

Original article

Hypersensitivity to *Forcipomyia taiwana* (biting midge): clinical analysis and identification of major For t 1, For t 2 and For t 3 allergens

Background: *Forcipomyia taiwana* is a tiny, blood-sucking midge that cause intense pruritis and swelling in sensitive individuals. It is distributed island-wide in rural Taiwan and Southern China.

Objective: This study aimed to study the allergic immune responses and identify *F. taiwana* allergens.

Methods: Crude whole body *F. taiwana* extracts were prepared with phosphate-buffered saline. The specific IgE antibody was determined by enzyme-linked immunoassay and immunoblotting. Protein was analyzed by electrospray ionization tandem mass spectrometry.

Results: Among the 372 subjects that were exposed to *F. taiwana* bites, 179 (48%) reported an immediate skin reaction with/without delay reaction and 41(11.1%) reported a solely delay reaction. The skin of 21 subjects was tested with *F. taiwana* extract. Of these 21 subjects, 12 (57.1%) produced immediate skin reactions and contained high levels of specific IgE antibody against *F. taiwana*. Immunoblotting revealed that 11 allergenic components are able to bind specific IgE. Allergens of 22, 24, 35, 36, and 64 kDa bound 50, 50, 75, 66.7, and 75% of IgE-containing sera tested, respectively. Tryptic fragments of the 24, 35, 36, and 64 kDa allergens were analyzed by ESI-MS/MS. Selected tryptic peptides of 24, 35, and 36, and 64 kDa allergens exhibited significant sequence identity with triosephosphate isomerase of *Anopheles merus*, *Tenebrio molitor*, *Ochlerotatus togoi*, and *Chrysops vittatus*, fructose 1,6-bisphosphate aldolase of *Antheraea yamamai* and *Homalodisca coagulata*, and a slow muscle myosin S₁ heavy chain of *Homarus americanus* and a protein with unknown function from *A. gambiae*, respectively. The 35 and 36 kDa proteins may represent different isoforms of the fructose 1,6-bisphosphate aldolase.

Conclusion: We conclude that immediate reaction to *F. taiwana* bites is IgE mediated and the 24 (For t 1), 35 (For t 2), and 64 kDa (For t 3) proteins are candidates for major *F. taiwana* allergens. Further studies are needed to confirm these allergens.

**Y.-H. Chen^{1,3}, M.-F. Lee^{2,3}, J.-L. Lan¹,
C.-S. Chen³, H.-L. Wang²,
G.-Y. Hwang³, C.-H. Wu²**

¹Department of Internal Medicine, Division of Allergy, Immunology and Rheumatology;

²Department of Education and Research, Taichung Veterans General Hospital; ³Department of Life Science, Tunghai University, Taichung, Taiwan

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Chii-Huei Wu
Department of Education and Research
Taichung Veterans General Hospital
160 Chang Kang Road
Section 3
Taichung 407
Taiwan

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Insects have been documented to be an important source of allergens in humans since antiquity. The body components of insects can become inhalant allergens causing allergic rhino-conjunctivitis and asthma. Stinging insects can cause allergic reactions by directly introducing saliva or venom into human skin (1, 2). The allergic reactions of stinging insects range from pruritic local reactions to fatal anaphylactic shock and continue to be a serious medical problem (3, 4).

Shiraki first reported the presence of *Forcipomyia* (*Lasiohelea*) *taiwana* (Shiraki) in central Taiwan in 1913 (5). *Forcipomyia taiwana* is a tiny (1–1.5 mm) blood-sucking midge that is distributed island-wide in urban and suburban Taiwan as well as in Southern China. Its larvae live in moderately moist soil around housing or shaded

areas of cultivated bamboo, tea, vegetable and betel nuts (6–8). Female *F. taiwana* midges attack exposed parts of the body during the day, causing intense pruritis and swelling in sensitive individuals. Following an outbreak in 1989, it spread rapidly and became abundant in certain areas of Taiwan, such as the Ta-Ken foothill area of Taichung, and has become one of the most abundant bloodsucking pests in Taiwan. Although local physicians have been aware of severe reactions to *F. taiwana* bites for a long time, there are no clinical data regarding *F. taiwana* allergy, and the antigenic and allergenic properties of *F. taiwana* have never been documented. In the present study, we report the immune responses to *F. taiwana* and the identification of 24, 35, and 64 kDa major allergens of *F. taiwana*. The 24, 35, and 64 kDa proteins, according to World Health

Organization (WHO) and the International Union of Immunological Societies (IUIS) nomenclature, are designated as For t 1, For t 2, and For t 3, respectively.

