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# IgE antibody reactivity to bacterial antigens in atopic dermatitis patients

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## Abstract

**Background—**Atopic dermatitis (AD) is a chronic inflammatory skin disease affecting up to 20% children and 9% adults worldwide. AD patients are often sensitized against a broad variety of allergens and more than 90% of them suffer from skin superinfections with *Staphylococcus aureus*.

**Objective—**In this study, we searched for the presence of specific IgE antibodies against *S. aureus* and *Escherichia coli* antigens in AD patients.

**Methods—**Sera from AD patients (n=79), patients suffering only from allergic rhinoconjunctivitis (n=41) or allergic asthma (n=37) were tested for IgE reactivity to nitrocellulose-blotted *S. aureus*, *E. coli* and gut bacterial antigens. IgE-reactive bacterial antigens were affinity purified and identified by mass spectrometry.

**Results—**More than 30% of AD patients but not patients suffering only from allergic rhinoconjunctivitis and asthma or non-allergic persons exhibited IgE binding to several protein antigens among them DNA-binding and ribosomal proteins and flagellin. Patients with severe skin manifestations showed more frequently IgE reactivity to *S. aureus* compared to AD patients with mild symptoms. Positive immediate and late skin test reactions could be induced in sensitized AD patients with *S. aureus* extract.

**Conclusion and Clinical Relevance—**Specific IgE reactivities against a variety of bacterial antigens were observed in a subgroup comprising a third of AD patients and may contribute to allergic inflammation.

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**CONFLICT OF INTEREST**

The authors state no conflict of interest.

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**Keywords**

Atopic dermatitis; bacterial antigens; *Staphylococcus aureus*; *Escherichia coli*; bacterial allergen

## INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease which affects up to 20.5% of children and between 0.2 to 8.8% of adults [1–4]. The clinical manifestations of AD vary and can range from dry skin and eczematous lesions to intense pruritus and lichenified flextures [5]. It has been reported that about 80% of AD patients exhibit elevated levels of serum IgE, and the IgE levels are often correlated with disease severity [6,7]. As AD is associated with other atopic diseases such as asthma and allergic rhinitis, patients with AD often have specific IgE antibodies and allergic symptoms to great variety of food and inhalant allergens [8,9].

Individuals suffering from AD show increased susceptibility to cutaneous bacterial, viral and fungal infections [10,11]. The predominant skin infection in AD is caused by *Staphylococcus aureus*, which affects between 29–100% of patients [12–14]. *S. aureus* is present at 100–1000 fold higher density (about $10^5$ cfu/mL) in the skin of AD patients compared to the skin of healthy individuals [15]. In contrast, only 5–8% of healthy persons harbor *S. aureus* which is usually concentrated in their mucosal cavities [16]. Density and frequency of *S. aureus* colonization is significantly correlated with the severity of eczema [14,17]. Furthermore, treatment of *S. aureus* skin infections with anti-staphylococcal antibiotics significantly reduces bacterial count and clinical severity of the disease [18,19].

*Escherichia coli* is not a common microflora in infected AD lesions. In a study by Brook, *E. coli* was isolated from secondary infected eczema lesions of 10% of AD patients, and the colonization was restricted to the leg and buttock regions [12]. This was in contrast to *S. aureus* which was detected in 29% of the patients in the same study, and was recovered from all body sites [12]. In another study, *E. coli* was isolated from the diaper area of between 0.3 – 1.1% of children with AD, which was much lower compared to *S. aureus* (4.2 – 10.8%) in the same study [20]. There have been no reports on the exacerbation of AD due to *E. coli* infection.

Beginning from the early 1980s, several groups reported that specific IgE against *S. aureus* proteins could be detected in the serum of AD patients [21–25]. Anti- *S. aureus* IgE titers were mostly observed in patients with moderate to severe AD [22,24] but no detailed information about the IgE reactive antigens were available except that both cellular proteins and cell wall components of *S. aureus* may be involved [24,26–30]. Furthermore, some of the toxins were shown to react with IgE antibodies [31–33].

In the present study, the prevalence of serum IgE binding to antigens from *S. aureus* and *E. coli* was studied in patients suffering from AD of different severity, allergic rhinoconjunctivitis or allergic asthma by IgE immunoblotting. The nature of the IgE reactive antigens was characterized by determination of their molecular weights, testing for anti-carbohydrate IgE reactivity and IgE inhibition experiments in different populations of AD patients. Additionally, effects of *S. aureus* and *E. coli* protein stimulation were evaluated by lymphoproliferations and measurements of cytokine secreted. IgE reactivity to proteins from seven most commonly occurring ileum and colon-colonizing bacteria were studied by immunoblotting. Furthermore, immune complexes consisting of IgE and bacterial antigens were affinity purified and subjected to mass spectrometry to identify IgE-reactive bacterial

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proteins. The allergenic activity of *S. aureus* antigens was investigated by skin testing in sensitized AD patients.

# METHODS

## Characterization of patients

Sera from 35 Austrian and 44 German patients who according to the criteria of Hanifin and Rajka [34] suffered from AD were analysed. Tables 1 and 2 summarize the demographic, clinical and serological data of these patients. For control purposes, sera from Austrian patients with allergic rhino-conjunctivitis but no AD (n=41) and allergic asthma without AD (n=37), and from 9 non-atopic individuals were included. To investigate possible associations between severity of AD, *S. aureus* skin superinfections and IgE reactivity profiles, AD patients from Germany were tested. Serum samples were from patients who had undergone routine clinical testing and were used in an anonymous manner, with approval from the respective local ethics committees.

## Preparation of bacterial total protein extracts

*S. aureus* (subspecies Rosenbach, ATCC 25923) and *E. coli* (strain Seattle 1946, ATCC 25922) were grown overnight in tryptic soy broth at 37°C. The other bacterial species (Supplementary Table 1) were cultured on trypticase soy agar plates with 5% sheep blood. All bacterial species used were well characterized type cultures, purchased from either ATCC or the German collection of microorganisms and cell cultures (DSMZ). The bacterial cells were harvested by centrifugation at 3220×*g*. The cells were washed twice with PBS, re-suspended in PBS and homogenized using an Ultra Turrax (IKA Labortechnik, Germany) and glass beads. The homogenates were then centrifuged at 20,000×*g* for 10 min and the supernatants stored at −20°C until use.

