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ORIGINAL ARTICLE

Clinical significance of a basophil activation test for Japanese beekeepers naturally sensitized to honey bee venom

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Abstract

Background: The basophil activation test (BAT) has high sensitivity and specificity for diagnosing Hymenoptera venom allergy and is useful for predicting the clinical sensitivity of bee venom–allergic patients after venom immunotherapy. Patients sensitized to Hymenoptera venom are at risk for systemic reactions (SRs) to subsequent stings. Therefore, a tool that can predict the occurrence of SRs and the severity of Hymenoptera stings is needed.

Objective: We performed BATs on Japanese beekeepers naturally sensitized to honey bee venom (HBV) and analyzed the positive threshold concentration for the occurrence of SRs following honey bee stings (HBS).

Methods: Sixty-one beekeepers were interviewed and blood samples were taken. Data including history of HBS and the occurrence and severity of SRs to HBS were recorded. Blood samples were exposed to HBV-specific IgE antibodies (sIgE) and BAT was performed. Participants with HBV-sIgE ≥ class 1 were considered sensitized to HBV. The positive threshold for BAT scored as 0.0001, 0.001, 0.01, 0.1, and 1 μg/ml was classified as classes 5, 4, 3, 2, and 1, respectively. Samples negative at 1 μg/ml were classified as class 0.

Results: About 40% of beekeepers with a positive BAT threshold ≤ 0.1 μg/ml had SRs after HBS. The mean score of the BAT positivity threshold for beekeepers who developed SRs was significantly lower than that for beekeepers with no history of SRs (2.6 ± 0.8 vs 1.4 ± 1.1, $P < 0.01$).

Conclusion: Analysis of the positive threshold of BAT in Japanese beekeepers naturally sensitized to HBV may be a useful tool for predicting the occurrence of SRs.

Key words: beekeepers, basophil activation test, honey bee allergy, adrenalin auto-injectors, honey bee venom–specific IgE antibodies

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Abbreviations:

sIgE	specific IgE antibodies
BAT	basophil activation test
SRs	systemic reactions
HBS	honey bee stings
HBV	honey bee venom

Introduction

The basophil activation test (BAT) has high sensitivity (85–100%) and specificity (83–100%) for diagnosing Hymenoptera venom allergy[1-3] and is useful for predicting the clinical sensitivity of bee venom–allergic patients after venom immunotherapy.[4] Patients sensitized to Hymenoptera venom have a 10–17% chance of systemic reactions (SRs) to subsequent stings.[5-7] Therefore, a tool that can predict the occurrence of SRs and the severity of Hymenoptera stings is needed. Beekeepers often become sensitized to honey bee venom (HBV) due to frequent honey bee stings (HBS), and many have a history of SRs.[8] However, no previous study has clarified the correlations between venom-specific IgE antibody titers and the occurrence or severity of SRs.

Therefore, in this study, we performed BATs on Japanese beekeepers naturally sensitized to HBV and analyzed the positive threshold concentration for the occurrence of SRs following HBS.

Methods

Participants

Between June and November 2023, 61 beekeepers were interviewed and blood samples were taken (**Figure 1**). Data including sex, age, 5-year history of HBS, time of most recent HBS, and the occurrence and severity of SRs at the time of HBS were recorded. Mueller grade was used for severity.[9] No participants received antiallergic drugs, oral steroids, or HBV extract–based allergen immunotherapy. They all completed questionnaires and underwent peripheral blood tests. This study was approved by the Research Ethics Committee of Saitama Medical Center, Dokkyo Medical University (authorization No. 23006), and written informed consent was obtained from each participant before enrollment.

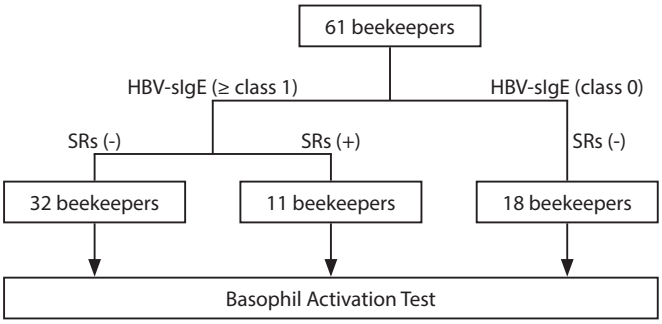


Figure 1. Overview of the 61 beekeepers according to HBV-sIgE class.