Methods

Subjects

During a two 5-month periods (May 2003–September 2003, and May 2004–September 2004), 372 subjects with history of *F. taiwana* bite were selected from the allergy clinic at Taichung Veterans General Hospital and a college in Ta-Ken, Taichung, Taiwan and interviewed by a nurse using a questionnaire regarding exposure and detailed reactions to *F. taiwana*. Reactions developed within 1 h of bites are defined as immediate reaction and that developed after 6 h or more as delayed reaction. Those presented both immediate and delay reactions were categorized into the immediate reaction group.

Preparation of whole body *F. taiwana* extract

Female midges were collected by human bait method using special collectors designed by Dr C. S. Chen's laboratory, Department of Life Science, Tunghai University, Taichung, Taiwan. One thousand females midges were ground and dissolved in 5 ml of phosphate buffered saline (PBS), ultrasonicated for 30 min at 4°C, and centrifuged at 8000 g for 15 min. The supernatant was collected, filtered through 0.45 µm filter, aliquoted, and stored at -70°C.

Skin testing and patients' sera

Crude *F. taiwana* extract at a concentration of 200 µg/ml in PBS containing 50% glycerol was used for skin testing using epicutaneous sterile disposable Sharp Test® applicators (Greer Laboratories, Lenoir, NC). Histamine (1 mg/ml) and 50% PBS-glycerol were used as positive and negative controls, respectively. All skin test results were read 20 min after placement. A response with a wheal or an erythema 3 mm larger in size than that produced by the negative control but less than 1/2 of the diameter of the positive control was graded as 1+, and a reaction ranging between 1/2 and equivalent size of the positive control was graded as 2+. A reaction compatible with the positive control was graded as 3+. A reaction larger than the positive control was grade as 4+. Sera were collected after skin test.

Allergen specific IgE

Optimal concentrations of antigen and conjugate were determined by checkerboard titration (9), and level of IgE specific to crude *F. taiwana* extract (3.0 µg/well) was measured using a commercial enzyme allergosorbent test (EAST) kit according to the manufacturer's instructions (Allergopharma, Reinbek, Germany). Reference disc coated with *Phleum pratense* allergen and reference sera containing 17.5, 3.5, 0.7, and 0.35 kU/l were used as positive controls, and sera from nonallergic subjects were used as negative controls.

Western blot analysis

Crude *F. taiwana* extract was subjected to standard SDS-PAGE under denatured conditions with a discontinuous buffer, and proteins were transferred onto PVDF membranes and immunodetected according to the method previously described (10).

Two-dimensional gel electrophoresis (2-D)

Crude *F. taiwana* extract was subjected to 2-D under denatured conditions. The first dimension of 2-D was performed with a Bio-Rad Protean II xi 2-D cell system (Bio-Rad) with pH gradient (Bio-Rad) from 3 to 10 for a total volt-hour of 14 200. The second dimension SDS-PAGE was then performed according to the methods recommended by the manufacturer and immunoblotting was performed.

Mass spectrometry

Proteins were visualized with silver stain after 2-D, and the target protein spots were excised for in-gel digestion using a sequencing-grade modified trypsin (Promega, Madison, WI). The tryptic peptide mixtures were analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS; Finnigan MAT, San Jose, CA, USA) and searched with a Mascot computer program (Matrix Science).

Results

Incidence and manifestations of hypersensitive reactions to *F. taiwana*

A total of 372 (153 males and 219 females) subjects who had been exposed to *F. taiwana* (Fig. 1) bites were interviewed by questionnaire. The mean age of subjects was 38.59 ± 15.25 years, with a range of 1–80 years. Of the 372 subjects, 59.1% (220/372) were found to have immediate and/or delay type reactions to *F. taiwana*. There were no significant differences in incidence of *F. taiwana* allergy with regard to sex, age or concomitant atopic diseases (Table 1). Large wheals developed within 1 h of bite, and the size of swelling varied from 2 cm to more than 10 cm (Fig. 2A). In some patients, delayed papular-vesicles developed following immediate reaction (Fig. 2B). In the solely delayed reactions, itching papules

