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Utility of the immunoglobulin E crosslinking-induced luciferase expression assay in buckwheat allergy diagnosis

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Abstract
Background: Buckwheat allergy carries a high risk of anaphylaxis. Although the oral food challenge (OFC) is the diagnostic gold standard, it can provoke severe reactions, leading many patients to adopt elimination diets. Conventional markers, including buckwheat-specific IgE (Bw-sIgE) and skin prick testing, have limited accuracy. The IgE crosslinking-induced luciferase expression (EXiLE) assay is a serum-based in vitro cell-activation test that has shown high accuracy for other food allergies. We evaluated its utility for buckwheat allergy.
Methods: This multicenter observational study in Japan enrolled participants with buckwheat-specific IgE ≥ 0.35 U_A/mL (n = 25), categorized as buckwheat allergy (BwA) or non-buckwheat allergy (non-BwA) based on OFC results. The EXiLE assay used crude buckwheat antigen at 0.1-1000 ng/mL. Diagnostic performance was compared using receiver operating characteristic (ROC) curves for EXiLE, Bw-sIgE, and the Bw-sIgE/total IgE ratio.
Results: Among 25 participants, the EXiLE assay demonstrated superior diagnostic performance compared to Bw-sIgE and the Bw-sIgE/total IgE ratio. The area under the ROC curve (AUC) was 0.96 at 10 ng/mL (1 ng/mL, 0.94), exceeding Bw-sIgE (0.68) and the Bw-sIgE/total IgE ratio (0.72). At optimal cutoffs, EXiLE achieved sensitivity 0.92, specificity 0.92, and LR+ 11.1 (versus LR+ 3.2 for Bw-sIgE and 2.5 for the IgE ratio), indicating fewer false positives and better overall accuracy.
Conclusion: The EXiLE assay provides high diagnostic accuracy for IgE-mediated buckwheat allergy and, as a serum-based in vitro assay, may reduce reliance on OFC and help avoid unnecessary elimination diets.

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Introduction

Buckwheat is traditionally consumed in several Asian countries and is also eaten in parts of Europe, particularly in Central and Eastern Europe and northern Italy.[1] In a multicenter Italian survey, buckwheat sensitization was detected in 3.6% of patients attending allergy clinics, with regional prevalences ranging from 2.2–4.5% and anaphylaxis accounting for over 20% of identified clinical cases.[2] Buckwheat is therefore recognized as an important allergen capable of causing severe, potentially life-threatening reactions, including anaphylaxis, with reports from Europe and Asia.[2-4] In Japan, a Consumer Affairs Agency survey found that buckwheat accounted for only 1.1% of immediate-type food allergies.[5] Nevertheless, a nationwide database of food-induced anaphylaxis ranked buckwheat as the fifth most common trigger.[4] This apparent discrepancy between prevalence and clinical severity underscores the need for accurate diagnostic tools and robust management strategies for buckwheat allergy.

The oral food challenge (OFC) is the gold standard for confirming the diagnosis of food allergy;[6] however, it carries the risk of severe allergic reactions, such as anaphylaxis.[6] One report noted that more than half of the patients who tested positive for buckwheat in the OFC developed anaphylaxis.[7] In clinical practice, many patients with buckwheat allergy avoid the OFC for fear of triggering severe symptoms, choosing instead to eliminate buckwheat from their diet.[8]

Serum buckwheat-specific immunoglobulin E (Bw-sIgE) testing has limited sensitivity (66.7%) and specificity (54.6%).[9] Although skin prick testing may have higher accuracy, there is a risk of systemic reactions, creating a need for safer and more accurate diagnostic methods.[9-11] Approaches such as quantifying Fag e 3 (a buckwheat allergen component) or calculating the ratio of Bw-sIgE to total IgE may improve diagnostic precision.[8,12] Among in vitro diagnostic methods, the basophil activation test (BAT) has been recognized as a highly sensitive assay that measures upregulation of activation markers (e.g., CD63 and CD203c) on patients' basophils by flow cytometry. However, because BAT requires fresh whole blood and intact basophil responsiveness, it must be performed on the same day as blood collection, which limits its practicality.[13]

In contrast, the EXiLE assay uses RS-ATL8 mast cells engineered to express human FcεRI and a luciferase reporter under the control of a nuclear factor of activated T cells (NFAT)-dependent promoter. Patient serum passively sensitizes the cells via bound IgE; upon stimulation with the relevant allergen, antigen-specific IgE is cross-linked, triggering FcεRI-dependent activation and leading to luciferase expression, which is quantified as chemiluminescence.[14,15] Thus, EXiLE provides a serum-based, batched or retrospective alternative that probes the same IgE-dependent activation axis without the need for fresh basophils. EXiLE, a cell-based functional IgE assay, has also been reported to be useful in diagnosing other IgE-mediated food allergies.[14,16] In this study, we investigated the potential utility of the EXiLE assay for diagnosing buckwheat allergy.

