiei* paramyosin is predominantly recognized by OS-infested individuals and not HDM allergic subjects, and subjects with OS have a specific IgE response to Sar s 11. The IgE binding observed in subjects with OS was also significantly higher than that of controls and no cross-reactivity with the dust mite homolog was observed.¹² Any serological test for scabies is going to be limited in its utility to diagnose scabies early. When compared, WMA extract and Sar s 14.3 showed high sensitivity and specificity for diagnosis of sarcoptic mange from weeks 8–16 postinfection.²⁶ Despite this, serology has a significant advantage over clinical

TABLE 2
Comparison of sensitivity and specificity of ELISA for Sspara2, Sspara3, and cocktail antigens

	Sspara2		Sspara3		Cocktail antigens	
	IgE	IgG	IgE	IgG	IgE	IgG
Cutoff	0.24	0.2	0.22	0.34	0.2	0.15
Sensitivity	98%	70%	84%	40%	94%	62%
Specificity	90%	96%	100%	87.5%	100%	92%
AUC	0.97	0.82	0.96	0.70	0.96	0.91

AUC = area under curve; ELISA = enzyme-linked immunosorbent assay.

diagnosis and/or skin scrapings that have poor specificity and sensitivity, respectively.^{12,13} Overall, it appears that paramyosin is an important immunogen in scabies and a potent allergenic protein in mite infestations.³⁵ A recombinant *S. scabiei* paramyosin (Sar s 11) could, therefore, potentially be used as an infection marker in immunodiagnosis of OS and in better control of the infestation in communities where scabies is endemic.

Received December 12, 2016. Accepted for publication May 5, 2017.

Published online July 10, 2017.

Acknowledgments: We thank S. M. Saqlan Naqvi of Department of Biochemistry, PMAS Agriculture University Rawalpindi for earlier suggestions in the work.

Financial support: This work was supported by International Research Support Initiative Program (IRSIP) from Higher Education Commission (HEC) of Pakistan to Shumaila Naz.

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