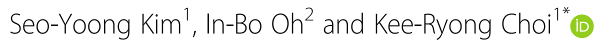
Kim et al. Journal of Ecology and Environment (2017) 41:33
DOI 10.1186/s41610-017-0052-1

Journal of Ecology
and Environment

RESEARCH Open Access

Comparison of IgE induction in mice by pollens from three pine tree species



Seo-Yoong Kim[1], In-Bo Oh[2] and Kee-Ryong Choi[1*]

Abstract

Background: Over the years, pine pollens have been excluded as an allergen due to its relatively large size, low protein content, and waxy hydrophobic layer, despite their abundance. However, recent studies suggest the possibilities of pine pollens being allergens, and it has been reported that allergy symptoms were highly prevalent in areas with considerably large pine forests and high possibility of exposure to the pollen. Therefore, we conducted a comparative analysis of the allergenicities of the pollens from the dominant species of Korean pines, red pine (*Pinus densiflora*), black pine (*Pinus thunbergii*), and pitch pine (*Pinus rigida*), in mice.

Methods: The protein composition of the pollens from the three pine species was compared via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The pine pollens and proteins extracted from the pollens were introduced to BALB/c mice by nasal inhalation and application to exposed skin and the IgE produced by the mice were extracted from blood and analyzed via ELISA.

Results: SDS-PAGE showed differing protein compositions of the pollens of the three pine species. Analysis of blood IgE compositions showed a similar amount of IgE produced when pollens were applied to skin. In contrast, when mice inhaled the pollens, *P. densiflora* was shown to induce significantly more IgE production than those of the other two species.

Conclusions: The experimental results demonstrate that the pollens of all three South Korean pine species induce IgE production, and this production was more pronounced when the pollens were inhaled than when they were applied to the skin. Of the three species, the pollen of *P. densiflora* was found to induce the highest level of IgE production.

Keywords: Pollinosis, Allergen, IgE, Red pine, Black pine, Pitch pine

Background

Several characteristics of pine pollen, such as its relatively large size (45–65 μm), low protein content, and waxy hydrophobic layer, have led researchers to exclude it as a possible cause of allergic reactions, despite its abundance (Howlett et al. 1981; Pettyjohn and Levetin 1997). It has even been used as a negative control in inhalation challenge tests (Frølund et al. 1986). However, some studies have suggested the possibility of pine pollens being allergens (Walker 1921; Rowe 1939; Newmark and Itkin 1967; Harris and German 1985; Cornford et al. 1988; Kalliel and Settipane 1988; Cornford et al. 1990). In addition, significantly high rates of allergy symptoms have been reported in areas with considerably large pine forests and, hence, a high possibility of exposure to pine pollen (Farnham and Vaida 1982; Farnham 1988; Fountain and Cornford 1991; Freeman 1993; Gastaminza et al. 2009). In South Korea, it has been reported that 7.31% of asthmatic patients and 16.9% of allergic coryza and conjunctivitis patients test positive to pine pollen as an allergen (Hong 2015).

There are over 90 species belonging to the *Pinus* genus worldwide (Lawrence 1951). Korean forests are composed of 40.5% coniferous forests, 27% broadleaf forests, and 29.3% mixed stand forests. These coniferous forests are primarily dominated by pines, including *Pinus densiflora* (56.1%), *Pinus rigida* (15%), *Pinus koraiensis* (8.3%), *Cryptomeria japonica*, and *Chamaecyparis obtusa* (3.4%) (Korean Forest Service 2011). It has also been

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determined that among the airborne pollens in South Korea, pine pollen disperses for the longest period at the highest abundance (Oh et al. 2000; Choi et al. 2011; Jung and Choi 2013; Choi et al. 2014). Studies have reported differences in the antigenicities of the pollens of various allergenic plants. Therefore, detailed information regarding the antigenicity of each species is needed (Calenoff et al. 1990; Park et al. 1999; Shahali et al. 2007; Cox et al. 2009, 2011). The purpose of this study was to investigate the antigenicity of pine pollen, especially *P. densiflora*, *P. thunbergii*, and *P. rigida* pollen, derived from Korean pine plants. To analyze the protein allergens of these pollens, three types of proteins were compared by SDS-PAGE. We also observed the changes in IgE expression induced by pine pollens in mice after nasal inhalation and skin application (Tamura et al. 1986; Tordesillas et al. 2015).