## IgE immunoblotting, chemical deglycosylation, IgE-immunoblot inhibitions

Bacterial extracts (*S. aureus, E. coli*) were boiled for 5 min with sodium dodecyl sulfate (SDS) sample buffer containing 5% v/v β-mercaptoethanol [35]. Approximately 200 μg/cm of each bacterial extract was separated on a 12.5% preparative SDS-PAGE [35]. A protein molecular weight marker (Rainbow-Marker, Amersham, UK or PageRuler Prestained Protein Ladder Plus, Fermentas) were used as a standards. The separated proteins were then transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by electroblotting [36]. Nitrocellulose strips were cut from the membranes and blocked twice for 10 min and once for 30 min in buffer A (50mM sodium phosphate buffer, pH 7.4, containing 0.5% w/v bovine serum albumin (BSA), 0.5% v/v Tween 20, and 0.05% w/v $NaN_3$) at room temperature. Strips were incubated overnight with patients' sera (diluted 1:10 in buffer A) at 4°C. The strips were then washed as described for blocking. Bound IgE was detected by incubating the strips with $^{125}$I-labeled anti-human IgE antibodies (Pharmacia, Uppsala, Sweden) diluted 1:10 in buffer A, overnight at room temperature. After repeated washes in buffer A, the strips were dried and exposed to Kodak X-OMAT S films at −70°C using intensifying screens (Kodak, Heidelberg, Germany).

For chemical deglycosylation, nitrocellulose-blotted bacterial extracts were incubated with 50 mM sodium acetate, pH 4.5 with or without 10 mM periodate for 2 h at 4°C. Strips were washed twice for 10 min and once for 30 min with buffer A, and were then incubated with patients' sera diluted 1:10 in buffer A overnight at 4°C. Bound IgE antibodies were detected as described for immunoblotting. For inhibition experiments, patients' sera (diluted 1:10 in buffer A) were pre-incubated with bacterial extracts overnight at 4°C (1000 μg bacterial extract per 1mL of diluted sera). The nitrocellulose-blotted bacterial extracts were then

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incubated with the pre-adsorbed sera overnight at 4°C, and bound IgE antibodies were detected as described for immunoblotting.

### Measurement of *S. aureus* enterotoxin specific IgE antibodies

Serum IgE antibodies against Staphylococcus enterotoxin (SE) A, SEB, SEC, SED, SEE, and toxic shock syndrome toxin (TSST) (Toxin Technology, Florida, USA) were measured using the CAP assay (Phadia, Uppsala, Sweden) [37].

### Specific lymphocyte proliferation and cytokine responses to *S. aureus* and *E. coli* protein extracts

PBMC were isolated from heparinized blood from four AD and five non-allergic individuals using Ficoll-Paque gradient centrifugation and were resuspended in Ultraculture medium (Bio Whittaker, Walkersville, MD, USA) supplemented with 2 mM glutamine, 50 mM β-mecarptoethanol and 0.1 mg/mL gentamycin. Cells were cultured in sterile 96 well plates (Nunc) at $2\times10^5$ cells/well and stimulated with *S. aureus* extract (50 µg/well), *E. coli* extract (50 µg/well), IL-2 (positive control) or medium (negative control) in triplicates at 37°C with 5% $CO_2$. Six days after initial stimulation, 0.5 µCi of $^3$H-thymidine (Amersham, Buckinghamshire, UK) was added to each well, and cells were incubated for 16 h. $^3$H-thymidine incorporation was measured by scintillation counting (Microbeta Trilux, Perkin Elmer) and are reported as stimulation index (SI), calculated as the ratio of the mean proliferation after antigen stimulation over medium control values. Cytokine levels (IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, GM-CSF, TNF-α and IFN-γ) were measured in supernatants collected from PBMC cultures at day 6, using the Bio-Plex Pro Human Cytokine Th1/Th2 assay kit (Bio-Rad, Hercules, CA, USA) and the assay was performed according to the manufacturer's instructions and fluorescent signals were read on a Bio-Plex system (Bio-Rad).

### Skin testing

Routine skin testing was performed by intradermal injection of aliquots of 20 µL of *S. aureus* extract, histamine, or saline into forearms of 22 AD patients in the Russian centre. Patients had to be in remission regarding AD and pyoderma and did not use any pharmacotherapy for at least 5 days prior to skin testing. Skin reactivities were recorded after 20 min and 24 h.

### Identification of IgE-reactive *S. aureus* and *E. coli* proteins by mass spectrometry

An anti-human IgE column was prepared by coupling 1.5 mg of monoclonal mouse anti-human IgE (BD Pharmingen) on a HiTrap NHS-activated HP column (GE Healthcare). The column was washed with PBS to remove unbound antibodies and loaded with serum IgE of AD patients. Then the column was washed with PBS and loaded with *S. aureus* or *E. coli* extract at a concentration of 1mg/mL. Unbound proteins were removed by washing with PBS and IgE immune complexes were eluted with 0.1M glycine pH 2.8, and neutralized immediately with 1M Tris pH 10. The eluted fraction was desalted using a PD10 column with a solution of 5mM $NaH_2PO_4$, pH 7.0. Eluted proteins were then digested overnight at 37°C with trypsin (Sigma), and subjected to mass spectrometry on Nano-LC system coupled to Bruker HCT Ultra ESI trap (Bruker Daltonics, Bremen, Germany). The peptides were fractionated on a C18 PepMap 100 column (3µm, 100Å) (LC Packings) using a 5%–80% acetonitrile solvent gradient. During elution, the mass spectra were recorded in the mass/charge range of 300 to 3000. Data were processed using DataAnalysis™ 3.4, and the peak list generated was searched against the Swiss-Prot database subset of eubacteria proteins using MASCOT (Matrix Science) search engine. The search setting included a missed cleavage site value of three, carboxymethylation of cysteine and variable oxidation of

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methionine, histidine and tryptophan. Protein matches with scores greater than 50 indicated relevant identity to known proteins ($p<0.05$).

