



Development of a light-initiated chemiluminescence assay for the quantitative detection of cow's milk allergen-specific IgE

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ABSTRACT

Background: Cow's milk allergy is a common food allergy in children. Clinically, cow's milk-specific IgE (CM-sIgE) antibody test is often used to diagnose milk allergy. An inexpensive light-initiated chemiluminescence assay (LICA), with fast detection speed and small sample volume demand, has application prospects in the field of quantitative detection of CM-sIgE.

Methods: Chemibeads coated with five major milk allergens, serum samples, biotinylated anti-human IgE antibodies, and streptavidin-coated sensibeads constitute a system to establish a LICA method for the quantitative detection of CM-sIgE. A series of experiments were performed to optimize its reaction conditions and evaluated its performance.

Results: The optimal conditions for LICA were 10:4 mass ratio of chemibeads to milk allergen, 20 µg/mL chemibeads, 1.0 µg/mL biotinylated anti-human IgE antibodies and a 1/10 dilution of serum for 30-min incubation. The limit of Quantitation (LoQ) was 0.22 kU_A/L. For repeatability, the CV ranged from 3.71% to 8.11%. For intermediate precision, the CV ranged from 4.08% to 14.71%. It was linear within 0.20–18.20 kU_A/L. This method did not interfere with common interfering substances and total IgE in serum, and there was no obvious cross-reaction with milk-specific IgG and non-milk-specific IgE.

Conclusion: We have established a method to quantitatively detect CM-sIgE based on light-initiated chemiluminescence assay, which has good analytical performance and could meet the needs of clinical laboratories.

1. Introduction

In the past 20 to 30 years, the prevalence of food allergy in the general population has continued to increase, especially among people in Western countries or highly industrialized areas. Compared with adults, food allergies are more common in children (Osborne et al., 2011; Sicherer and Sampson, 2018). Cow's milk allergy is the most common type of food allergy in infants and children (Boyce et al., 2010a; Matricardi et al., 2016). According to statistics, up to 7.5% of children suffered from milk allergy (Luyt et al., 2014). Most patients presented mild milk allergy symptoms, including urticaria, vomiting, coughing, and dizziness, but in a few cases, some patients also presented more severe clinical allergic reactions, such as anaphylactic shock (Bock et al., 2007; Fiocchi et al., 2010). About 60% of the adverse reactions caused by milk

are caused by IgE antibodies and may affect one or more organs. Typical IgE-mediated milk allergy symptoms often appeared within 1–2 h of milk intake, and in some severe cases, multiple systems could be affected at the same time, including skin, breathing, gastrointestinal system, and even systemic allergies reactions (Fiocchi et al., 2010; Kattan et al., 2011). According to guidelines, expert panels and systematic reviews, the detection of serum food-sIgE antibodies is the recommended immunoassay for the diagnosis of allergy (Boyce et al., 2010b; Muraro et al., 2014; Sampson et al., 2014). The quantitative detection of serum-sIgE antibody is one of the important methods for diagnosing food allergy, and it has guiding significance for patients (especially children) with persistent allergy and desensitization (Dolen, 2003; Furuya et al., 2016; Savage et al., 2016). Since IgE was discovered in 1967 (Johansson and Bennich, 1967), the in vitro detection of allergen sIgE antibodies

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was achieved for the first time through the radioallergosorbent test (RAST). Since then, a series of second-generation detection systems, such as fluorescence enzyme immunoassay (FEIA) and enzyme immunoassay based on membrane, appeared one after another and had been widely used. These methods effectively solved the problem of in vitro detection of sIgE antibodies with the advantages of strong binding capacity and non-radioactive labeling. However, they also have the disadvantages of complicated operation, long incubation time, and a large volume of serum required (Lee et al., 2009; Shyur et al., 2010).

Light-initiated chemiluminescence assay (LICA) is an immunoassay based on microspheres. There are two types of microspheres in the detection system, namely chemibeads and sensibeads. Under the interaction of antigen-antibody, biotin-streptavidin, the chemibeads and sensibeads are brought closer, to realize the transmission of high-energy reactive oxygen species and induce light-initiated chemiluminescence process (Ullman et al., 1994; Ullman et al., 1996; Wu et al., 2016). The LICA analysis system is a homogeneous immunoassay and provides a liquid phase three-dimensional reaction environment. It has the characteristics of high sensitivity, high throughput, and easy operation (Szekeres et al., 2008). It was reported that the detection of egg-sIgE antibodies based on LICA could achieve better performance characteristics (Bian et al., 2018). However, there is few studies about the detection of CM-sIgE antibodies by using LICA. In addition, there is few verification of the performance of LICA in detecting the allergen-sIgE from the perspective of reagent developers. Thus, in this study, we established a method for the detection of CM-sIgE based on LICA and evaluated its performance in accordance with CLSI (Clinical and Laboratory Standards Institute) standards.

2. Materials and methods

2.1. Samples

Sera from patients allergic to cow's milk ($n = 47$) were obtained from Tianjin Children's Hospital and Tianjin Port Hospital. The diagnosis of IgE-mediated cow's milk allergy was based on a convincing clinical history related to milk consumption, severe and/or acute reactions (urticaria, gastrointestinal or respiratory symptoms), and increased specific IgE levels (cow's milk-sIgE >0.35 kU_A/L). Sera from 50 non-allergic individuals (no history of allergic symptoms, negative serum cow's milk sIgE level) were used as negative controls. The clinical characteristics of the participants are shown in Table 1. All samples were stored at -80 °C until use. The study was approved by the Ethics Committee of Tianjin Children's Hospital and Tianjin Port Hospital. Informed consent was obtained from the patients' parents.

