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Chironomid midges as allergens: evidence from two species from West Bengal, Kolkata, India

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Background & objectives: **Arthropods of different taxonomic identity including chironomid midges are known to induce allergic response in humans. The present study was done to access two common chironomid species *Chironomus circumdatus* and *Polypedilum nubifer* for their sensitizing potential as an allergen in atopic patients and controls.**

Methods: **Following preparation of allergenic extracts of the two chironomid species separately, 198 atopic patients attending an allergy clinic and 50 age matched controls were tested along with a routine panel of allergens to assess sensitization.**

Results: **The skin prick test (SPT) results revealed that 189 of the 198 patients (95.4%) demonstrated sensitization to both the chironomid species. Higher levels of total IgE was observed in atopic subjects than in the control group.**

Interpretation & conclusions: **The results suggest that the chironomid midges *Chironomus circumdatus* and *Polypedilum nubifer* can elicit sensitization in humans. A potential risk for allergic reactions by susceptible individuals exists due to these chironomid species, owing to their abundance and chances of contact with human beings. Further studies may be initiated to characterize the nature of the allergens and to assess their clinical relevance.**

Key words Allergy - chironomid midges - IgE - skin prick test (SPT)

Insects like chironomid midges (Diptera: *Chironomidae*) are potent allergens that elicit allergic reactions[1-4], predominantly in farmers and fish food handlers[5]. Different chironomid species under the genera *Chironomus*, *Polypedilum*, *Cladotanytarsus* and *Glyptotendipes* have been recorded to cause allergic reactions in human, worldwide[6-10]. Many of these species are common in various parts of India, including West Bengal[11,12]. The life cycle of the chironomid midges includes aquatic larval stages and aerial adult stage, with varying durations depending on the species and environmental factors[13,14]. In many instances it has been shown that the haemoglobin of chironomid larvae is the main molecule to bring about hypersensitive reactions in human[15,16]. The contact with the adult or larval chironomid causes allergic reactions and is

considered as a public health problem in many parts of the world. Data from India are lacking. Invertebrates like snails have been related to cercarial dermatitis[17]; and prawns[1] and mites[18] have been established as allergens.

Chironomid midges are ubiquitous in almost all kind of aquatic habitats in India, exhibiting a wide range of species diversity[11]. The aquatic food web is strongly influenced by the abundance of these insects reflecting their importance[12-14]. Increased abundance of chironomid increases the chance of contact with human being, leading to allergic manifestation in susceptible individuals. Considering the prevalence of chironomid larvae in vast type of water bodies, the assessment of sensitization in human is necessary. In the present study, sensitization to two chironomid species (*Chironomus circumdatus* and *Polypedilum nubifer*) was carried out using conventional allergy test on allergy prone and control individuals.

Material & Methods

This study was conducted during June 2010 to March 2011. The skin prick test (SPT) and blood collection for total IgE from 198 patients and 50 healthy control subjects was done at Allergy and Asthma Research Center, Kolkata, India. Collection and rearing of chironomid larvae and analysis of data were done in Entomology and Wild life Biology Laboratory in Kolkata, India. The research work was approved by Institutional Ethics Committee of Allergy and Asthma Research Center, Kolkata.

Adults and larvae of *Chironomus circumdatus* and *Polypedilum nubifer* were used separately to prepare allergenic extracts[4,6,15] in technical collaboration with Creative Drug Agency, Mumbai, India. The species of chironomids selected for the preparation of allergenic extracts was mainly based on their relative abundance in the study area. Egg masses of the two species were collected from ponds near Ballygunge Science College campus and allowed to hatch as instar I larva in sterilized Petri dishes (90 mm containing 15 ml of deionized water). Randomly selected instar I larvae were reared in six well tissue culture plates (Nunc™, Thermo Fisher, USA) using 10 ml of deionized water and finely ground fish food (Tokyu™, Thailand) (@1 mg/day in each well) to yield pupae. The pupae were placed individually in 10 ml glass vials (20 X40 mm) and allowed to emerge as adults (P-generation). Following oviposition of the adults, the resultant eggs (F_1 generation) were reared in the same way. In the course of development of F_1 individuals, randomly selected instar IV larvae were separated using a pipette, thoroughly rinsed in deionized water and placed on filter paper. These IV instar larvae and the adults obtained from F_1 generation were dried in room temperature (27 - 30°C) and placed in dry ice in sterilized homogenizer vials (Tarsons®, India). The vials were forwarded to Mumbai (Creative Drug Agency) for preparation of allergen.