## RESULTS

### A third of AD patients exhibit specific IgE reactivity to various protein antigens from *S. aureus* and *E. coli*

IgE reactivities to nitrocellulose-blotted *S. aureus* and *E. coli* antigens (Figures 1 and 2) were analysed using sera from 35 AD patients from Austria (Table 1) and 44 AD patients from Germany (Table 2). From the total of 79 patients, 39 were male, and the mean age on the patients was 39.3 years (range 18 to 80 years). The majority of patients also presented allergic manifestations other than AD, such as rhinitis and bronchial asthma, and were sensitized to multiple allergen sources. The patients displayed a wide range of total serum IgE levels (5.9 to 66286 kU/L).

Ten of the 35 AD patients from the Austrian center showed distinct IgE reactivities to bands of different molecular weight (10–70 kDa) in extracts of *S. aureus* (Figure 1A). Elevated total serum IgE levels were often associated with IgE reactivity to *S. aureus* but were not a pre-requisite for IgE binding. In fact, not all patients with high total serum IgE (e.g., A13, A18, A20, A21) contained *S. aureus*-specific IgE (Table 1; Figure 1A). Similar results were obtained for the German AD patients (Table 2, Figure 2A), where some patients (e.g. H10, H17, H32) with high total IgE levels showed no *S. aureus*-specific IgE. When the serum from a non-atopic individual was used (panel N, Figures 1A and 2A), no IgE binding was observed.

The same sera were also probed with nitrocellulose-blotted *E. coli* extract (Figure 1B). Twelve of the 35 patients showed IgE reactivity to at least one band in *E. coli* extracts. Some of the IgE-reactive bands in *E. coli* extracts had similar molecular weights as those in *S. aureus* whereas others were different. Interestingly, certain patients showed IgE reactivity only to *E. coli* antigens (e.g., A13, A18, A21) but not to *S. aureus* antigens. Similarly in the German population, some patients (e.g., H9 and H27) had specific IgE reactivity only to *E. coli*, but not *S. aureus* proteins (Figure 2B). Again IgE reactivity was often associated with high total serum IgE but there were also patients with high total serum IgE lacking IgE reactivity to *E. coli* antigens (e.g., A20, H47, H1, H26). A slight anti-IgE-reactive band at approximately 34–35 kDa was observed with all sera, including the non-allergic persons (panel N) serum as well as the buffer control (data not shown). Hence it was a result of binding of the anti-IgE conjugate to this band. For control purposes, sera from 37 individuals with asthma and from 41 individuals with rhino-conjunctivitis but without allergic skin manifestations were also tested but no IgE reactivity to bacterial extracts was observed with these sera (data not shown).

Nineteen sera from the 35 AD patients were also tested for IgE reactivity to *S. aureus* toxins, of which 13 contained toxin-specific IgE (Table 1). Again toxin-specific IgE was mainly found in sera containing high total serum IgE levels but there were also sera (e.g., A7) which contained low total IgE levels and toxin-specific IgE (Table 1).

Periodate deglycosylation assay was used to determine if the IgE-reactive antigens were proteins or carbohydrates. Pre-treatment of nitrocellulose-blotted *S. aureus* antigens with periodate did not lead to a relevant reduction of IgE-binding capacity of the bacterial antigenic structures, compared to treatment with buffer alone. Periodate treatment of *E. coli* antigens also did not show any change in IgE binding capacity in five of the six patients tested. In one patient, periodate treatment even revealed additional IgE-reactive bands of approximately 25 kDa and 20 kDa (data not shown).

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### Patients with severe dermatitis symptoms show more frequently IgE reactivity to bacterial antigens than patients with mild symptoms

Based on the disease severity score of AD (SCORAD) for the German AD patients (Table 2), we classified these patients as having mild (0–24), moderate (25–50) or severe (>50) disease. Interestingly, 0%, 48% and 77% of these patients showed positive *S. aureus* IgE reactivity in the mild, moderate and severe groups, respectively. A similar relationship between *E. coli*-IgE reactivity and SCORAD was observed in this population (i.e. 10%, 48% and 77% for mild, moderate and severe AD respectively). Hence, AD patients with increased disease severity showed more frequently specific IgE reactivity to *S. aureus* and *E. coli* proteins, compared to AD patients with mild symptoms.

Next, we were interested to investigate if there was any association between *S. aureus* skin superinfections and IgE reactivity to bacterial antigens. For this purpose, sera from AD populations with and without skin superinfections (defined as clinically visible bacterial infection) obtained from the German center was studied (Figure 2). IgE-reactivity to *S. aureus* antigens were observed in 33% (3 of 9) of patients with skin superinfections, and 43% (15 of 35) of patients without skin superinfections (Figure 2A). Similarly, 44% (4 of 9) and 43% (15 of 35) patients had IgE-reactivity to *E. coli* proteins in this group of patients (Figure 2B). Hence, the frequency of IgE reactivity to bacterial antigens was not related to the presence of skin superinfections in AD patients.

### IgE reactivity to gut bacteria

In this study, more than a third of the AD patients showed IgE-reactivity to *E. coli* proteins. The route of *E. coli* sensitization occurs most likely from the gut, and we therefore studied if AD patients also show IgE binding to antigens of other gut bacterial species. Nitrocellulose-blotted protein extracts of seven of the most commonly occurring gut bacterial species of the ileum or colon region (Supplementary Table 1) [38] were tested with remaining serum samples from AD patients with and without IgE-reactivity to *E. coli* proteins (Figures 3A, B). We found that both groups of AD patients showed IgE reactivity to ileum- and colon-colonizing bacteria (Figures 3A,B)

### Low IgE cross-reactivity between *S. aureus* and *E. coli* antigens

*S. aureus* and *E. coli* extracts contained IgE-reactive bands of similar molecular weights. We therefore searched for the presence of IgE cross-reactive structures in *S. aureus* and *E. coli* in patients who displayed IgE reactivity to both extracts and for whom sufficient serum was available (Figure 4).