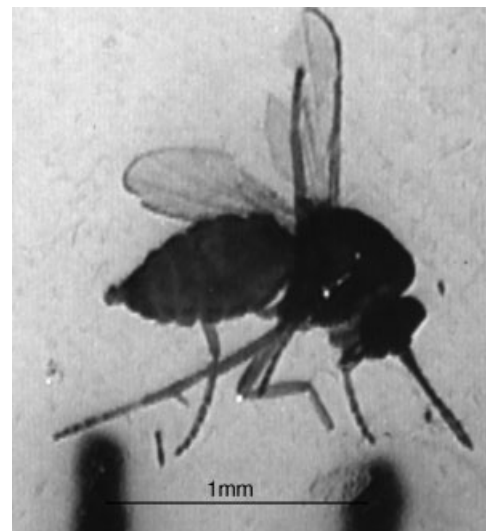


Figure 1. Picture of *Forcipomyia taiwana*, under 10× microscope.

or vesicles developed in 6–24 h, and the intense pruritic lesions become centrally necrotic later in some subjects (Fig. 2C). The delayed skin lesions persisted for weeks, or even months in some individuals.

Allergy skin test and IgE antibodies

Twenty-one subjects with a history of hypersensitive reaction to *F. taiwana* bites were randomly selected and skin tested with *F. taiwana* extract after informed consent. Ten individuals with no reaction to *F. taiwana* bites showed negative skin reaction and no specific IgE antibody against *F. taiwana*. Of the 21 patients, 57.1% (12/21) gave a positive immediate skin reaction and 42.9% (10/21) gave a solely delayed reaction. IgE antibody for patients with immediate and solely delayed reactions gave a mean value of 17.75 ± 6.72 and 3.58 ± 2.84 kU/l, respectively, as defined by EAST. The results are summarized in Table 2.

SDS-PAGE and immunoblotting

Resolution of proteins of *F. taiwana* by SDS-PAGE revealed at least 33 proteins, with molecular weights (MW) ranging from 10 to 70 kDa, that were visible following silver staining (data not shown). Immunoblotting revealed that 11 allergenic components are able to bind specific IgE (Fig. 3, lanes a–l). Allergens of 10, 20, 22, 24, 29, 30, 31, 35, 36, 37, and 64 kDa bound 8.3% (1/12), 33.3% (4/12), 50% (6/12), 50% (6/12), 33.3%

(4/12), 116.7% (2/12), 33.3% (4/12), 75% (9/12), 66.7% (8/12), 8.3% (1/12), and 75% (9/12) of the sera tested, respectively. Except for nonspecific binding to a band of around 29 kDa found in all of the sera, sera from *F. taiwana*-negative atopic and nonatopic subjects (Fig. 3, lanes m and n) and patients with solely delayed skin reaction (patients 13–21, data not shown) did not display any IgE binding to any *F. taiwana* proteins.

The 2-D and ESI-MS/MS

The isoelectric points (pI) of the 24 (For t 1), 35, 36, and 64 kDa (For t 3) *F. taiwana* allergens were 5.8, 8.6, 8.7, and 5.2, respectively as revealed by 2-D (data not shown). The stable signals obtained from tryptic peptide mixtures of the For t 1, 35, 36 kDa and For t 3 allergens

Table 2. Clinical characteristics of hypersensitive reactions to *F. taiwana*

Subject	Age	Sex	Symptoms	Skin test/kU/L	Atopy	Autoimmune disease
1	42	F	I, D	4+/16.39	AR	–
2	23	F	I, D	1+/9.28	–	SLE
3	28	M	I, D	4+/22.89	–	–
4	37	M	I	3+/11.94	–	–
5	14	M	I, D	2+/16.43	AR	–
6	24	F	I, D	1+/8.25	–	–
7	53	M	I, D	4+/10.56	–	SLE
8	39	F	I, D	3+/19.34	–	–
9	18	M	I	2+/21.9	AR, AS, AD	–
10	37	F	I, D	1+/22.56	–	–
11	29	F	I, D	2+/29.98	–	SLE
12	60	F	I	2+/11.5	–	–
13	45	F	D	–/0.65	–	–
14	24	F	D	–/8.25	–	–
15	36	F	D	–/1.40	AR	–
16	50	F	D	–/3.04	AR	–
17	29	M	D	–/6.78	–	–
18	45	F	D	–/0.72	–	–
19	22	F	D	–/5.32	AR	–
20	37	F	D	–/4.94	–	–
21	24	F	D	–/1.12	AR	–

I, immediate reaction; D, delayed reaction; AS, asthma; AR, allergic rhinoconjunctivitis; AD, atopic dermatitis; SLE, systemic lupus erythematosus.