Methods

Collection of pine pollens from *P. densiflora*, *P. thunbergii*, and *P. rigida* and preparation of pollen antigens

The pine pollens used in this study were collected from *P. rigida*, *P. densiflora,* and *P. thunbergii* samples from the University of Ulsan (St. A), Mt. Munsu (St. B), Guyu-dong (St. C), and Yeonam-dong (St. D), South Korea, respectively from April to May, 2014 (Fig. 1).

Male flowers were collected and dried in the shade for 7 days. From these, pollen was extracted through a sieve with a pore size of 150 μm. Next, 5 g of each pollen sample was added to 5 volumes of ethyl ether (*w*/*v* 1:5), stirred twice every 12 h at 4 °C, and air-dried for 12 h to completely remove ethyl ether. The completely defatted sample was then added to phosphate-buffered saline (PBS; pH 7.8) in a *w*/*v* ratio of 1:5 and stirred for 24 h at 4 °C, after which it was centrifuged for 30 min at 21,000*g* at 4 °C. The protein-containing supernatant was extracted and dialyzed by adding the sample to a dialysis tube and placing the tube in distilled water at 4 °C for 48 h, during which the water was changed 2–3 times. Then, the samples were filtered with a 0.45-μm Millipore filter and stored at − 20 °C. Using the Thermo Scientific Pierce bicinchoninic acid (BCA) Protein Assay, the presence of proteins in the sample was confirmed by a change in color, and the amount of protein was quantified and standardized based on the absorbance of each sample.