As shown in Figure 4A, we found no (e.g., A4, A6) or limited cross-reactivity (e.g., A36, H12, H40) to nitrocellulose-blotted *S. aureus* extract when patient's sera was pre-incubated with *E. coli* extract. Three of the four patients' sera (A4, A21 and H49) showed no IgE cross-reactivity between *S. aureus* extract on nitrocellulose-blotted *E. coli* proteins. Sera of patient H48 showed only limited cross-reactivity, to a protein band of approximately 32 kDa (Figure 4B).

### Lymphoproliferation and cytokine responses of AD patients to bacterial antigens

We found that *S. aureus* but not *E. coli* induced lymphoproliferative responses in AD as well as in non-allergic persons (Figure 5A). We also measured the levels of cytokines released into the cell culture supernatants after stimulation with bacterial extracts (Figure 5B). The stimulation of lymphocytes with proteins from *S. aureus* resulted in the release of elevated levels Th1 and Th2 cytokines, when compared to stimulation with *E. coli* extract or medium alone.

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### *S. aureus* antigens can induce immediate type skin reactions in sensitized AD patients

Figure 6 shows the immediate and late skin reactions of 22 patients to *S. aureus* extract. All but one of the 19 patients with IgE antibodies to *S. aureus* proteins had both immediate and late phase inflammations upon intradermal skin testing with *S. aureus* extract. None of the patients who did not mount IgE against *S. aureus* IgE showed any skin reaction. The results show that *S. aureus* extracts can induce both specific immediate and late phase skin inflammation in patients with IgE antibodies against *S. aureus* components.

### Identification of IgE-reactive bacterial antigens by mass spectrometry

In order to identify IgE-reactive bacterial antigens, serum IgE from AD patients reacting with bacterial antigens was specifically immobilized on an anti-IgE column and incubated with bacterial antigens. Immune complexes consisting of IgE and bacterial antigens were then purified and subjected to MS/MS analysis. Supplementary Table 2 displays proteins from *S. aureus* and *E. coli* for which matching peptides could be identified by mass spectrometry in immune complexes obtained four reactive AD patients with a probability of >0.05. These proteins included among other proteins, DNA-binding proteins, ribosomal proteins and flagellin.

## DISCUSSION

In this study, we report that a subgroup comprising more than a third of AD patients exhibits specific IgE reactivity against a variety of protein antigens of diverse molecular weights in *S. aureus* and *E. coli*. IgE binding was directed to protein bands in the molecular weight range of 20–100 kDa, with a high IgE recognition frequency to antigens in the 30–66 kDa range. The specificity of the IgE reactivity to the bacterial antigens was underlined by the fact that there was no clear relationship between total serum IgE and presence of specific IgE to antigens of *S. aureus* or *E. coli*. Elevated total serum IgE was associated with IgE reactivity to bacterial antigens, but was not a pre-requisite for this reaction. For the majority of patients, no or limited IgE cross-reactivity between antigens of *S. aureus* and *E. coli* was observed.

IgE reactivity to *S. aureus* has already been reported in earlier studies, but the antigens have not been characterized. We found that approximately 30% of AD patients had IgE against *S. aureus* antigens, whereas in earlier studies the prevalence of anti- *S. aureus* IgE in AD patients showed a great variation from 7– 70%. This variation might be explained by the antigen preparation (whole cells [21,25,27,39], protein extracts [23,26] or purified components [24–27,29,30]), choice of detection systems (RAST [21–27,30,39], immunoblots [30] , ELISA [29]), and also the patient population.

We investigated if the antigens of *S. aureus* and *E. coli* contain protein or carbohydrate epitopes. Based on the results of our chemical deglycosylation study, we did not detect a significant difference between IgE reactivity to periodate treated or untreated nitrocellulose-blotted antigens from *S. aureus* and *E. coli*, and hence conclude that the bacterial antigens comprise of mainly protein moieties. In fact the analysis of IgE-reactive bacterial proteins by mass spectrometry identified several of these proteins, among them DNA-binding proteins, ribosomal proteins and flagellin.

A quite unexpected finding of this study was the presence of anti-*E. coli* IgE reactivity. Up to 38% of the AD patients tested had specific IgE antibodies to *E. coli* antigens, although *E. coli* is not a common microflora in the infected skin lesions of AD patients. Based on the inhibition immunoblot assay, the IgE antibodies to *E. coli* proteins were mainly not cross-reactive to *S. aureus* antigens. With regards to the route of sensitization for *E. coli*, it is possible that it occurs via the skin, although there is little colonization. Alternatively, the gut

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may be a more likely entrance and sensitization point for *E. coli*. Based on this, IgE-reactivity to protein extracts of commonly occurring gut bacterial species of the ileum and colon regions were investigated and IgE binding to gut bacterial proteins was detected. Based on these results it may be interesting to investigate in a clinical study whether IgE antibodies against *E. coli* can induce food allergy-like symptoms.

In the case of *S. aureus*, the most probable route of sensitization is the skin route. In fact, we observed that patients suffering from severe forms of AD and with higher SCORAD values, showed more frequently IgE-reactivity to bacterial antigens compared to patients with mild AD symptoms and it is thus possible that patients with more severe skin damage experience higher loads of bacterial antigen exposure capable of boosting anti-bacterial IgE. The production of anti-microbial peptides, such as β-defensins and cathelicidins is reduced in AD patients compared to healthy persons [40,41]. Additionally, it is known that many individuals with AD also have a defective skin barrier, and the combination of these two factors allows for the increased colonization of bacteria on the diseased skin, leading to increased penetration of *S. aureus* antigens through the skin [42] and elevated specific IgE production.

Specific anti- *S. aureus* IgE was detected exclusively in patients with AD, but not those suffering from other clinical manifestations of allergy, such as allergic asthma and rhino-conjunctivitis. This was also the case for another microorganism that was found to colonize the skin of AD patients, *Malassezia sympodialis* [40]. This finding could be a result of the continuous sensitizations resulting from the lack of innate immune function and disrupted skin barrier in AD patients. In contrast, patients with allergic asthma or rhino-conjunctivitis have healthy skin, with intact innate immune mechanism, and are therefore able to better defend themselves against microorganism colonization.