HBV-sIgE, honey bee venom–specific IgE antibody; SRs, systemic reactions

Measurements for HBV-specific IgE antibodies

Blood samples were exposed to HBV-specific IgE antibodies (sIgE), using the ImmunoCAP allergy testing system (Thermo Fisher Scientific, Waltham, MA). Participants with HBV-sIgE ≥ class 1 (≥ 0.34 UA/ml) were considered sensitized to HBV.

Measurements for BAT

BAT was performed using Allergenicity Kit (Beckman Coulter, Brea, CA), following a previous report.[10,11] Whole blood (100 μl) with ethylenediaminetetraacetic acid was incubated with anti-human IgE antibody (10 μg/ml) as a positive control, with phosphate-buffered saline as a negative control, or with various concentrations (0.0001–1 μg/ml final concentration) of non-standardized HBV extract (*Apis mellifera*) (Citeq Biologics, Groningen, Netherlands) with three colors of fluorescence-conjugated antibodies (Anti-CD294-FITC, Anti-CD203c-PE, Anti-CD3-PC7) for 10 min at 37°C. After stopping the activation reaction by the addition of a stop solution, a hemolysis fixative was added and the mixture was incubated for 10 min at room temperature. After centrifugation at 200×g for 5 min, cells were washed and fluorescence was measured with a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Basophils were detected based on forward and side scatter and the expression of CD294 and CD203c without the expression of CD3. The basophils of all participants showed clear positive results when stimulated with anti-IgE antibodies as positive controls. The change in threshold sensitivity was evaluated by basophil CD294 and CD203c response at submaximal concentrations. The analysis was performed using the software BD CellQuest Pro ver. 6.1 (Becton Dickinson). In this study, a negative activation rate plus 6% or more was considered positive.[11] The lowest concentration that resulted in a positive test was defined as the positive threshold. Flow cytometry results for a representative beekeeper (No. 40 in **Table 1A**) are shown in **Figure 2**. The positive threshold for BAT was scored as 0.0001, 0.001, 0.01, 0.1, and 1 μg/ml, respectively classified as classes 5, 4, 3, 2, and 1. Samples negative at 1 μg/ml were classified as class 0. Five and three concentrations were measured for beekeepers with and without a history of SRs, respectively.

Statistical analysis

Data are presented as mean ± standard deviation. The Mann–Whitney *U* test was used for statistical analysis of the occurrence of SRs in HBS and the positive threshold of BAT, or HBV-sIgE. Spearman's rank correlation coefficients were plotted between SRs (Mueller grade) and BAT scores as well as between BAT scores and the values of HBV-sIgE. Fisher's exact test was used to analyze the occurrence of SRs in HBS at each positive threshold of BAT. Receiver operating characteristic (ROC) curve analysis was performed to evaluate the sensitivity, specificity, positive predictive value, negative predictive value, and area under the curve of the occurrence of SRs from 1 to 0.01 ug/ml positive threshold for BAT. A P value < 0.05 was considered to be significant. All statistical analyses were performed using SPSS Statistics 29.0 (IBM Corp., Armonk, NY), GraphPad Prism 9, and EZR.[12]

Results

BAT results in beekeepers sensitized to HBV

Of the 61 beekeepers, 43 had HBV-sIgE ≥ class 1 and had had multiple HBS in the past 5 years and were considered sensitized to HBV. An overview of the 61 beekeepers according to HBV-sIgE class is shown in **Figure 1**. The BAT results are shown in **Table 1A**. Eleven of these 43 beekeepers developed SRs immediately after HBS. Despite not having any SRs in the past 5 years, beekeeper No. 9 had had a Mueller grade I reaction once, > 10 years ago, just after becoming a beekeeper. The other 31 beekeepers had no lifetime history of SRs to HBS. Of the 11 beekeepers with SRs, 7 had a grade I reaction, two had grade II, and one each had grade III and grade IV. **Table 1B** summarizes the participant background characteristics by presence or absence of SRs. The BAT score of beekeeper No. 35 could not be evaluated because of the high negative control value and was thus excluded from the analysis. The mean age of beekeepers who developed SRs was 61.3 ± 14.4 years, the male to female ratio was 5:5, and the mean value of HBV-sIgE was 34.8 ± 39.3 UA/ml. The mean age of beekeepers with no SRs was 56.8 ± 18.2 years, the male:female ratio was 28:4, and the mean value of HBV-sIgE was 6.2 ± 9.1 UA/ml. Beekeepers with a history of SRs had significantly higher levels of HBV-sIgE compared with the controls ($P < 0.01$); all 6 healthy participants with no history of HBS had negative HBV-sIgE and negative BAT at 100 μg/ml. There were very weak correlations ($R = 0.37$, $P < 0.0147$) between the levels of HBV-sIgE and BAT scores (**Figure 3A**). In addition, we analyzed the difference in BAT scores by dividing the beekeepers according to whether their most recent sting was within the past 6 months, but no significant ($P = 0.958$) difference was found (data not shown).