Table 1. Incidence of hypersensitive reactions to *F. taiwana*

Reactions	No. of subjects (n = 372)	Percent (%)
None	152	40.9
Immediate reaction	179	48.0
Solely delay reaction		
Papules	20	5.4
Vesicles	13	3.5
Lymphadenopathy	1	0.3
Fever	3	0.8
Others	4	1.1



Figure 2. (A) Immediate reaction to *Forcipomyia taiwana* bites. Large wheals usually develop within 1 h of bites and the sizes vary from 2 cm to more than 10 cm. (B) Immediate reaction plus delayed papular-vesicles. Note the large local swelling at right foot designated by thick arrow and small vesicles designated by thin arrow. (C) Solely delayed reactions. Note the erythematous papules and vesicles over the lower leg of the patient.

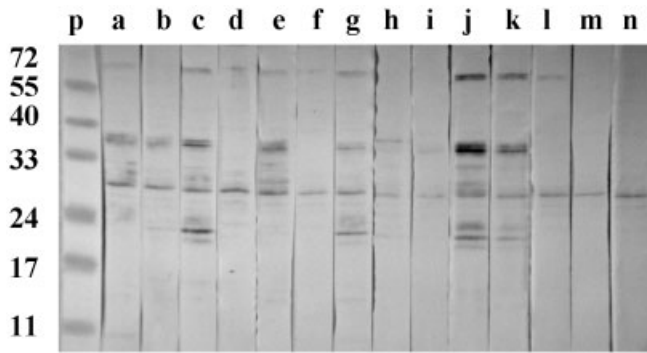


Figure 3. Immunoblot patterns of crude *F. taiwana* extract with immediate reaction to *F. taiwana* subjects 1–12 (lanes a–l), *F. taiwana*-negative atopic (lane m) and nonatopic (lane n) subjects. Numbers at left indicate size of standard proteins in kDa (lane p).

were analyzed by ESI-MS/MS and identified as triosephosphate isomerase (TPI) of *Anopheles merus* (fly), *Tenebrio molitor* (yellow mealworm), *Ochlerotatus togoi* (fly) and *Chrysops vittatus* (deer fly), fructose 1,6-bisphosphate aldolase of *Homalodisca coagulata* (glassy-winged sharpshooter) and *Antheraea yamamai* (Japanese oak silkworm) and a slow muscle myosin S₁ heavy chain of *Homarus americanus* (American lobster), respectively (Table 3). The 35, 36 kDa (For t 2) proteins may represent different isoforms of the fructose 1,6-bisphosphate aldolase. Moreover, using the same technique, 13 tryptic peptides obtained from the For t 3 allergen, showed sequence identity with an unpublished protein sequence of the African malaria mosquito (*A. gambiae* str. PEST; 151.6 kDa, pI 5.57; GenBank gi:31235885).

Table 3. Identification of *F. taiwana* allergens by ESI-MS/MS

Allergen*	pI*	Matching identity	Species	GenBank access. No.	MW/pI (GenBank)	Matching residue sequence
For t 1 (24 kDa)	5.8	Triosephosphate isomerase (EC 5.3.1.1)	<i>A. merus</i>	gi:1857455	21.86/5.33	¹⁰⁴ VIACIGETLQER ¹¹⁵ ¹⁵⁶ TATPEQAQEVHAAALRK ¹⁷¹ ¹⁸⁷ IQYGGSVTAANCR ¹⁹⁹
			<i>T. molitor</i>	gi:22090453	26.66/6.0	⁸⁴ DVGADWVILGHSERR ⁹⁷ ¹²² VIACIGETLEER ¹³³ ¹⁴⁸ AIAAKVNDWSNVVVIAYEPVWAIGTGK ¹⁷³
			<i>O. togoi</i>	gi:1857457	21.91/5.30	¹⁰⁴ VIACIGETLQER ¹¹⁵ ⁶⁶ DVGANWVIGHSER ⁷⁹
			<i>C. vittatus</i>	gi:1857453	16.42/5.39	¹⁷¹ KWFTDNVSADVSASIRIQYGGSVTAANCR ¹⁹⁹ ¹⁰⁴ VIACIGETLQER ¹¹⁵
For t 2 (35 and 36 kDa)	8.6	Fructose 1,6-bisphosphate aldolase (EC 4.1.2.13)	<i>H. coagulata</i>	gi:46561746	39.68/7.56	¹⁵⁷ NTPSYOAILLENANVLR ¹⁷³
			<i>A. yamamai</i>	gi:45330818	39.67/7.59	¹⁵⁷ NTPSYOAILLENANVLR ¹⁷³ ¹⁸³ IVPIVEPEVLPDGEHDLDR ²⁰¹
For t 3 (64 kDa)	5.2	Slow muscle myosin S1 heavy chain	<i>H. americanus</i>	gi:37925239	58.53/5.22	¹⁶ QRIATELEDMQIEVER ³¹ ³⁵² AKKAMVDAAR ³⁶¹ ⁴⁷⁸ QIEEAEIIALNLAK ⁴⁹²

*Experimental molecular weight and pI.