Tests for sensitization against chironomid species was carried on 198 atopic patients clinically diagnosed as suffering from either of the allergic complaints like urticaria, allergic rhinitis and bronchial asthma and recommended for routine allergy skin prick test (SPT) for other allergen sources by the Physicians. An assessment of potential of chironomid midges as allergen was made using SPT and total serum IgE level in collaboration with Allergy and Asthma Research Centre, Kolkata. Nine potential sources of allergens commonly used in routine allergy tests (*viz.* grains, pulses, cereals, vegetables, fruits, fish and meat, pollen, moulds, fibres, and house dust), and the two different species of chironomids (allergens prepared from both larvae and adults) were used to evaluate sensitization of the patients. The results of SPT were obtained in terms of no response, low, medium and high response depending upon the diameter of wheals (Table I). Fifty healthy persons (25 male and 25 female), selected from the same area of Kolkata, belonging to the same age group as patients with no personal and /or family history of atopy were selected to serve as control subjects. All patients and control subjects were categorized into three age group, A (15-30 yr), B (31-45 yr) and C (>45 yr).

Skin prick test was applied on the flexor side of forearms. One small drop (10 µl) of the test solution was applied on the flexor side of the arm. The wheal size was recorded by circling the reaction, 20 min after application of the antigens. The wheal diameter was graded as 1^+, 2^+, 3^+, and 4^+ as compared to positive control. In the present study, the interpretation of results was done on the basis of comparison of reaction against a negative (phosphate buffer saline, PBS) or a positive (Histamine phosphate) reference as suggested by Aas and Belin[19], and graded following the method suggested by American College of Allergists[20,21] (Table I).

The total serum IgE in patients as well as control subjects was measured by using enzyme immuno assay (EIA; Omega Diagnostic, Scotland, UK) technique[21].

A Kolmogorov-Smirnov goodness of fit test was used to judge whether the proportional response to the chironomid allergens remained equal or not. Data on IgE was subjected to Shapiro-Wilks's test to judge compliance for normal distribution, following which Kruskal-Wallis ANOVA was performed to conclude about the differences in IgE with relation to the age and sex of patients. All statistical analyses[22] were carried out using SPSS ver. 10 software[23].

Results

A total of 198 patients and 50 control subjects were selected for skin prick test. The patient group comprised 104 male and 94 female patients with a history of different allergic symptoms. The results of SPT for 198 patients against common allergens of clinical importance revealed that almost all the patients showed sensitivity to at least one of the allergens tested. The higher degree of sensitivity was observed towards cotton (94.95%) followed by kapok (89.9%), *Cocos nucifer* (79.8%), brinjal (78.8%), *Cyanodon dactylon* (75.75%), banana, *Chironomus circumdatus* adult (75.25% each) and prawn (72.72%). Moderate response was observed against *Polypedilum nubifer* larva and papaya (56.06% each), and *Hilsa* fish (*Ctenuolosa ilisha*) (55.05%). In case of chironomids, the sensitivity was highest towards *Chironomus circumdatus* adult (CC adults) allergen (75.25%) followed by allergen of *Chironomus circumdatus* larva (CC larva) (63.13%), *Polypedilum nubifer* adult (PN adult) (62.12%) and *Polypedilum nubifer* larva (PN larva) (56.06%) (Table I). The Kolmogorov-Smirnov test for goodness of fit was significant (P = 0.002) suggesting that the response in the patients, due to the four chironomid species differed significantly. However, in case of control subjects, the reaction was not significant both qualitatively and quantitatively.