Table 1. A: Background of individual beekeepers with the results of HBV-specific IgE antibody and BAT.

No.	Sex	Age (years)	Most recent honey bee stings period (ago)	Systemic reactions (yes or no) in the past 5 years (if yes, then time of stings and *Mueller Grade)	Specific IgE antibody (UA/ml)	BAT (%)						
								Concentration of honey bee venom				
						Negative control	Positive control	1 μg/ml	0.1 μg/ml	0.01 μg/ml	0.001 μg/ml	0.0001 μg/ml
1	Male	20	1 month	No	16.7	2.7	78.9	85.5	86.4	60.5	3.5	N.D.
2	Male	31	1 week	No	9.6	2.1	57.9	66.1	31.9	3.2	1.4	N.D.
3	Male	56	14 month	No	11.8	3.1	67.8	56.9	36.8	21.0	1.5	N.D.
4	Male	43	12 month	Yes (36 month ago, Grade I)	21.8	1.3	54.7	70.9	47.8	3.2	3.1	N.D.
5	Male	73	1 month	No	4.5	2.4	66.5	1.8	1.4	1.4	2.4	N.D.
6	Male	57	2 month	No	1.7	0.8	85.4	27.0	1.7	0.7	0.5	N.D.
7	Male	47	2 week	No	2.1	5.2	41.3	44.6	51.6	15.8	5.4	N.D.
8	Male	35	2 month	No	2.7	2.5	74.0	75.9	72.2	37.5	3.4	N.D.
9	Male	71	1 week	No	1.4	0.5	13.1	4.8	1.7	1.3	1.3	N.D.
10	Male	21	1 month	No	20.5	2.4	65.8	52.9	31.2	3.5	1.4	N.D.
11	Female	50	9 month	No	0.6	1.5	48.3	20.4	25.4	2.0	N.D.	N.D.
12	Male	34	24 month	No	0.4	0.8	61.9	12.6	0.8	0.3	N.D.	N.D.
13	Male	74	1 week	No	3.2	1.5	68.7	67.8	53.2	2.6	N.D.	N.D.
14	Male	37	1 week	No	0.4	2.4	75.7	3.2	1.5	2.9	N.D.	N.D.
15	Male	66	22 week	No	1.8	2.1	69.7	84.2	66.3	6.5	N.D.	N.D.
16	Female	66	2 day	Yes (48 month ago, Grade I)	5.8	0.8	39.5	54.9	69.3	34.6	0.5	0.2
17	Female	40	6 week	Yes (6 week ago, Grade I)	above 100.0	2.4	14.4	20.1	26.0	1.7	1.3	1.4
18	Male	61	1 week	No	28.8	3.0	48.2	62.9	77.8	40.4	N.D.	N.D.

Table 1. A (Continued)