Materials and Methods

Study participants

This multicenter observational study was conducted at four hospitals in Japan and performed in accordance with the Declaration of Helsinki. Ethical approval was obtained from the Fujita Health University Ethics Committee (Reference number: HM25-045), which covered all participating sites.

Among the cases in which informed consent for sample use was obtained from the patient and/or their parents, individuals with Bw-sIgE levels ≥0.35 U_A/mL were divided into a buckwheat allergy group (BwA) and a non-buckwheat allergy group (non-BwA) (Table 1). The BwA group included individuals who met at least one of the following criteria: (i) an immediate allergic reaction confirmed by a buckwheat OFC; or (ii) clear immediate-type allergic reactions occurring within ≤2 h of buckwheat ingestion during the 5 years preceding sample collection. The non-BwA group consisted of those who could ingest ≥1200 mg of buckwheat protein without developing symptoms during an OFC. No significant differences in age or total IgE levels were observed between the BwA and non-BwA groups. Most participants in both groups had a history of allergy-related conditions, such as atopic dermatitis, asthma, allergic rhinitis, or other food allergies.

Table 1 Characteristics of the study participants in the BwA and non-BwA groups.

	BwA (n = 12)	Non-BwA (n = 13)	P
Sex (F/M): number	4/8	4/9	1.00
Age (y): median [range]	7.5 [4–21]	9 [3–14]	0.83
Atopic dermatitis % (number)	50 (6)	84.6 (11)	0.10
Asthma % (number)	50 (6)	30.8 (4)	0.43
Allergic rhinitis % (number)	75 (9)	100 (13)	0.10
Other food allergies % (number)	75 (9)	92.3 (12)	0.32
Total IgE (IU/mL): median [range]	1051.6 [198–2740.4]	2080 [139–2818]	0.15

BwA, buckwheat allergy group; F, female; IgE, immunoglobulin E; M, male; non-BwA, non-buckwheat allergy group; y, years. Data are presented as n (%) for categorical variables and as median [range] for continuous variables. P values were obtained using Fisher's exact test for categorical variables and the Mann–Whitney U test for continuous variables; two-sided $P < 0.05$ was considered statistically significant. Percentages were calculated using group-specific denominators.

Bw-sIgE was measured by ImmunoCAP and reported in U_A/mL; total IgE was reported in IU/mL.

After collection, sera were promptly aliquoted and then stored at −30°C until testing. Each aliquot underwent no more than two freeze–thaw cycles. The median interval between the most recent OFC (or index reaction for BwA without OFC) and serum sampling was 10 months (IQR 5–16 months; range 1 week–51 months). EXiLE measurements were performed on frozen aliquots.

Antigen extraction

Antigens were extracted as described by Yamada et al.[17] Buckwheat flour harvested from Nagano Prefecture, Japan, was the antigen source. One gram of flour was mixed with 50 mL of diethyl ether and shaken at room temperature for 2 h to defat the flour. The defatted powder (1 g) was then mixed with 10 mL of Coca solution (containing 85 mM NaCl, 32.7 mM $NaHCO_3$, 42.5 mM phenol, and distilled water up to 1 L) and stirred at room temperature for 48 h. After centrifugation for 30 min, the supernatant was filtered through a 0.45 μm filter, dialyzed against phosphate-buffered saline (PBS; 10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4), and lyophilized.

EXiLE method

The EXiLE assay was performed using crude buckwheat antigen following the procedure described by Nakamura et al.[15] Rat mast cells (RS-ATL8) stably expressing the high-affinity human IgE receptor (FcεRI) were seeded into tissue culture flasks (CORNING Flask 430372, Corning Incorporated, Corning, NY, USA) and incubated at 37°C under 5% CO_2. Cultured cells (1.0×10^6 cells/mL) were incubated with patient serum at a 1:100 dilution. Next, 1.0×10^4 cells in 50 μL/well were seeded into 96-well white plates with transparent bottoms (IsoPlate-96 TC, PerkinElmer, Waltham, MA, USA) and incubated overnight at 37°C under 5% CO_2.