Discussion

Seasonal allergic dermatitis caused by biting midges in horses, sheep, cattle, and donkey (11–14), have been documented, but clinical manifestations and identification of allergenic components of *F. taiwana* have not been reported in humans. Here we found that 220 out of 372 subjects (59.1%) reported reactions to *F. taiwana* bites. Among these 220 subjects, 179 (81.4%) had immediate large local reactions or immediate large local reactions followed by delayed reactions to *F. taiwana* bites, whereas 33 subjects (15.0%) had delayed cutaneous reactions with either itching papules or vesicles and four subjects (1.8%) had systemic reactions such as fever or lymphadenopathy. Among the 21 selected individuals with history of hypersensitive reaction to *F. taiwana* bites, the mean level for *F. taiwana*-specific IgE was significantly higher (Student's *t*-test, $P < 0.001$) in subjects with immediate reaction than in those with solely delayed reaction. Hypersensitive reactions to mosquito bites are usually IgE-mediated and lymphocyte-mediated (15–17). It is conceivable that the delayed reaction to *F. taiwana* may be caused by IgG or lymphocyte-mediated immune responses.

Triosephosphate isomerase, a glycolytic enzyme, catalyses the isomerization of dihydroxyacetone phosphate into D-glyceraldehyde 3-phosphate, and TPI had been identified as wheat flour (18) and latex (19, 20) allergens. Fructose 1,6-bisphosphate (FBP) aldolase (ADL, D-fructose 1,6-bisphosphate D-glyceraldehydes 3-*P*-lyase; EC 4.1.2.13) catalyzes the reversible aldol condensation of glyceraldehydes 3-phosphate and dihydroxyacetone phosphate yielding FBP. In class-I aldolase, which is found in animals and higher plants, catalysis depends upon Schiff-base formation with the substrate (21), whereas in class-II aldolase, which is found in fungi and prokaryotes, divalent cations are required for activity (22). Using

amino acid sequentiation, ADL (37 kDa) was reported to be a wheat allergen causing bakers' asthma (23, 24). Crustacean muscles are biochemically characterized as fast-twitch, slow-twitch (S₁) and slow-tonic (S₂) types (25). Fiber-type-specific isoforms of myosin heavy and light chains, tropomyosin, paramyosin, and troponin-I, -T and -C are expressed in lobster and crayfish muscles (26). SMM S₁HC from the American lobster was able to bind to the anti-troponin T (TnT) antibody (27), and showed 66% sequence identity with an unknown function protein from the African malaria mosquito. The TnT is a member of the troponin complex, consisting of troponins-I, -C and -T, which is responsible for binding tropomyosin to regulate calcium binding and contractility of vertebrate striated muscles. Tropomyosin is an important allergen found in crustaceans, mollusks and other invertebrates such as lobsters, crabs, shrimps, squids, snails, oysters, house dust mites, and cockroaches (28), and may be the cause of clinical cross-sensitivity among invertebrates (29). These and our results open the possibility that TPI, ADL, and SMMS₁HC may have a tendency to cause allergic diseases and are worthy for further investigation.

In summary, we describe the humoral immune response to *F. taiwana* allergens in selected individuals with immediate and delayed allergic reactions. We

identified several allergenic components from the crude extract using sensitive and specific immunoassay. Skin testing revealed that all 12 tested subjects gave a positive reaction, and immunoblotting revealed a prevalence rate of 50, 75, and 75% for For t 1, For t 2, and For t 3 allergens, which indicates that these proteins are candidate as major allergens of *F. taiwana* (at least 50% according to WHO-IUIS). To our knowledge, this is the first study to investigate the antigenic and allergenic properties of *F. taiwana*. Further study is required to characterize and confirm these allergens, to investigate the lymphocyte-mediated reactions to *F. taiwana* and to elucidate the mechanisms of *F. taiwana* hypersensitivity.

Acknowledgments

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