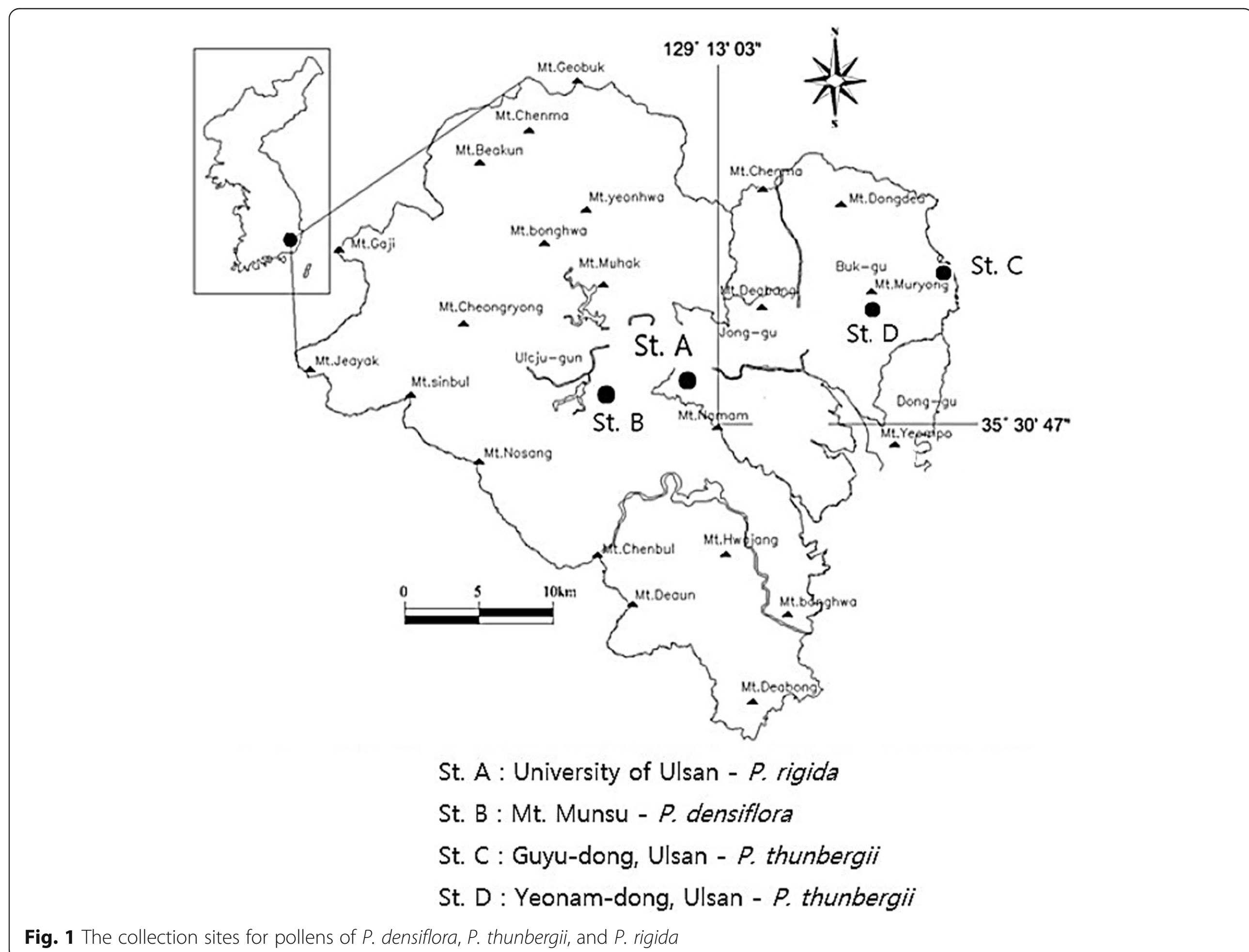

Fig. 1 The collection sites for pollens of *P. densiflora*, *P. thunbergii*, and *P. rigida*

SDS-PAGE of pine pollen antigens

Polyacrylamide gels were made at 10%, 15% (separating gels), and 5% (stacking gels), and the protein samples were mixed with SDS gel-loading buffer (50 mM Tris·Cl, 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 1% β-mercaptoethanol) and boiled for 3 min to prepare for electrophoresis. Standard size markers and the samples were added to the wells and run at 80 V for 3 h in a Tris-glycine electrophoresis buffer system (25 mM Tris, 250 mM glycine, and 0.1% SDS). Then, the gel was stained in a staining solution (0.25% Coomassie Brilliant Blue R250 and 10% glacial acetic acid in methanol: H_2O 1:1 *v*/*v*) and destained with a destaining solution (methanol: glacial acetic acid:H_2O 3:1:6) that was changed 3 times. SDS-PAGE was used to conduct a comparative analysis of the proteins of the pine pollens of *P. densiflora*, *P. thunbergii*, and *P. rigida*. Gels of different concentrations (5, 10, and 15%) were used to broaden the size range of the proteins that could be analyzed.

Mice

Female Balb/c mice aged 7 weeks were purchased from the Dae-Han Bio Link Co. Ltd. They were maintained under specific pathogen-free (SPF) conditions in the animal facility of the University of Ulsan and were used at 8–10 weeks.

Detection of changes in IgE expression induced by pine pollens in mice

In order to assess the induction of IgE expression in a mouse system by pine pollens, the procedure described in Fig. 2 was carried out. That is, for nasal inhalation tests, 0.05 g of pine pollen from *P. densiflora*, *P. thunbergii*, or *P. rigida* was added to 1 mL of sterilized distilled water and mixed by vortexing. This produced a homogeneous mixture of a 0.05 g/mL solution; 25 μL (1.25 g) was injected directly into the nasal cavity of each mouse (n = 5) daily for 15 days. Another method involved a skin test during which each anesthetized mouse had its back fur removed with clippers. Then, 100 μL of pine pollen proteins from *P. densiflora*, *P. thunbergii*, or *P. rigida* was applied to the skin daily for 15 days. In the control group, 25 μL of sterilized distilled water was injected into the nasal cavity of each mouse once daily for 15 days. On days 5, 10, and 15 after introduction of pine pollens, capillary tubes were used to extract blood from the capillary vessels of the eyes of mice in the control group, nasal inhalation test group, and skin test group. The blood samples were centrifuged at 800*g* for 15 min, and the supernatant was extracted to