After washing the cells three times with PBS, various concentrations of buckwheat antigen solution (0.1–1000 ng/mL) were added to the sensitized cells (50 μL/well), followed by incubation for 3 h at 37°C under 5% CO_2. After stimulation, 50 μL of luciferase substrate solution (ONE-Glo, Promega Corp., Tokyo, Japan) was added to each well, and chemiluminescence was measured using a Centro XS^3 LB 960 microplate luminometer (Berthold Technologies, Bad Wildbad, Germany). Luciferase expression levels were calculated by first subtracting the blank (no cells) from the measured luminescence in each well and then calculating the ratio of the luminescence in each antigen-containing well to that of non-stimulated control cells (cells + serum only, no antigen). Each sample was measured in duplicate, and the assay was repeated three times, with the mean values used for analysis.

Statistical analysis

Continuous variables were summarized as medians with ranges and compared using the Mann–Whitney U test. Categorical variables were summarized as counts and percentages and compared using Fisher's exact test. Receiver operating characteristic (ROC) curves were generated for Bw-sIgE, the Bw-sIgE/total IgE ratio, and the EXiLE assay at each antigen concentration. Areas under the ROC curve (AUCs) and their 95% confidence intervals were estimated using DeLong's method. The optimal cutoff for each marker was determined by maximizing Youden's J statistic (J = sensitivity + specificity − 1). At the selected cutoff, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (LR+), and the corresponding contingency counts were calculated. When a zero-cell occurred in the contingency table, the Haldane–Anscombe correction (adding 0.5 to each cell) was applied for likelihood-ratio calculations. All tests were two-sided, and $P < 0.05$ was considered statistically significant. Analyses were performed using GraphPad Prism version 10.5 (GraphPad Software, San Diego, CA, USA) for data visualization and basic summaries, and R (R Foundation for Statistical Computing, Vienna, Austria) with the pROC package (via EZR, Saitama Medical Center, Jichi Medical University, Saitama, Japan) for ROC analyses and DeLong procedures. In the EXiLE assay, "fold" was defined as the ratio of the stimulated luminescence value to the nonstimulated control value.

Results

No significant differences were observed between the two groups in Bw-sIgE levels or in the Bw-sIgE/total IgE ratio. In contrast, EXiLE assay values were significantly higher in the BwA than in the non-BwA group across antigen concentrations of 0.1–1000 ng/mL, with the largest between-group differences at 1 and 10 ng/mL (Figure 1).

Diagnostic performance was compared using ROC curves for Bw-sIgE, the Bw-sIgE/total IgE ratio, and the EXiLE assay (Figure 2). Across all tested antigen concentrations (0.1–1000 ng/mL), the AUC of the EXiLE assay exceeded those of both Bw-sIgE and the Bw-sIgE/total IgE ratio. Among the tested concentrations, 10 ng/mL yielded the largest AUC (0.96), followed by 1 ng/mL (0.94). Using optimal cutoffs derived from ROC curves, we calculated sensitivity, specificity, PPV, NPV, and LR+ for each test (Table 2). At 1 and 10 ng/mL, the EXiLE assay cutoffs were 1.07-fold and 1.16-fold, respectively, producing identical sensitivity and specificity (both 0.92) with PPV 0.92, NPV 0.92, and LR+ 11.1. For context, LR+ was lower for Bw-sIgE and for the Bw-sIgE/total IgE ratio (3.2 and 2.5, respectively; Table 2).