We found that *S. aureus* induced lymphoproliferative responses and the secretion of Th1 and Th2 cytokines in cultured PBMC both from AD and non-allergic persons, which is not too surprising given that both populations may have had contact with *S. aureus*. Interestingly, we did not observe relevant lymphoproliferative response or cytokine secretion when PBMC were stimulated with *E. coli* proteins, which may be explained as an effect of selective tolerance to gut bacteria. The non-specific activation of skin T- cells by *S. aureus* superantigens has been proposed as one pathomechanism by which *S. aureus* contributes to skin inflammation in AD. The presence of specific IgE reactive antigens could represent another pathomechanism in AD because they cross-link mast cells and induce specific T-cell responses. Our data demonstrate that *S. aureus* can induce immediate and late phase skin reactions indicating that IgE and T-cell recognition of *S. aureus* allergens may contribute to skin inflammation in AD. Therefore *S. aureus* should be considered as an allergen source for AD patients and future studies may investigate the allergenic potential of gut bacteria as well.

Our results thus provide another explanation how bacteria may contribute to the pathogenesis of AD and could open new avenues for diagnostic and therapeutic strategies. A better understanding of the individual antigens of *S. aureus* should provide component-resolved recombinant diagnostics, which may help to identify patients who benefit from antibiotic treatment and lead to the development of therapies targeting bacteria involved in the pathogenicity of AD.

## Supplementary Material

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## ABBREVIATIONS

| | |
|---|---|
| **AD** | atopic dermatitis |
| **RAST** | radioallergosorbent test |
| ***S. aureus*** | *Staphylococcus aureus* |
| ***E. coli*** | *Escherichia coli* |
| **SE** | staphylococcal enterotoxin |
| **TSST** | toxic shock syndrome toxin |
| **PBS** | phosphate buffered saline |
| **IgE** | immunoglobulin E |
| **PBMC** | peripheral blood mononuclear cell |

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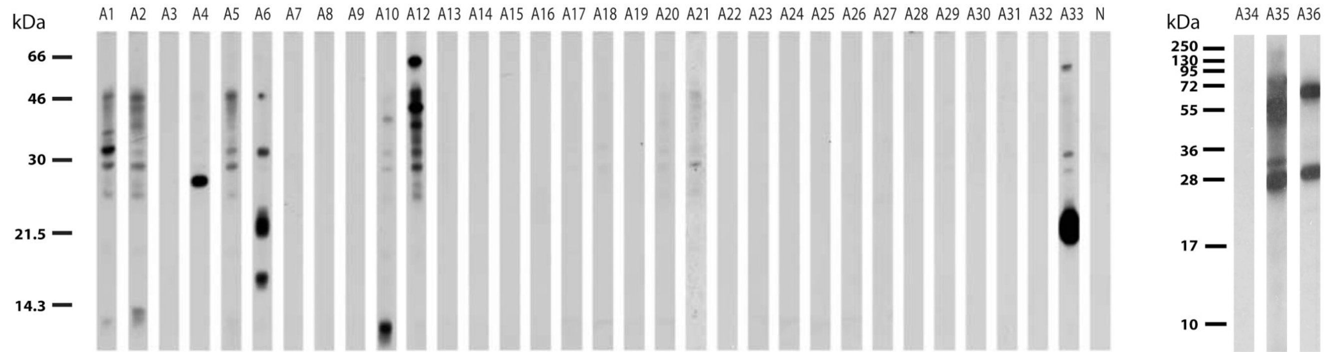


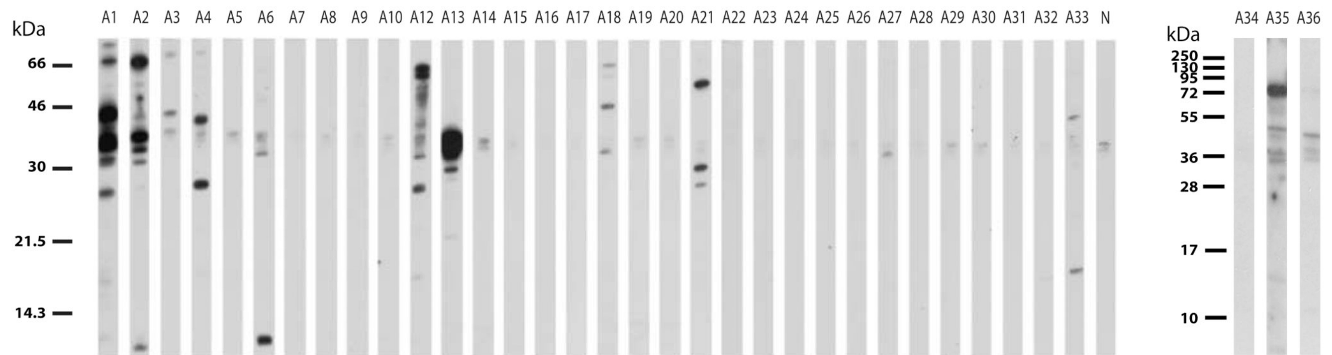


**Figure 1. IgE reactivity of AD patients to nitrocellulose-blotted *S. aureus* and *E. coli* protein extracts**

Total protein extracts of *S. aureus* (A) and *E. coli* (B) were separated under reducing conditions by SDS-PAGE, blotted on to nitrocellulose membrane and probed with sera from 35 patients with AD. As a control, serum from a non-atopic individual (panel N) was used. Molecular weights (kDa) are indicated in the left margin.

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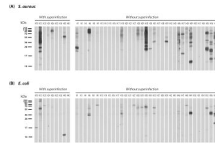

**Figure 2. IgE reactivity of AD patients with and without skin superinfections to nitrocellulose-blotted *S. aureus* and *E. coli* protein extracts**

Total protein extracts of *S. aureus* (A) and *E. coli* (B) were separated under reducing conditions by SDS-PAGE, blotted on to nitrocellulose membrane and probed with sera from the German AD patient populations with or without skin superinfections. As a control, serum from a non-atopic individual (panel N) was used. Molecular weights (kDa) are indicated in the left margin.