No.	Sex	Age (years)	Most recent honey bee stings period (ago)	Systemic reactions (yes or no) in the past 5 years (if yes, then time of stings and *Mueller Grade)	Specific IgE antibody (UA/ml)	BAT (%)						
								Concentration of honey bee venom				
						Negative control	Positive control	1 μg/ml	0.1 μg/ml	0.01 μg/ml	0.001 μg/ml	0.0001 μg/ml
19	Female	60	1 month	No	7.91	2.0	15.9	28.9	9.7	1.2	N.D.	N.D.
20	Male	78	1 day	Yes (58 month ago, Grade I)	11.9	3.3	8.5	12.0	11.6	1.1	1.1	0.6
21	Male	76	12 month	No	10.5	2.2	7.0	2.7	1.6	2.2	N.D.	N.D.
22	Female	71	2 month	No	3.3	0.9	42.5	30.0	2.7	1.3	N.D.	N.D.
23	Female	66	2 month	No	2.2	1.5	89.8	66.8	14.7	1.8	N.D.	N.D.
24	Male	78	1 month	No	0.8	1.2	81.8	31.4	15.5	1.4	N.D.	N.D.
25	Male	73	1 month	No	1.8	1.1	7.2	4.9	1.0	0.8	N.D.	N.D.
26	Male	74	1 day	No	2.0	2.6	61.9	23.6	5.8	1.7	N.D.	N.D.
27	Male	86	1 month	Yes (36 month ago, Grade I)	5.5	8.8	13.6	15.2	18	11.2	5.7	6.1
28	Male	70	3 week	No	1.0	2.7	15.1	16.9	4.0	4.4	N.D.	N.D.
29	Female	54	5 month	Yes (5 month ago, Grade III)	9.8	2.8	74.2	81.8	79.7	69.6	9.6	2.1
30	Male	60	2 month	Yes (24 month ago, Grade I)	68.1	4.0	9.7	10.5	15.3	3.9	2.7	2.7
31	Male	53	1 month	No	0.9	2.3	79.4	46.9	15.0	2.4	N.D.	N.D.
32	Female	53	1 month	Yes (18 month ago, Grade II)	24.3	1.8	61.0	79.4	77.9	56.2	5.0	1.0
33	Male	69	1 month	No	0.6	3.0	76.4	38.3	16.1	2.9	N.D.	N.D.
34	Male	72	1 month	No	9.5	2.7	34.3	2.9	0.6	1.4	N.D.	N.D.
35	Male	64	1 month	Yes (30 month ago, Grade II)	2.9	16.6	14.8	21.6	16.9	19.1	20.3	14.4
36	Male	33	2 day	No	0.5	2.4	68.1	3.1	2.1	1.7	N.D.	N.D.
37	Male	75	1 day	No	1.2	1.7	2.4	3.2	1.0	2.0	N.D.	N.D.
38	Male	71	2 week	No	2.1	3.8	5.5	4.8	4.5	3.3	N.D.	N.D.
39	Male	71	3 month	No	4.9	1.3	49.0	2.7	2.3	2.5	N.D.	N.D.
40	Female	66	5 month	Yes (5 month ago, Grade I)	above 100.0	7.9	65.0	64.6	65.5	51.2	17.3	8.7
41	Male	29	1 week	No	40.9	2.9	81.7	78.1	24.7	1.1	N.D.	N.D.
42	Male	67	51 month	Yes (51 month ago, Grade IV)	0.4	1.9	95.1	20.3	20.7	3.6	1.3	1.6
43	Male	42	2 week	No	1.5	4.3	47.0	25.3	7.3	4.5	N.D.	N.D.

N.D., no data; *Mueller grade was used for severity [9].

Table 1. B: Beekeeper background characteristics and the results of HBV-specific IgE antibody and BAT based on the presence or absence of SRs. N = 42 [SR (+), 10; SR (−), 32], excluding beekeeper No. 35.

	SRs (+)	SRs (-)
Number (Male/Female)	10 (5/5)	32 (28/4)
Age (years)	61.3 ± 14.4 (Range:40-86)	56.8 ± 18.2 (Range:20-78)
Most recent honey bee stings period (ago)	Range: 1 day-51 month	Range: 1 day-24 month
Specific IgE antibody (UA/ml)	34.8 ± 39.3 (Range; 0.4-above 100)	6.2 ± 9.1 (Range; 0.4-40.9)
BAT Positive (%, number; positive/total)		
1 μg/ml	100.0% (10/10)	68.8% (22/32)
0.1 μg/ml	100.0% (10/10)	50.0% (16/32)
0.01 μg/ml	40.0% (4/10)	15.6% (5/32)
0.001 μg/ml	20.0% (2/10)	0.0% (0/9)
0.0001 μg/ml	0.0% (0/9)	N.D

SRs; Systemic reactions in the past 5 year. HBV; honey bee venom, N.D; no data.