Discussion

In this study, we focused on patients with detectable Bw-sIgE (≥0.35 U_A/mL) and compared the diagnostic utility of the EXiLE assay with that of Bw-sIgE and the Bw-sIgE/total IgE ratio using ROC curves. The EXiLE assay demonstrated superior diagnostic performance compared to Bw-sIgE and the Bw-sIgE/total IgE ratio, with the largest AUC at 10 ng/mL (0.96), followed by 1 ng/mL (0.94), and may reduce false positives, thereby minimizing unnecessary

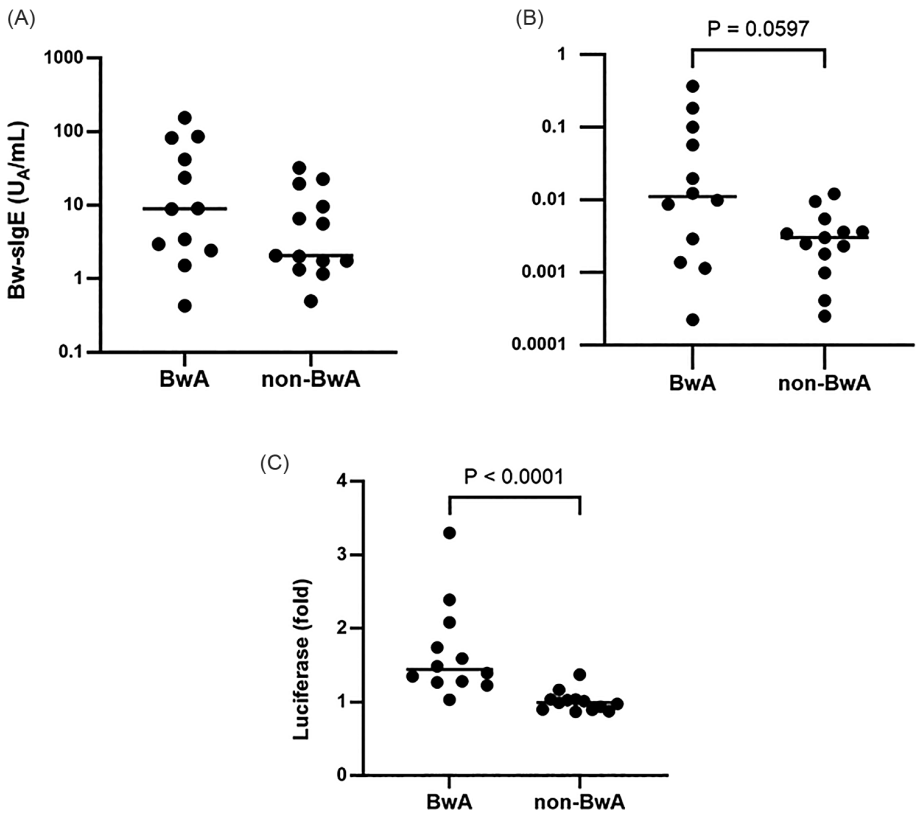

Figure 1 (A) Bw-sIgE, (B) the Bw-sIgE/total IgE ratio, and (C) EXiLE assay luminescence (fold) in the BwA and non-BwA groups. The EXiLE assay used RS-ATL8 cells sensitized with 1:100 diluted serum and stimulated with crude buckwheat antigen at concentrations ranging from 0.1 to 1000 ng/mL; panel c shows the responses at 10 ng/mL. Each dot represents one participant; horizontal bars indicate medians. P values were calculated using the two-sided Mann–Whitney U test; $P < 0.05$ was considered significant. "Fold" denotes the ratio of the stimulated luminescence value to the nonstimulated control value. BwA, buckwheat allergy; Bw-sIgE, buckwheat-specific serum IgE; EXiLE, IgE crosslinking-induced luciferase expression; non-BwA, non-buckwheat allergy.

buckwheat elimination and improving patients' quality of life (QOL).[18]

The EXiLE assay utilizes rat mast cells (RS-ATL8) stably expressing the human high-affinity IgE receptor FcεRI and a luciferase reporter gene activated by IgE crosslinking. Compared with conventional enzyme immunoassays, it is less prone to false-positive results.[14,16] In addition, because EXiLE relies on cultured rat mast cells rather than fresh whole blood, it is not influenced by nonresponsive basophils (as in BAT). It can be performed using frozen serum, allowing for retrospective analyses.

In the non-BwA group, only one case exceeded the EXiLE cutoff at 1 and 10 ng/mL and was considered a false positive. One possible cause of a false positive result is the presence of IgG in the serum, which acts as a neutralizing antibody and prevents antigen binding to IgE.[19] In our study, when this sample was retested without the usual washing steps, which likely preserved neutralizing antibodies, luminescence decreased at all tested antigen concentrations (0.1–1000 ng/mL), and the values fell below the cutoff at 1 and 10 ng/mL (Figure 3). These findings suggest that neutralizing antibodies may have influenced the initial false-positive results.