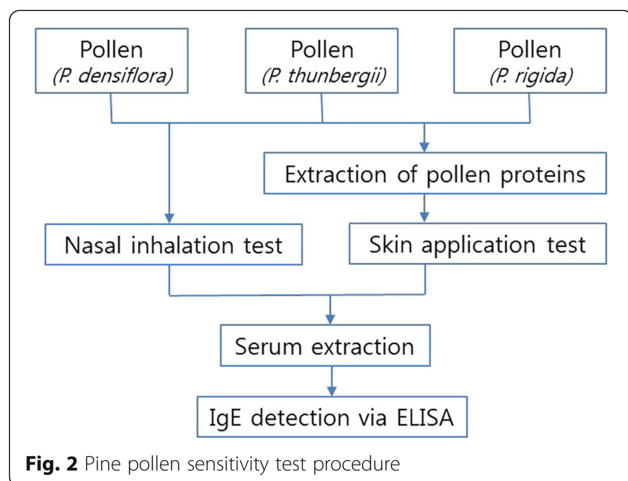

Fig. 2 Pine pollen sensitivity test procedure

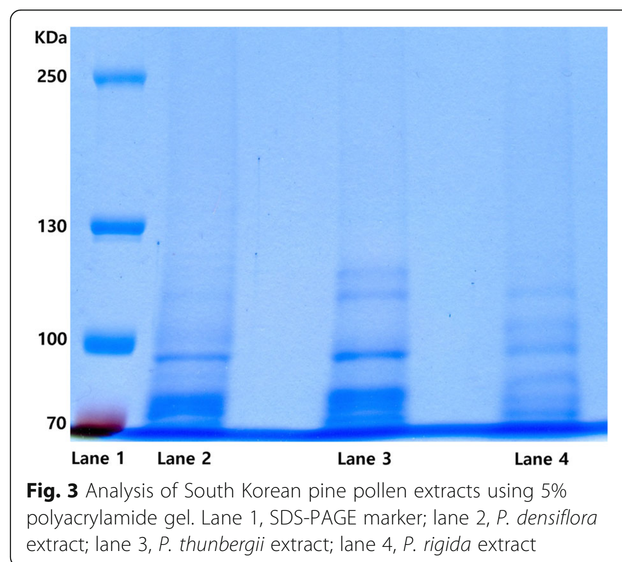

Fig. 3 Analysis of South Korean pine pollen extracts using 5% polyacrylamide gel. Lane 1, SDS-PAGE marker; lane 2, *P. densiflora* extract; lane 3, *P. thunbergii* extract; lane 4, *P. rigida* extract

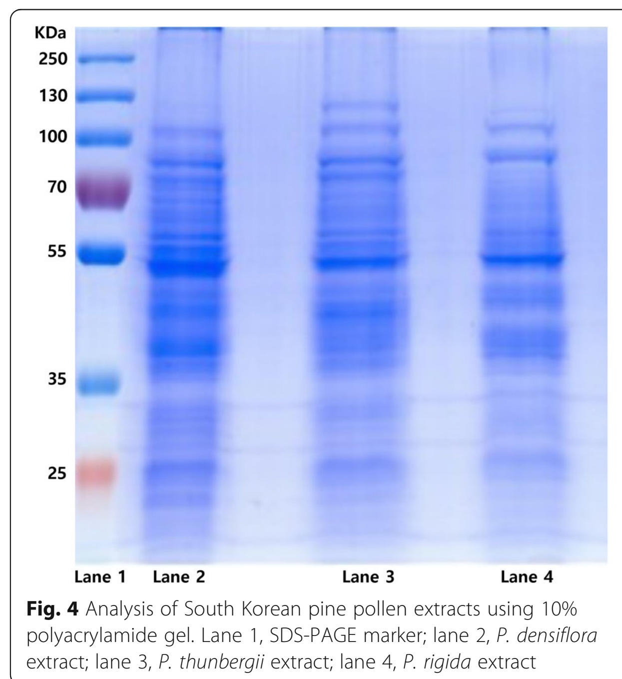

Fig. 4 Analysis of South Korean pine pollen extracts using 10% polyacrylamide gel. Lane 1, SDS-PAGE marker; lane 2, *P. densiflora* extract; lane 3, *P. thunbergii* extract; lane 4, *P. rigida* extract

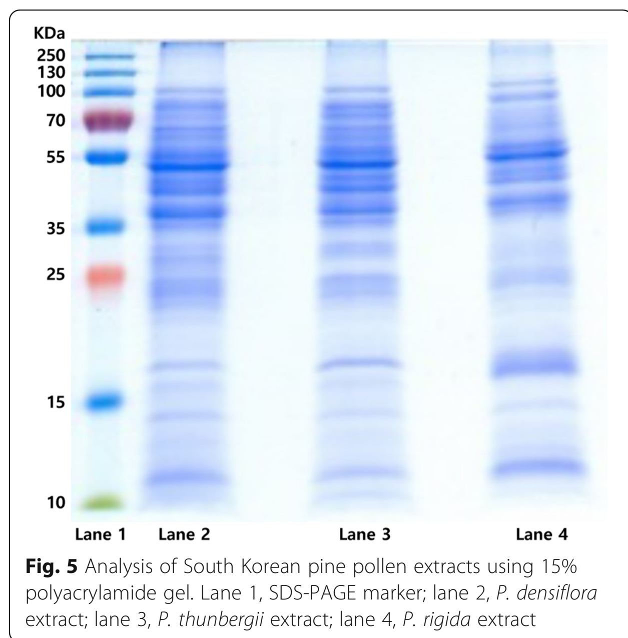

Fig. 5 Analysis of South Korean pine pollen extracts using 15% polyacrylamide gel. Lane 1, SDS-PAGE marker; lane 2, *P. densiflora* extract; lane 3, *P. thunbergii* extract; lane 4, *P. rigida* extract