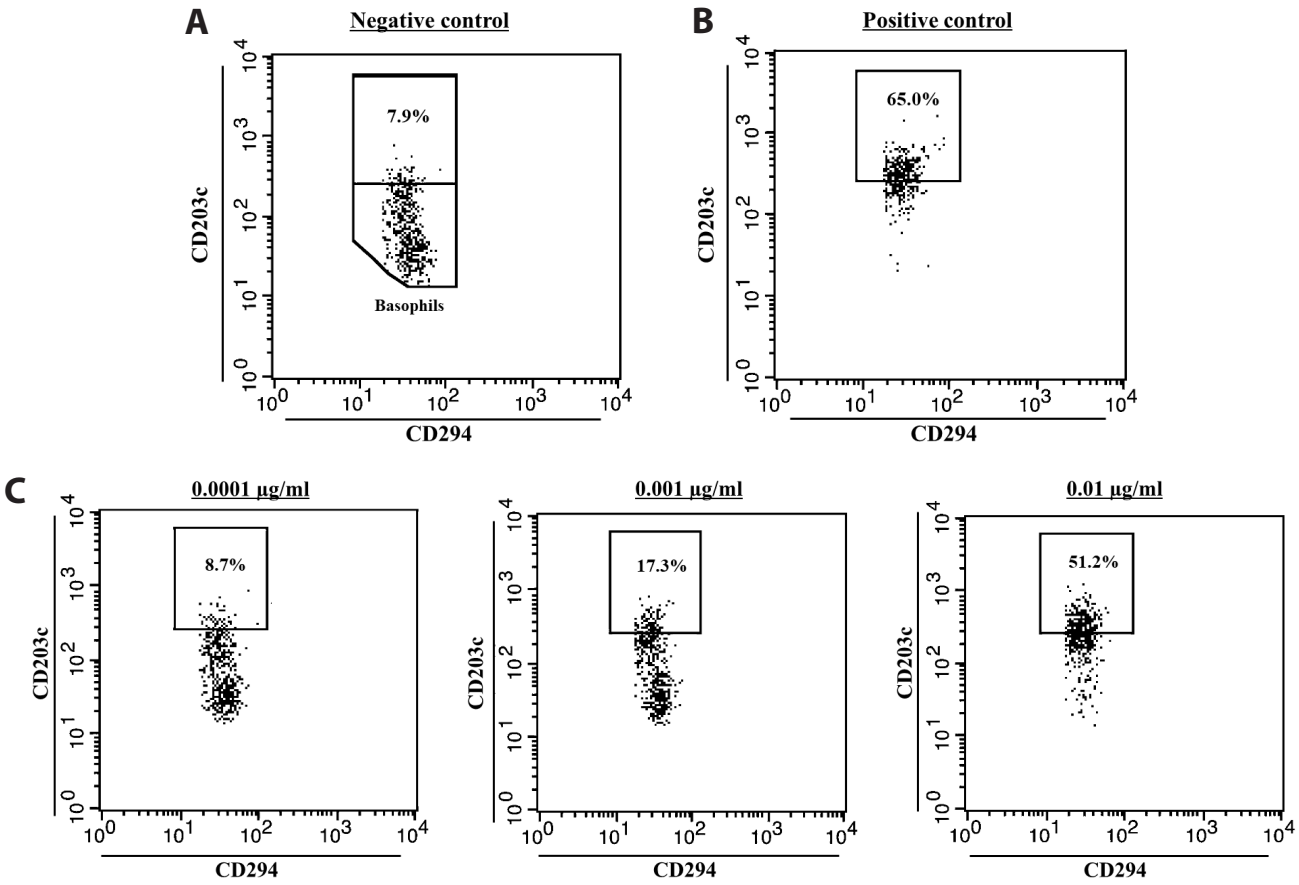
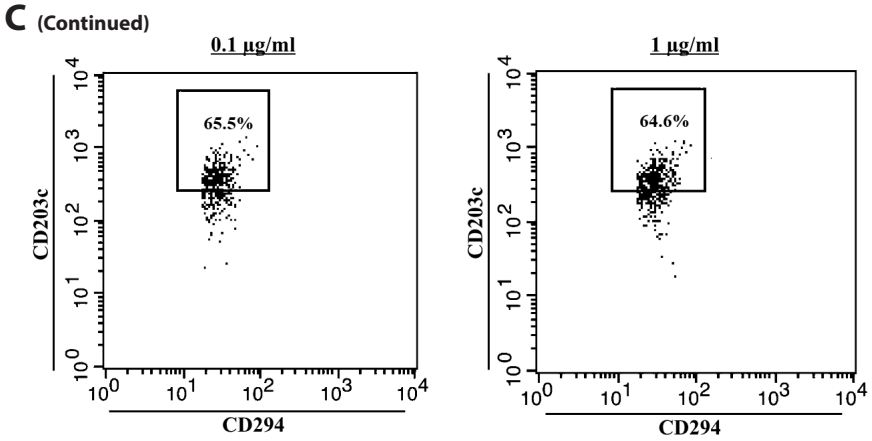


Figure 2. Flow cytometric analysis of a representative beekeeper (example of a positive threshold of 0.001 μg/ml). The gating strategy is shown.
A: Negative control. Dots show the expression of CD294 and CD203c without the expression of CD3.
B: Positive control. Dots show the expression of CD295 and CD203c without the expression of CD3.
C: Dots show the expression of CD295 and CD203c without the expression of CD3 for each honey bee venom concentration (0.0001–1 μg/ml).