In the BwA group, only one case showed values below the EXiLE cutoff at 1 and 10 ng/mL and was deemed a false negative. Although the luminescence at 1 and 10 ng/mL did not exceed the cutoff, it was still higher than that observed in the nonstimulated control. Furthermore, the luminescence at 0.1, 100, and 1000 ng/mL exceeded the cutoff value. A possible explanation for this is that low Bw-sIgE levels reduce the reactivity in the EXiLE assay.[15] In this case, the Bw-sIgE was relatively low (1.52 U_A/mL); however, some BwA patients in our study with even lower Bw-sIgE levels tested positive by EXiLE, indicating that the relatively low IgE level of this patient may not fully explain the false-negative results. Clinically, this patient tolerated up to 15 g of buckwheat before experiencing Grade 2 respiratory symptoms. This threshold was not particularly low, and the symptoms were not severe. Further studies are needed to clarify whether variations in the concentration at which luminescence peaks, the threshold for symptom induction, and clinical severity correlate with EXiLE luminescence patterns.

Kajita et al. reported that the Bw-sIgE/total IgE ratio (cutoff 0.0058) achieved an AUC of 0.89 with 90% sensitivity and 82% specificity, indicating higher diagnostic utility than Bw-sIgE alone.[12] In the present study, the same ratio (cutoff 0.0055) showed a lower AUC (0.72), sensitivity (0.85), and specificity (0.67). Although the median Bw-sIgE levels in our cohort were almost identical to those reported by Kajita et al. (BwA: 9.01 U_A/mL vs. 9.38 U_A/mL; non-BwA: 2.08 U_A/mL vs. 3.58 U_A/mL), total IgE values

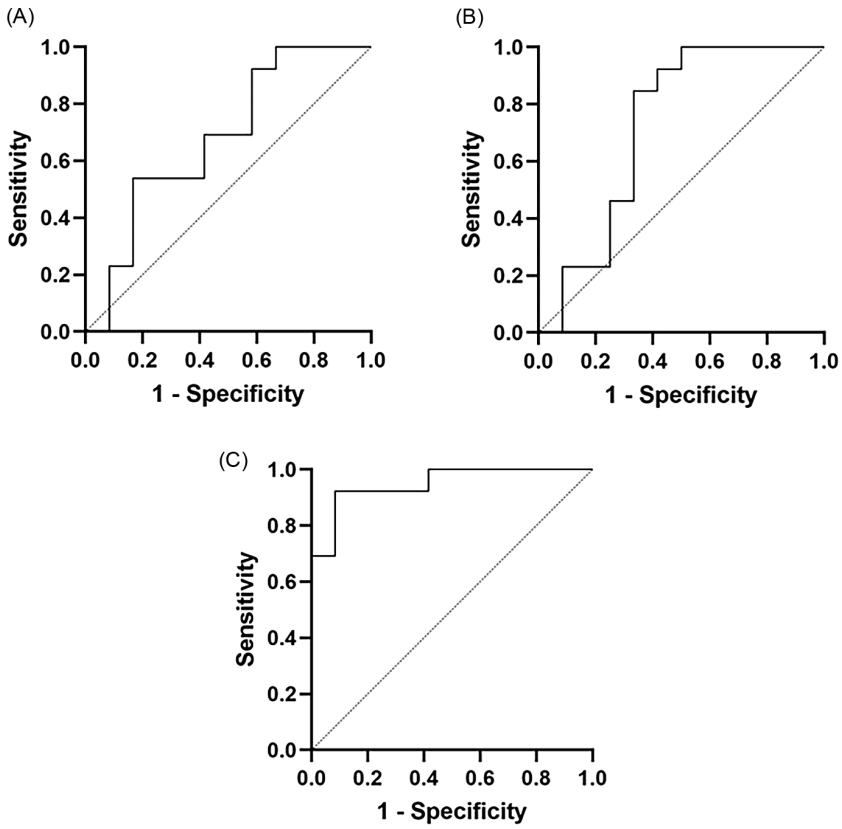

Figure 2 ROC curves discriminating BwA from non-BwA for (A) Bw-sIgE, (B) the Bw-sIgE/total IgE ratio, and (C) the EXiLE assay. The EXiLE assay used RS-ATL8 cells sensitized with 1:100 diluted serum and stimulated with crude buckwheat antigen; panel C shows the ROC curve for 10 ng/mL. The diagonal line denotes chance performance. AUC (95% CI) values are summarized in Table 2. ROC, receiver operating characteristic; AUC, area under the ROC curve; BwA, buckwheat allergy; Bw-sIgE, buckwheat-specific serum IgE; CI, confidence interval; EXiLE, IgE crosslinking-induced luciferase expression; non-BwA, non-buckwheat allergy.