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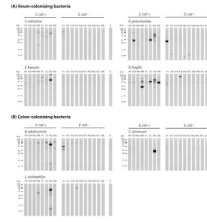

**Figure 3. IgE reactivity of AD patients to nitrocellulose-blotted protein extracts from gut-colonizing bacteria**

Nitrocellulose-blotted protein extracts of *S. salivarius, K. pneumoniae, E. faecalis* and *B. fragilis* which are ileum-colonizing bacteria (A) and *B. adolescentis, C. ramosum* and *L. acidophilus* which are colon-colonizing bacteria (B) were probed with sera from patients with AD, with IgE to *E. coli* proteins (*E. coli* +) or without (*E. coli* −). Lane B denotes buffer control. Patient numbers are as in Tables 1 and 2. Molecular weights (kDa) are indicated in the left margin.

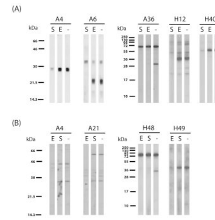


**Figure 4. Low cross-reactivity between IgE-reactive antigens from *S. aureus* and *E. coli***
Nitrocellulose-blotted *S. aureus* extracts (A) and *E. coli* extracts (B) were probed with sera from selected AD patients, which were pre-adsorbed with protein extracts of either *S. aureus* (lanes S), *E. coli* (lanes E), or with bovine serum albumin (BSA; lanes -). Patient numbers are as in Tables 1 and 2. Molecular weights (kDa) are indicated in the left margin.

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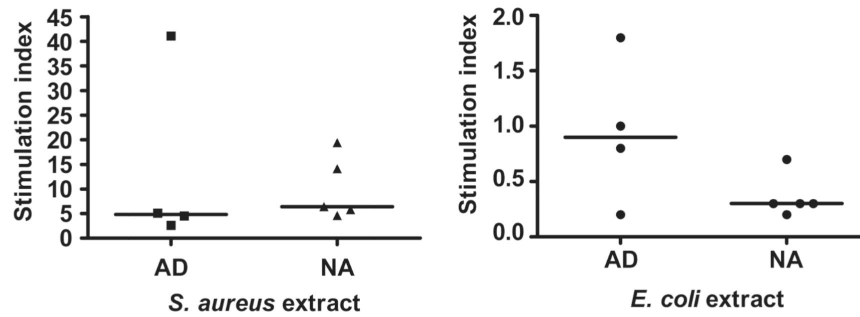


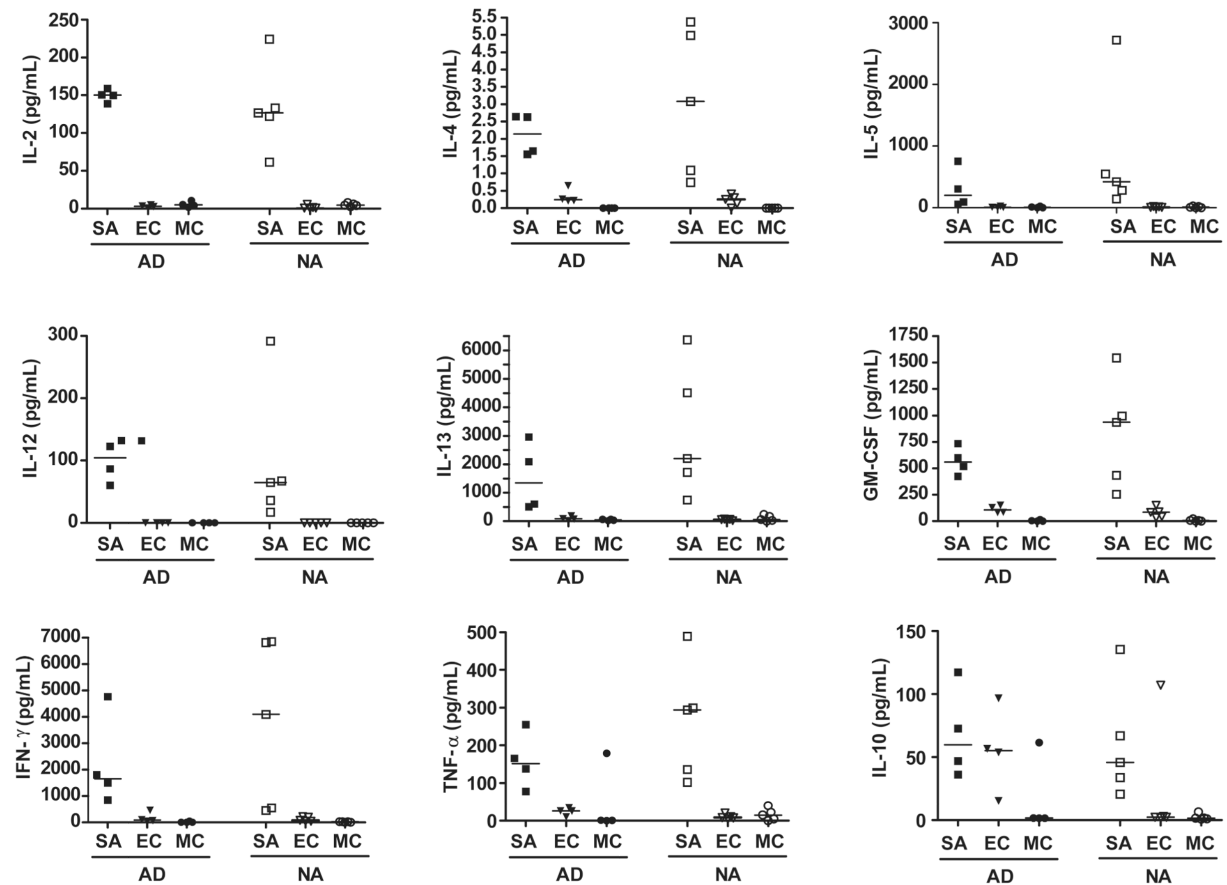


**Figure 5. Lymphocyte proliferation and cytokine releases induced in PBMC cultures of AD patients and non-allergic individuals by stimulation with *S. aureus* and *E. coli* protein extracts**

(A) PBMCs from AD or non-atopic (NA) individuals were stimulated with *S. aureus* extract, *E. coli* extract or medium (MC). Mean lymphoproliferative responses of triplicate determinations were measured by $^{3}$H-thymidine incorporation and are displayed as stimulation indices. Horizontal lines denote the median value of each group. (B) The amounts of released cytokines are presented and horizontal lines denote the median value of each group.