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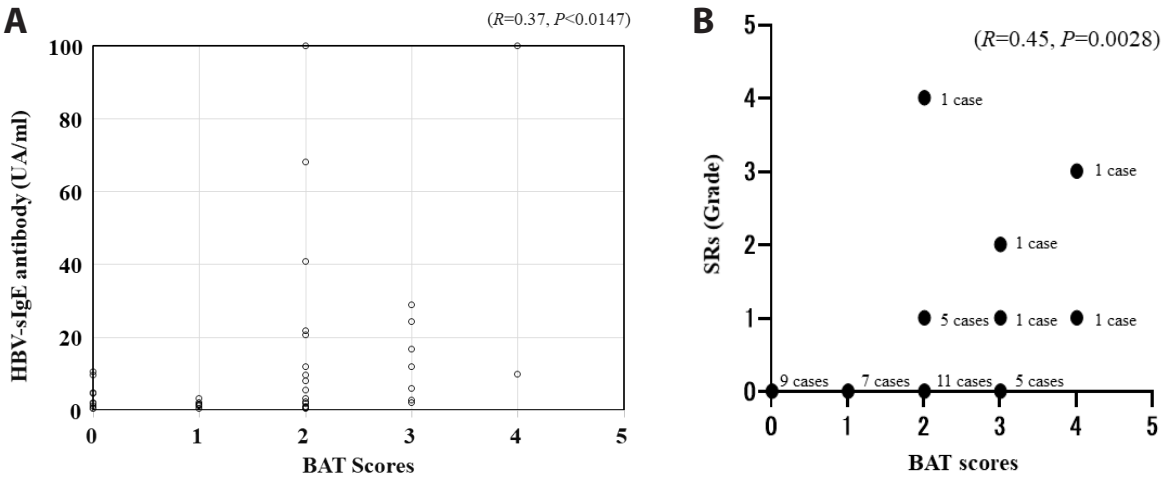

Figure 3.
A: Correlations between the levels of HBV-sIgE and BAT scores. *P* values < 0.05 were considered significant. N = 42.
B: Correlation coefficients between severity (Mueller grade) of SRs and BAT scores. *P* values < 0.05 were considered significant. N = 42 (SR+, 10; SR−, 32).

Correlation coefficient between severity of SRs and BAT scores

There was a significant positive correlation coefficient (R = 0.45, P = 0.0028) between severity (Mueller grade) of SRs and BAT scores (**Figure 3B**).

ROC analysis of SRs with different BAT positivity thresholds

Table 2 shows the ROC analysis of SRs with different (1 μg/ml, 0.1 μg/ml, and 1 μg/ml) BAT positivity thresholds. At a BAT concentration of 0.1 μg/ml (95% confidence interval; 60.4–89.6, P = 0.018), the sensitivity was 100%, the specificity was 50.0%, the positive predictive value was 38.5%, and the negative predictive value was 75.0%.

Table 2. ROC analysis of SRs with different (1 μg/ml, 0.1 μg/ml, and 0.01 μg/ml) BAT positivity thresholds. *P* values < 0.05 were considered significant. N = 10.

ROC analysis of SRs with different BAT positive with thresholds			
	BAT positive with thresholds		
	Concentration of honey bee venom		
	1 μg/ml	0.1 μg/ml	0.01 μg/ml
Sensitivity, %	100	100	40.0
Specificity, %	31.3	50.0	84.4
Positive predictive value, %	31.3	38.5	44.4
Negative predictive value, %	100	100	81.8
Area under the curve, %	65.5	75.0	62.2
95% Confidence interval	48.5-82.8	60.4-89.6	40.9-83.5
P value	0.140	0.018	0.249