Table 2 Diagnostic performance of each test for buckwheat allergy.

Test	AUC (95% CI)	P	Cutoff	Sens	Spec	PPV	NPV	LR+
ImmunoCap (RW)	0.68 (0.46–0.90)	0.14	2.08 (U_A/mL)	0.54	0.83	0.63	0.78	3.2
Bw-sIgE/total IgE	0.72 (0.50–0.95)	0.060	0.0055	0.85	0.67	0.80	0.73	2.5
EXiLE (BW, 0.1 ng/mL)	0.88 (0.75–1.00)	0.0015	1.02 (fold)	0.77	0.92	0.79	0.91	9.2
EXiLE (BW, 1 ng/mL)	0.94 (0.85–1.00)	< 0.001	1.07 (fold)	0.92	0.92	0.92	0.92	11.1
EXiLE (BW, 10 ng/mL)	0.96 (0.89–1.00)	< 0.001	1.16 (fold)	0.92	0.92	0.92	0.92	11.1
EXiLE (BW, 100 ng/mL)	0.87 (0.70–1.00)	< 0.001	1.12 (fold)	0.85	1.00	0.86	1.00	21.3
EXiLE (BW, 1000 ng/mL)	0.78 (0.58–0.99)	0.016	1.26 (fold)	0.69	0.92	0.73	0.90	8.3

AUC, area under the ROC curve; Bw-sIgE/total IgE, ratio of buckwheat-specific IgE to total IgE; CI, confidence interval; ImmunoCAP (BW), serum buckwheat-specific IgE measured by ImmunoCAP; LR+, positive likelihood ratio; NPV, negative predictive value; PPV, positive predictive value; Sens, sensitivity; Spec, specificity.
Data are shown as point estimates (95% CI) for AUCs and sensitivity, specificity, PPV, NPV, and LR+. For AUC, 95% CIs were calculated using DeLong's method. LR+ values were computed from exact contingency counts, and when a zero-cell occurred in the contingency table, the Haldane–Anscombe correction (adding 0.5 to each cell) was applied. "Fold" denotes the ratio of the stimulated luminescence value to the non-stimulated control value. P values were obtained using Fisher's exact test for categorical variables and the Mann–Whitney U test for continuous variables; two-sided $P < 0.05$ was considered significant.

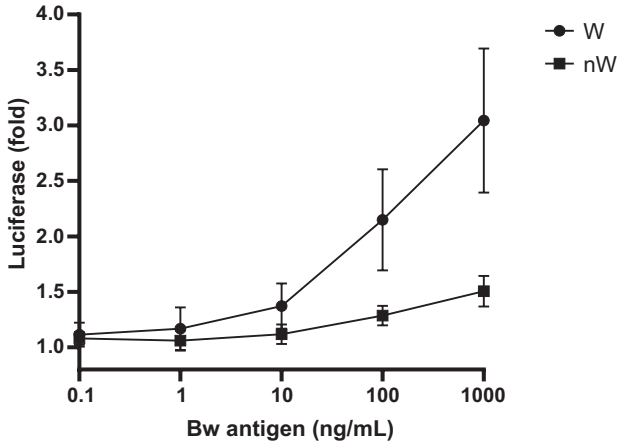

Figure 3 EXiLE assay luminescence for a representative sample that initially yielded a false-positive result under standard washing (W) and no-wash (nW) conditions. RS-ATL8 cells were sensitized with serum diluted 1:100 and stimulated with crude buckwheat antigen across 0.1–1000 ng/mL. The y-axis shows luminescence as fold relative to the non-stimulated control; points indicate mean ± SD from three independent experiments (each in duplicate). Signals were lower under nW, consistent with retention of neutralizing antibodies; at 1 and 10 ng/mL, the responses fell below the respective cutoffs (1.07-fold and 1.16-fold), converting the classification from positive to negative. EXiLE, IgE crosslinking-induced luciferase expression; W, standard washing; nW, no-wash.