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**Figure 6. Immediate and late type skin reactions to *S. aureus* extract in AD patients**

Twenty two patients with AD, with or without IgE-reactivity to *S. aureus* proteins, were injected intradermally with *S. aureus* extract or saline buffer. Average wheal diameter was measured 20 minutes (immediate reaction) and 24 hours (late reaction) after the challenge.

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## Table 1

Demographic, clinical and serological characterization of AD patients from Austria[1]

| Patient | Sex | Age | Symptoms | Allergies | | | Total IgE (kU/L) | SA IgE | EC IgE | SA enterotoxin RAST |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Pollen | Food | Other | | | | |
| A1 | M | 56 | AD, RC | t, g, w | | mi | 1720 | + | + | SEA (1), SEB (2), SEC (2), SED (1), SEE (2), TSST (2) |
| A2 | M | 38 | AD, RC | t, g, w | s, n, ap, c | a, mi | >2000 | + | + | SEA (2), SEB (2), SEC (2), SED (2), SEE (2), TSST (2) |
| A3 | M | 39 | AD, RC | t, g, w | | mi | 1060 | − | + | SEA (0), SEB (0), SEC (0), TSST(1) |
| A4 | F | 61 | AD, AS | t, g | s | mi | ND | + | + | SEA (1), SEB (2), SEC (2), SED (1), SEE (1), TSST (1) |
| A5 | F | 45 | AD | t, g | mk | a, mi | ND | + | − | ND |
| A6 | F | 80 | AD, AS | t, g | | a | >1000 | + | + | SEB (1), SEC (1), TSST (2) |
| A7 | F | 44 | AD | | ap, n | | 165 | − | − | SEA (0), SEB (2), SEC (0), TSST (0) |
| A8 | F | 46 | AD | | c | a, mi | 271 | − | − | SEA (0), SEB (0), SEC (0), TSST (0) |
| A9 | F | 43 | AD, RC | t, g | ap, n | a | 802 | − | − | SEA (0), SEB (2), SEC (0), TSST (1) |
| A10 | F | 73 | AD | g | | mi | >2000 | + | − | SEA (2), SEB (2), SEC (2), SED (2), SEE (2), TSST (3) |
| A12 | M | 52 | AD, RC, AS | | | a, mi | >2000 | + | + | SEA (2), SEB (2), SEC (2), SED (2), SEE (2), TSST (2) |
| A13 | M | 67 | AD | | | mi | >5000 | − | + | SEA (1), SEB (2), SEC (2) |
| A14 | F | 29 | AD, RC | t, g, w | | mi | 46 | − | − | SEA (0), SEB (0), SEC (0) |
| A15 | F | 36 | AD, AS | t, g, w | | a, mi | 580 | − | − | ND |
| A16 | F | 38 | AD, RC | t, g, w | | a | 270 | − | − | SEA (0), SEB (0), SEC (0) |
| A17 | M | 66 | AD, RC | t, g, w | n, ap | mi | 932 | − | − | ND |
| A18 | M | 34 | AD, RC | t, g, w | | | >2000 | − | + | ND |
| A19 | F | 37 | AD, RC | t, g, w | n, ap, k | | 339 | − | − | ND |
| A20 | M | 36 | AD, RC | t, g, w | ap, ca | | >2000 | − | − | SEA (2), SEB (1), SEC (1), SED (1), SEE (1), TSST (2) |
| A21 | M | 48 | AD, RC, AS | t, g, w | n, ap, k, ca | mi | >2000 | − | + | TSST (2) |
| A22 | F | 54 | AD, RC, AS | t | ap, ca, c | | 187 | − | − | SEA (0), SEB (0), SEC (0) |
| A23 | M | 49 | AD, RC, AS | g | | mi | 196 | − | − | ND |
| A24 | F | 34 | AD, RC | t | n, ap, c | a | 189 | − | − | SEA (0), SEB (0), SEC (0) |
| A25 | F | 29 | AD, RC, AS | t, g, w | | mi | 294 | − | − | ND |
| A26 | M | 37 | AD, RC | t, g, w | | | 380 | − | − | ND |
| A27 | M | 41 | AD, RC | t, g, w | | a, mi | 290 | − | − | SEA (0), SEB (0), SEC (0) |
| A28 | M | 34 | AD, RC | t, g, w | ap | a | 309 | − | − | ND |

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| Patient | Sex | Age | Symptoms | Allergies: Pollen | Allergies: Food | Allergies: Other | Total IgE (kU/L) | SA IgE | EC IgE | SA enterotoxin RAST |
|---|---|---|---|---|---|---|---|---|---|---|
| A29 | M | 36 | AD, RC | t, g, w | n, ap, k | | 512 | − | − | ND |
| A30 | M | 51 | AD, RC | t, g, w | k | mi | 80 | − | − | ND |
| A31 | M | 38 | AD, RC | t | | | 27 | − | − | ND |
| A32 | F | 34 | AD, RC, AS | g | n, k, ca | | 112 | − | − | ND |
| A33 | F | 30 | AD, RC | t, g, w | | a, mi | >2000 | + | + | SEA (1), SEB (2), SEC (3), SED (2), TSST (2) |
| A34 | M | 27 | AD | t, g, w | n, ap, p | a | 205 | − | − | ND |
| A35 | F | 43 | AD, RC | t, g | n, ap, p, mk | a, mi | 19464 | + | + | ND |
| A36 | F | 31 | AD | t | ap | a | 2923 | + | + | ND |

[1] a, animals; AD, atopic dermatitis; ap, apple; AS, asthma; c, celery; ca, carrot; EC, *E. coli* ; F, female; g, grass; k, kiwi; kU/L, kilo units per liter; M, male; mi, mites; mk, milk; n, nuts; ND, not determined; p, peach; RC, rhinoconjunctivitis; s, seafood; SA, *S. aureus* ; SE, staphylococcal enterotoxins; t, trees; TSST, toxic shock syndrome toxin; w, weeds; +, positive reaction; −, negative reaction