isolate serum, which was stored at − 20 °C. A Mouse IgE ELISA kit (ebioscience mouse IgE ELISA Ready-SET-Go) was used to detect changes in IgE composition in blood serum samples of each group. A 96-well microplate was coated with IgE-capture antibody (Ab) and incubated at 4 °C for 18 h. To each well, 200 μL of PBS-T (Tween 20, 0.05%) was added and removed three times for washing. Then, 100 μL of 2% bovine serum albumin (BSA) was added to each well and incubated at 20 °C for 2 h to prevent unspecific binding. After washing each well three times with 200 μL PBS-T, 50 μL uniformly diluted mouse serum was added to each well and incubated for 2 h. After washing each well three times with 200 μL PBS-T, IgE-detection Ab was added and incubated at 20 °C for 1 h. After washing each well three times with 200 μL PBS-T, horseradish peroxidase-bound secondary Ab was added and incubated at 20 °C for 30 min. After washing each well three times with 200 μL PBS-T, 100 μL of a substrate for horseradish peroxidase, TMB (3,3′,5,5′-tetramethylbenzidine), was added per well to induce a color change. Then, 100 μL of 0.16 M sulfuric acid stop solution was added to each well to stop the color change, and the absorbance was read at 450 nm using a microplate reader.

Results and discussion

Analysis of pine pollen protein

After 5% SDS-PAGE, analysis of protein bands above 70 kDa showed different band patterns at approximately 80 and 120 kDa for *P. densiflora*, *P. thunbergii*, and *P. rigida* (Fig. 3). Next, 10% SDS-PAGE was run to analyze proteins of 35–70 kDa. Even though an exact comparison of the protein bands of the pine species was difficult owing to the large number of proteins in this size range, it was clear that *P. densiflora*, *P. thunbergii*, and *P. rigida* all showed similar band patterns (Fig. 4). Finally, 15% SDS-PAGE was run to analyze proteins under 35 kDa. The pollens from the three species showed different band patterns at approximately 30 and 17 kDa (Fig. 5). According to previous data, the major antigens in pine pollen are of 42 and 6–8 kDa (Weber 2005). In addition, in the case of *Pinus radiata*, it has been reported that the main allergens are of 140, 85, 70, 55, 42, 32, 22, 19, and 6–8 kDa (Cornford et al. 1988; Gastaminza et al. 2009). The three species that were used in this experiment showed different band patterns at approximately 17, 30, 80, and 120 kDa. This indicates that the three