tended to be higher in both groups (BwA median 1051 IU/mL; non-BwA 2080 IU/mL). Furthermore, the prevalence of atopic dermatitis and bronchial asthma was higher in our patients (68 and 40%, respectively) than in the previous report (38 and 19%, respectively). Because atopic dermatitis and asthma elevated the total IgE,[20] inflation of the denominator in the Bw-sIgE/total IgE ratio likely lowered the ratio, thereby increasing false negatives and reducing AUC and specificity. Consistent with this interpretation, all four false-negative cases in the present study had atopic comorbidities and high total IgE levels.

Compared with previously reported markers,[8,12] the buckwheat EXiLE assay at 10 ng/mL achieved the highest AUC (0.96), followed by 1 ng/mL (0.94). Both exceeded the previously reported AUCs for Fag e 3-specific IgE and the Bw-sIgE/total IgE ratio (each 0.89). These findings suggest that, among patients who test positive for Bw-sIgE, the buckwheat EXiLE assay, particularly at 10 ng/mL, could be highly useful for diagnosing buckwheat allergy.

In previous studies, participants with suspected buckwheat allergy underwent the OFC, and allergy-positive and -negative groups were compared. Here, we specifically examined patients who were already positive for Bw-sIgE, due to concerns regarding false-positive serological results. As we did not investigate patients who were negative for Bw-sIgE, our target population differed from previous studies, making direct comparisons somewhat challenging. Despite this, we believe that our study design more closely reflects real-world clinical practice. Because SPT carries a low yet well-documented risk of systemic reactions,[9,10] we did not perform additional SPT in this study. In future prospective work, we plan to include SPT for non-BwA participants once informed consent is obtained. Previous studies reported higher sensitivity and specificity for Fag e 3-specific IgE than for Bw-sIgE in diagnosing buckwheat allergy.[12] In future studies, we aim to adapt the EXiLE assay to include Fag e 3-specific IgE levels. Beyond these design considerations, this study is limited by its modest sample size (n = 25) and the absence of an a priori power calculation, which widens confidence intervals and reduces the precision of performance estimates; therefore, any subgroup analyses should be regarded as exploratory. Finally, our cohort comprised sIgE-positive patients recruited at Japanese centers, which may limit generalizability to other clinical settings, referral patterns, or regions; external validation in independent cohorts will be required to establish the robustness and transportability of the proposed EXiLE cutoffs.

From an implementation standpoint, the EXiLE assay requires access to the RS-ATL8 FcεRI-expressing reporter mast cell line, standard cell culture facilities, a luminometer, and assay standardization or quality control.[14,15] In our setting, batched testing allowed reporting of EXiLE results within 2 days once serum was available, whereas sIgE immunoassays and BAT results were typically available on the same day. BAT additionally requires the same-day fresh whole blood and is influenced by basophil responsiveness.[13] Costs vary by setting; EXiLE may exceed single-analyte sIgE yet be comparable to BAT or multicomponent panels when staffing and logistics are considered. Wider availability of EXiLE will depend on technology transfer and harmonized protocols.[14,15]

Conclusion

The EXiLE assay using crude buckwheat antigen demonstrated superior diagnostic accuracy to both Bw-sIgE and the Bw-sIgE/total IgE ratio for diagnosing IgE-mediated buckwheat allergy. Its high sensitivity and specificity, and its ability to use frozen serum samples, have significant advantages for clinical applications. This assay may minimize unnecessary dietary restrictions, improving patients' QOL and contributing to more efficient allergy management.

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Mandatory Disclosure on Use of Artificial Intelligence

The authors declare that AI-assisted tools were used as follows: ChatGPT (OpenAI; GPT-4o model) for English editing and readability. All references have been manually verified for accuracy and relevance.

Author Contributions

Toya Kono conceived and designed the study, prepared the buckwheat extract, acquired, analyzed, and interpreted the data, and drafted the manuscript. Kaoru Okamoto, Yuji Mori acquired and interpreted data and contributed to manuscript drafting and critical review. Tetsushi Yoshikawa interpreted data and critically reviewed the manuscript. Yasuto Kondo contributed to study design, data interpretation, and manuscript drafting and critical review. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no potential conflicts of interest with respect to research, authorship and/or publication of this article.

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