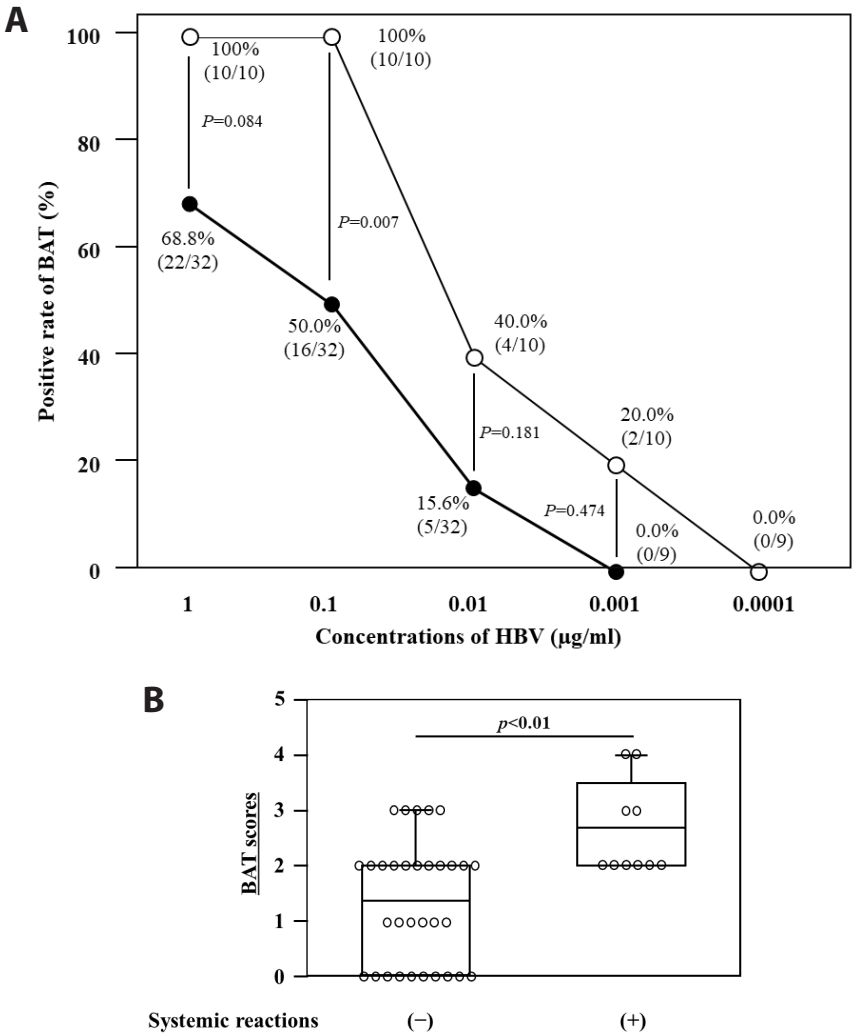

Figure 4.

A: BAT positive rate at each HBV concentration (0.0001, 0.001, 0.01, 0.1, and 1 μg/ml) for beekeepers with and without SRs. The number in parentheses is the total number of positive cases/measurements. White circles, SRs (−); black circles; SRs (+). *P* values < 0.05 were considered significant. N = 42.

B: Results of BAT on the occurrence of systemic reactions due to honey bee stings in beekeepers sensitized to honey bee venom. Systemic reactions (+); N = 10, systemic reactions (−); N = 32. Data are presented as mean ± standard deviation. *P* values < 0.05 were considered significant. Scored positivity threshold to honey bee venom; Score 0: negative with 1 μg/ml, Score 1: 1 μg/ml, Score 2: 0.1 μg/ml, Score 3: 0.01 μg/ml, Score 4: 0.001 μg/ml, Score 5: 0.0001 μg/ml. Circles are shown as individual data.

BAT positive rate at each HBV concentration for beekeepers with and without SRs

Figure 4A shows the BAT positive rate at each HBV concentration (0.0001, 0.001, 0.01, 0.1, and 1 μg/ml) for beekeepers with and without SRs. A significant difference (P = 0.007) in the positive rate of BAT was observed depending on the presence (100%; 10/10 total number) or absence (50.0%; 16/32 total number) of SRs at an HBV concentration of 0.1 μg/ml.

Comparisons between the occurrence of SRs and BAT scored with positive threshold

Figure 4B compares the occurrence of SRs and BAT scored with positive threshold. The mean score (2.6 ± 0.8 vs 1.4 ± 1.1) in BAT positivity threshold for beekeepers who developed SRs was significantly lower than that for beekeepers with no history of SRs (P < 0.01). In addition, all 10 beekeepers who developed SRs had positive BAT thresholds below 0.1 μg/ml.

BAT score of 18 beekeepers with class 0 HBV-sIgE

Of the 61 beekeepers who participated in this study, the results of 18 with class 0 HBV-sIgE who were thought to be non-sensitized or desensitized to HBV had no history of SRs. In addition, the positive BAT threshold was ≥ 1 μg/ml for 17 beekeepers and 0.1 μg/ml for 1 beekeeper; this beekeeper had been stung by a honey bee 2 weeks before, and the sIgE level was 0.14 UA/ml, suggesting that either the sIgE was consumed in an antigen–antibody reaction or possibly that the beekeeper was sensitized.