Table I. Results of skin prick test (SPT) using selected chironomid allergens

Allergen	No. (%) of patients sensitized (n=189)
P. nubifer adult (PN adult)	123 (65.08)
P. nubifer larvae (PN larva)	111 (58.73)
C. circumdatus adult (CC adult)	149 (78.84)
C. circumdatus larvae (CC larvae)	125 (66.14)

The intensity of skin reaction against four allergens of interest (CC adult, CC larva, PN adult and PN larva) tested varied between 0 and 3^+. Of the 198 patients tested, 189 (95.45%) showed positive response towards any of the chironomid allergens tested, either alone or in different combinations. The degree of reactivity varied considerably in individual patient. In case of CC adult, 43 (21.72%), 90 (45.45%), 17 (8.59%) patients showed 1^+, 2^+, 3^+ reactions, respectively while 48 (24.24%) did not show any reaction towards this allergen. In contrast, 125 (27.78% with 1^+, 33.33% with 2^+ and only 2% with 3^+ reaction) patients reacted positively against CC larval extract. In case of PN adult, 123 patients showed positive response, however, the intensity of reactions varied greatly 39 (19.7%), 78 (39.39%), 6 (3.03%) patients showed 1^+, 2^+, 3^+ reactions, respectively. Among 112 positive patients against PN larval extract, 54 (27.27%), 54 (27.27%) and 4 (2.02%) gave 1^+, 2^+, 3^+ reactions, respectively. The high degree (4^+) of reactivity was not shown by any of the patients towards any of the chironomid species. Of the 198 patients, 47 (24%) showed sensitization for chironomid only, 5 (3%) for prawn only, 4 (2%) to none, and 141 (71%) exhibited sensitization for both prawn and chironomid. Sensitization to both prawn and chironomid by a significantly by (P<0.001) higher proportion of patients indicated possibilities of cross-reactivity for the two arthropod organisms, since tropomyosin a common allergen, is present in both prawn[24] and chironomid[25].

Analysis of SPT results against PN adult (A), PN larva (B), CC adult (C) and CC larva (D) extracts revealed that 42 (21.2%) individuals responded to all of the four allergens, 68 (34.34%) to at least three, though in different combinations, 40 (20.2%) to at least two (in different combinations) and 39 (19.7%) to one allergen only. The Kolmogorov-Smirnov test for goodness of fit remained significant (P = 0.002) suggesting that the response to the allergens remained significantly different for the different categories, *i.e.* single, two, three or four allergens.

The total serum IgE levels in 189 patients and 50 control subjects varied between 338.43 ± 10.44 IU/ml (range 60 to 812 IU/ml) and 56 ± 34 IU/ml (range 15 to 120 IU/ml), respectively. Data on total serum IgE levels of the patient group did not follow normal distribution as revealed through Shapiro-Wilk test (P<0.001). Only 6.57 per cent (n=12) patients of study group had serum IgE level within normal limit, while the remaining

Table II. Total serum IgE level in different age group of patients

Age of patients (yr)	No. (%) of patients (n=198)	Range of IgE (IU/ml)	Mean IgE ± SE (IU/ml)
15-30	98 (49)	109-623	328.21 ± 13.51
31-45	75 (38)	60-812	364.17 ± 18.86
>45	25 (13)	115-620	301.28 ± 27.30

93.43 per cent (n=177) patients showed elevated serum IgE level. The mean value of serum total IgE levels in male patients (104, 52.53%) ranged between 83-812 IU/ml with a mean value of 333.39 ± 13. 51 IU/ml and in case of females (94, 47.47%) the corresponding value was 60-640 IU/ml with a mean value of 344.01 ± 16.18 IU/ml. Deviations from normal distribution for the IgE values was observed in both males (P<0.001) and females (P<0.004). Further analysis revealed that the lower and upper limits of total serum IgE in 98 (49%) patients within 15-30 yr age group were 109 and 623 IU/ml and mean value was 328.21 ± 13.51 IU/ml. In the middle age group, 31-45 yr (n=75, 38%), the range of total serum IgE level was between 60 and 812 IU/ml with a mean value of 364.17 ± 18.86 IU/ml. The range of serum IgE level of the age group >45yr in 25 (13%) patients varied between 115 and 620 IU/ml and the mean value was 301.28 ± 27.30 IU/ml (Table II). The mean values of total serum IgE level of patients in the three age group were significantly different (P<0.05), and the mean value of total serum IgE level in the middle age group (31-45 yr) was significantly higher (P<0.01) than the older age group (>45 yr).