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**Table 2**

Demographic, clinical and serological characterization of AD patients from Germany[2]

| Patient | Sex | Age | Symptoms | SCORAD | Allergies | | | Total IgE (kU/L) | SA IgE | EC IgE | Bacterial infection |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Pollen | Food | Other | | | | |
| H1 | M | 35 | AD, AS, AR | 45 | t, g, w | so | a, f, mi, asp | 12499 | + | − | N |
| H3 | M | 32 | AD, AS, AR | 44 | t | | | 68.7 | − | − | N |
| H4 | F | 40 | AD, AR | 24 | t, g, w | | a, f, mi | 1119 | − | − | N |
| H6 | M | 72 | AD | 33 | t, g, w | | a, f, mi, asp | 3668 | + | + | N |
| H8 | F | 25 | AD, AS, AR | 42 | t, g, w | | a, f, mi, asp | 1498 | − | − | N |
| H9 | F | 47 | AD, AS, AR | 31 | t, g, w | | a, mi, asp | 1020 | − | + | N |
| H10 | M | 25 | AD, AS, AR | 31 | t, g, w | | a, f, mi, asp | 2262 | − | + | Y |
| H12 | F | 22 | AD, AS, AR | 61 | t, g, w | n, so, mk, s, wh | a, f, mi, asp | 66286 | + | + | Y |
| H13 | F | 27 | AD | 22 | | | mi | 36 | − | − | N |
| H14 | F | 29 | AD | 12 | ND | ND | ND | 38.5 | − | − | N |
| H15 | F | 29 | AD, AS, AR | 13 | ND | ND | ND | 43.9 | − | − | N |
| H16 | M | 22 | AD, AR | 16 | t, g | | a, mi | 1308 | − | − | N |
| H17 | M | 40 | AD | 28 | | | f | 2298 | − | − | N |
| H18 | F | 37 | AD, AS, AR | 51 | t, g, w | n, so | a, mi, asp | 26200 | + | + | N |
| H20 | M | 48 | AD | 30 | | | mi | 184 | − | − | N |
| H21 | F | 34 | AD | 24 | | | a, f, mi | 1310 | − | − | N |
| H23 | F | 26 | AD | 52 | w | | a, f, asp | 843 | − | + | Y |
| H24 | F | 38 | AD | 51 | t, g | | a, f, mi, asp | 2661 | − | − | Y |
| H26 | M | 20 | AD, AR | 54 | g | | a, f, mi, asp | 5643 | + | − | Y |
| H27 | M | 30 | AD, AS, AR | 20 | t, g, w | so | a, f | 1042 | − | + | N |
| H28 | M | 43 | AD, AS, AR | 60 | t, g, w | e, s, wh, n, so | a, f, mi, asp | 15119 | + | + | N |
| H29 | M | 62 | AD | 56 | t, g, w | wh, n, so | a, f, mi, asp | 11544 | + | + | N |
| H30 | M | 49 | AD, AS | 46 | t, g, w | | a, f, mi, asp | 29240 | + | + | N |
| H32 | F | 28 | AD, AS, AR | 50 | t, g, w | | a, mi | 1815 | − | − | Y |
| H34 | F | 46 | AD, AS, AR | 53 | t, g, w | | a, f, mi, asp | 11403 | + | − | N |
| H35 | M | 60 | AD, AS, AR | 41 | | | a, mi, asp | 12841 | + | + | N |
| H36 | F | 19 | AD | 48 | | | a, f, asp | 830 | − | − | Y |

| Patient | Sex | Age | Symptoms | SCORAD | Allergies | | | Total IgE (kU/L) | SA IgE | EC IgE | Bacterial infection |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Pollen | Food | Other | | | | |
| H37 | F | 23 | AD, AR | 10 | t, g | | a, mi | 861 | − | − | N |
| H38 | M | 37 | AD | 22 | ND | ND | ND | 13.8 | − | − | N |
| H39 | M | 25 | AD, AR | 31 | t, g, w | | a, f, mi, asp | 3964 | + | − | N |
| H40 | M | 32 | AD, AS | 25 | t, g, w | | a, f, mi, asp | 13113 | + | + | Y |
| H42 | F | 33 | AD, AS, AR | 61 | t, g | so | f, mi | 1216 | − | − | N |
| H43 | F | 20 | AD | 54 | ND | ND | ND | 5.9 | − | − | Y |
| H45 | F | 44 | AD | 47 | ND | ND | ND | 23.4 | − | − | N |
| H46 | M | 36 | AD, AS | 49 | t, g, w | k, s, e, mk, wh, n, so | a, f, mi, asp | 19790 | + | + | N |
| H47 | M | 44 | AD, AS, AR | 43 | t, g, w | | a, f, mi, asp | 19545 | + | − | N |
| H48 | M | 53 | AD, AS | 62 | t, g, w | | a, f, mi, asp | 21428 | + | + | N |
| H49 | M | 34 | AD, AS, AR | 56 | t, g, w | | a, f, mi, asp | 33700 | + | + | N |
| H50 | F | 18 | AD, AS, AR | 28 | | | mi | 31.5 | − | − | N |
| H51 | F | 39 | AD, AS, AR | 47 | t, g | n | a, f, mi, asp, l | 463 | − | − | N |
| H54 | F | 26 | AD, AS, AR | 8 | t | | f | 68.5 | − | − | N |
| H55 | M | 43 | AD, AR | 49 | t, g, w | | a, f, mi, asp, l | 11950 | + | + | N |
| H56 | F | 54 | AD, AS, AR | 45 | g, w | | a, f, mi, asp, l | 9400 | + | + | N |
| H57 | M | 25 | AD, AS, AR | 63 | t, g, w | | a, f, mi, asp, l | 40200 | + | + | N |

[2] a, animals; AD, atopic dermatitis; AS, asthma; asp, *Aspergillus fumigatus* ; AR, allergic rhinitis; e, egg; EC, *E. coli* ; f, fungus; F, female; g, grass; k, kiwi; kU/L, kilo units per liter; l, latex; M, male; mi, mites; mk, milk; n, nuts; ND, not determined; s, seafood; SA, *S. aureus* ; so, soy; t, trees; w, weeds; wh, wheat; +, positive reaction; −, negative reaction



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