Discussion

We showed that approximately 70.5% (43/61 beekeepers) of these Japanese beekeepers were sensitized to HBV. In addition, 25.6% (11/43 beekeepers) of beekeepers sensitized to HBV had experienced SRs. A significant difference in the BAT positive rate was observed depending on the presence or absence of SRs at an HBV concentration of 0.1 μg/ml. At this BAT concentration, sensitivity was 100% and specificity was 50.0%. Furthermore, there was a positive correlation between the severity of SRs and BAT scores. The mean scores in BAT positivity threshold for beekeepers who developed SRs was significantly lower than that for beekeepers with no history of SRs. In addition, beekeepers who developed SRs had positive BAT thresholds below 0.1 μg/ml. In contrast, the positive BAT threshold was ≥ 1 μg/ml in 17 beekeepers who were thought to be non-sensitized or desensitized to HBV and had no history of SRs.

Approximately 27% of beekeepers sensitized to HBV experienced SRs, similar to our previous study.[8] Women experienced SRs more frequently than men, likely because women do less fieldwork and thus get stung less and may not become desensitized. The high levels of HBV-sIgE in beekeepers who had SRs was attributed to their lack of desensitization to HBV. Previous studies measured the stimulating concentration of HBV in BAT from 1 to 0.01 μg/ml,[13,14] whereas we measured it from 1 to 0.0001 μg/ml, and the positive threshold for all beekeepers was ≥ 0.001 μg/ml; the positive threshold for all beekeepers with a history of SRs was ≤ 0.1 μg/ml. About 40% of beekeepers with a positive BAT threshold ≤ 0.1 μg/ml had SRs after HBS. In addition, we showed that at a concentration of 0.1 μg/ml in BAT, there was a difference in the incidence of SRs in HBS and that the sensitivity was high. For beekeepers with a positive BAT threshold ≤ 0.1 μg/ml, there may be a connection with the occurrence of SRs due to HBS. All beekeepers with a positive BAT threshold ≥ 1 μg/ml had no history of SRs due to HBS. Furthermore, the BAT positivity threshold for each beekeeper with a grade II or III reaction was ≤ 0.001 μg/ml. Our analysis suggests that there may be a correlation between severity and BAT. Thus, the BAT threshold may be related to the severity and the presence or absence of SRs.

Many beekeepers with a negative BAT threshold of 1 μg/ml had multiple HBS each year. Therefore, the positive threshold may have increased due to natural desensitization to HBV, similar to allergen immunotherapy using HBV extract. About half of the beekeepers with no history of SRs due to HBS had positive BAT thresholds of 0.01 and 0.1 μg/ml. Compared with beekeepers with a history of SRs due to HBS, the results were considered individual differences in the amount of histamine released from basophils and reactivity to histamine receptors. Furthermore, we found that a beekeeper with class 0 HBV-sIgE had a BAT positivity threshold of 0.1 μg/ml. This beekeeper had been stung by a honey bee 2 weeks before, and the specific IgE antibody level was 0.14 UA/ml, suggesting either that the sIgE was consumed in an antigen–antibody reaction or that the beekeeper was possibly sensitized. In addition, we analyzed the difference in BAT scores by dividing the beekeepers according to whether their most recent sting was within the past 6 months, but no significant difference was found. As a result, it was considered that the BAT scores may not be affected by the timing of the most recent HBS (within the last 6 months).

Allergen immunotherapy is effective in patients with HBV allergies, but discontinuing it increases the risk of developing anaphylaxis following Hymenoptera stings.[15] Because beekeepers may experience HBS on a daily basis, they often become desensitized to HBV similar to allergen immunotherapy.[16] Similarly, beekeepers who have not experienced HBS for a period of time may develop anaphylaxis when they are stung again. BAT has been reported to be useful for confirming the effectiveness of allergen immunotherapy in patients with venom allergies.[4] By measuring BAT regularly, beekeepers may be able to take measures to prevent anaphylaxis (e.g., prescribing an adrenalin auto-injector, and implementation or resumption allergen immunotherapy) if a change in the positive threshold is detected. In this study, we reported the retrospective results of investigating the presence or absence of a history of SR onset before BAT measurement. In the future, it is necessary to investigate the prospective relationship with the occurrence of SR after BAT measurement.

In conclusion, analysis of the positive threshold of BAT in Japanese beekeepers naturally sensitized to HBV may be a useful tool for predicting the occurrence of SRs.

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Authors' contributions

- H. Hirokawa and H. Hirata carried out the study, analyzed the data, and wrote the manuscript.
- M.A. designed the study and helped to write the manuscript.
- Y.H. performed the statistical analysis.
- Y.F. designed the study and reviewed the manuscript.
- All authors read and approved the final manuscript.

Ethical approval

This study was approved by the Research Ethics Committee of Saitama Medical Center, Dokkyo Medical University (authorization No. 23006).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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