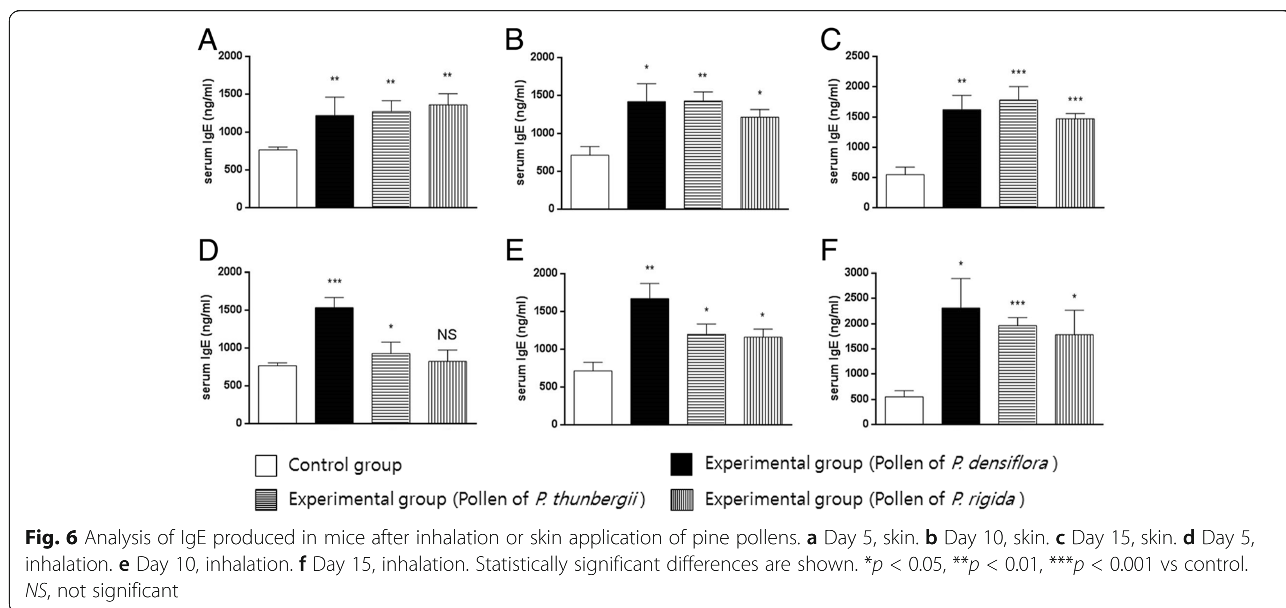

Fig. 6 Analysis of IgE produced in mice after inhalation or skin application of pine pollens. **a** Day 5, skin. **b** Day 10, skin. **c** Day 15, skin. **d** Day 5, inhalation. **e** Day 10, inhalation. **f** Day 15, inhalation. Statistically significant differences are shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs control. *NS*, not significant

species exhibit differences in antigenicity. It is necessary to identify the region of each of these proteins that acts as an allergen by binding to IgE. Future studies should also identify which protein band patterns cause allergic reactions using sera extracted from patients showing allergic reactions after exposure to pine pollens.

Comparison of IgE induction by pine pollens

Considering that the dispersion of pine pollens is most intense for about 2 weeks, IgE induction experiments were conducted on mice for 15 days to induce allergic reactions via inhalation and skin application (Tamura 1986; Conford et al. 1990; Maejima et al. 2000; Mahler et al. 2000; Seitzer et al. 2003a, b; Mondoulet et al. 2010, 2012; Tordesillas et al. 2015). Control mice showed no change in the IgE compositions of blood sera collected on days 5, 10, and 15 (Fig. 6). The experimental groups that underwent nasal inhalation and skin tests showed higher levels of IgE than the control group, and these levels increased with the duration of exposure (Fig. 6). In the skin test group, pollen from all three species induced IgE production in mice on the 5th day, and levels increased by similar amounts until the 15th day (Fig. 6a–c). In the nasal inhalation test, mice that inhaled the pollen of *P. densiflora* showed significant increases in IgE production on the 5th and 10th days, but those that inhaled the pollens of the other two species showed only very small increases in IgE production (Fig. 6d, e). However, mice that inhaled each of the three species showed significant increases in IgE production by the 15th day, such that all three groups showed a similar amount of IgE produced by the end of the experiment (Fig. 6f) In addition, on the 15th day, all groups that had inhaled pine pollen exhibited higher IgE levels than those in which pollen was applied to the skin (Fig. 6c, f). Thus, our results indicate that *P. densiflora*, *P. thunbergii*, and *P. rigida* pollen from South Korea can cause allergic reactions in animal models.

Conclusions

The experimental results demonstrate that the pollens of all three South Korean pine species induce IgE production, and this production was more pronounced when the pollens were inhaled than when they were applied to the skin. Of the three species, the pollen of *P. densiflora* was found to induce the highest level of IgE production.

Abbreviations

Ab: Antibody; BCA: Bicinchoninic acid; BSA: Bovine serum albumin; ELISA: Enzyme-linked immunosorbent assay; IgE: Immunoglobulin E; *P. densiflora*: *Pinus densiflora*; *P. rigida*: *Pinus rigida*; *P. thunbergii*: *Pinus thunbergii*; PBS: Phosphate-buffered saline; PBS-T: Phosphate-buffered saline Tween 20; SDS: Sodium dodecyl sulfate; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; SPF: Specific pathogen-free; TMB: 3,3′,5,5′-tetramethylbenzidine; *w/v*: Weight-to-volume

Acknowledgements

This research was supported by the Environmental Health Center of Ulsan University Hospital of the Ministry of Environment, Korea.

Funding

This work was supported by the 2016 Research Fund of the University of Ulsan.

Availability of data and materials

Not applicable.

Authors' contributions

SY conducted the experiments, analyzed the data, and wrote the manuscript. IB participated and designed the research. KR mainly designed the research and revised the paper totally. All authors read and approved the final manuscript.

Ethics approval

Mouse experimentation was carried out with the approval of the animal experimental ethics committee at the University of Ulsan. (Approval number: KRC-16-010; Title: Study of allergenicity of pine trees pollen: in mice).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 5 April 2017 Accepted: 7 September 2017
Published online: 16 September 2017

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