Discussion

The ability of the two chironomid species *Chironomus circumdatus* and *Polypedilum nubifer* to elicit sensitization in human varied considerably as reflected from the skin prick test and the level of total serum IgE. The SPT results indicated that, among four different allergens used in the present study, *C. circumdatus* adult allergens were the most potent chironomid-borne allergen. Although the possible key allergen molecules, like tropomyosin[25] or haemoglobin[3] of the chironomid species were not characterized, the SPT results indicated potential risk upon contact with these chironomid species. Sensitization for both larval and adult forms suggests chironomid midges can act both as contact and air-borne allergen, depending on the life form.

Since the 1950s, reactions of hypersensitivity to different species of chironomid midges have been reported, primarily in United States[3], Sudan[4,10], Spain[5], Japan[6], United Kingdom[9], Sweden[16] and Korea[25]. Chironomid midges are most common allergens by inhalation in certain areas in these countries, often causing rhinocojunctivitis and asthma. Allergy to chironomids has occasionally been observed in people who handle fish food and remain in contact with aquaria or water bodies in their workplace[2,5]. In Spain, a case of urticaria-angioedema has been reported due to contact in the workplace with larvae of chironomid midge *(C. thummi thummi)* in a patient with a history of pollen allergy[5]. No previous studies from India has substantiated the role of chironomid midges in different allergic disorders. Sensitization to chironomid species *C. circumdatus* and *P. nubifer* in population of Kolkata, India, was demonstrated in the present study. The intensity of response was higher against adult than larval form of chironomid midges, perhaps, due to different molecules acting as allergens. Empirical evidences suggest that haemoglobin[3,4,15] of larva and tropomyosin[25] of adult are responsible molecules eliciting sensitivity of human against larval[2,3,5,7,15,16] and adult[8-10,25] chironomid species. Tropomyosin of chironomids bear homology with crustaceans and other insects, which can be a cause for cross-reactivity[25]. In the present study, possibilities of cross reactivity was reflected through high proportion of individuals responding to both chironomid and prawns, but the proportion of individuals responding to chironomids but not to prawns established chironomids as potential risk as allergen to the populations of Kolkata, India.

A wide range of variations was observed in the total serum IgE levels of patients, which may possibly due to the individual health status with allergic tendency towards one or more allergens. Studies[26,27] have suggested that the elevated total serum IgE levels increase the probability of sensitization, particularly in younger age group between 20 and 44 yr. Total serum IgE can serve as a better discriminator between multisensitized and non-sensitized but not between monosenistized and non-sensitized pairs[26]. In atopic patients with insect allergy, asthma, and rhinitis, total serum IgE decreases with age[27]. This has been observed in the present context, where the mean value of total serum IgE level in the middle age group (31-45 yr) was significantly higher than the older age group (>45 yr). Although not confirmatory for chironomid allergens

tested, higher mean IgE levels were observed in patients in comparison to control subjects, comparable with earlier findings[2,6,7,15] on different chironomid species. Evaluation through specific immunological tests like ELISA and immunoblotting can help correlate chironomid allergen induced changes in IgE level. Bronchial challenge test is required to confirm chironomid allergen as an independent risk factor for asthma and other allergic disorders in Indian population. Although sensitization to *C. circumdatus* and *P. nubifer* substantiates chironomid midges as insects of public health concern in India, further studies are required to characterize the potent allergen molecule to facilitate subsequent therapy.

In conclusion, attention is drawn to the importance of chironomid midges as a potential allergen hidden in environment, besides an established facilitator of sustenance of cholera pathogen[28]. Considering the diversity of chironomid midges in India, and West Bengal in particular, sensitization to other chironomid species needs to be demonstrated by *in vivo* and *in vitro* methods.

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