Values are expressed as numbers or medians (range).

2.2. Reagents and equipment

Five major cow's milk allergen proteins (α -lactalbumin, β -lactoglobulin, α s-casein, β -casein, κ -casein) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). The biotinylated mouse anti-human IgE antibody was purchased from Abcam (Cambridge, UK). The chemibeads and sensibeads coupled with streptavidin were obtained from Beyond Biotech (Shanghai, China). The high throughput

chemiluminescence analyzer instrument used to carry out the LICA tests was from Beyond Biotech (Shanghai, China).

2.3. Coupling of milk allergens to chemibeads

First, the chemibeads were washed twice with ddH₂O and resuspended in 0.05 M carbonate buffer (pH 9.6). Then, five milk allergens were added in equal proportions and mixed with chemibeads overnight at 37 °C. After reduction with NaBH₄ and blocking with glycine solution, the beads were resuspended in the storage solution (25 mM HEPES + 1% BSA + 0.1% ProClin-300) to a final concentration of 10 mg/mL and stored at 4 °C in the dark.

2.4. LICA procedure

The assay was conducted in 1% BSA dissolved in PBS (pH 8.0). The detection procedures were as follows: First, 25 μ L of human serum, biotinylated anti-human IgE antibody, and chemibeads coated with milk allergens were added to the microplates and incubated at 37 °C for 30 min. Then, 175 μ L of streptavidin-conjugated sensibeads was added to plates and incubated at 37 °C for 15 min in the dark. The results were read on the LICA instrument. Samples were performed in duplicate. The reaction principle of this method is shown in Fig. 1.

2.5. Optimization of reaction conditions

To obtain a highly effective CM-sIgE detection method, the reaction conditions of LICA required optimization. As factors that affect the binding of antigen and antibody, the coating ratio of milk allergens, the working concentration of chemibeads, the working concentration of biotinylated anti-human IgE antibodies, and the dilution of serum were optimized. First, two sera pools containing high and low levels of CM-sIgE were collected. The concentrations of the two sera pools were about 17.50 and 0.50 kU_A/L, respectively. The high serum pool was used to evaluate the high point of the linear range, and the low serum pool was used to evaluate the sensitivity of the method. Sera from non-allergic individuals served as negative controls. The CM-sIgE of each sample was detected according to LICA procedure under different reaction conditions. The positive sera pool and negative sera pool were designated as P and N, respectively. Positive to negative (P/N) ratios were calculated.

2.6. Establishment of the calibration curve

Because it is difficult to obtain the standard of CM-sIgE, the patient serum with a CM-sIgE concentration of 35.0 kU_A/L was selected as the calibrator. The serum was diluted to 17.5 kU_A/L, 3.5 kU_A/L, 0.7 kU_A/L,

Table 1

Characteristics of the participants according to cow's milk-allergic and non-allergic individuals.

Characteristic	Cow's milk allergic individuals ($n = 47$)	Non-allergic individuals ($n = 50$)
Age(y)	4 (1–14)	2 (1–11)
Sex: male, n (%)	24 (51)	23 (46)
Specific IgE to cow's milk (kU _A /L)	3.10(0.38–35.17)	0.00 (0.00–0.07)

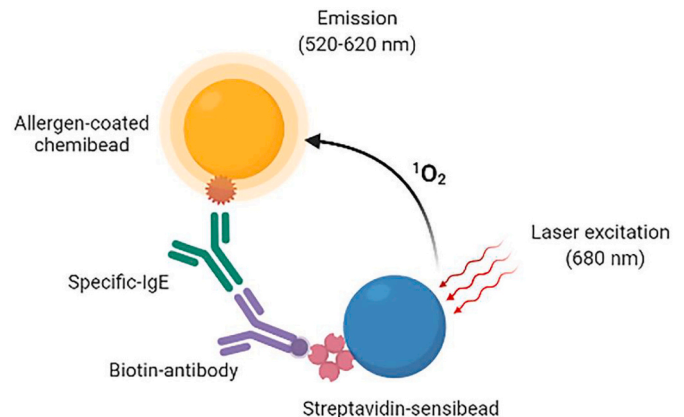


Fig. 1. The reaction principle of LICA for measuring CM-sIgE.

0.35 kU_A/L by non-allergic serum according to the method of ImmunoCAP to establish a calibration curve.

2.7. Performance validation

The method was validated according to key elements and recommendations defined by CLSI.

2.7.1. Imprecision

According to the clinical guideline documents CLSI-EP5-A2 (CLSI, 2004a, 2004b), two serum samples with different concentrations of CM-sIgE were selected for precision verification. For the repeatability assay, two serum samples were measured 10 consecutive times a day. For intermediate precision, two serum samples were repeatedly measured for 10 consecutive days. Two batches were tested every day, with two replicates for each batch, for a total of 40 replicates. The mean, standard deviation (SD), and coefficient of variation (CV) were calculated.

2.7.2. Limit of blank, limit of detection, limit of quantitation

According to the clinical guideline documents CLSI-EP17-A (CLSI, 2004), four non-allergic serums were used as blank samples to be tested 15 times within three days to get limit of blank (LoB). To obtain the limit of detection (LoD), four low-level samples with CM-sIgE concentration between LoB and 4 LoB were tested 15 times respectively within 3 days. The limit of quantitation (LoQ) was defined as the lowest concentration which could be measured with a precision of <20% CV. The serum sample with CM-sIgE concentration of 0.56 kU_A/L was diluted by non-allergic serum in a ratio of 1/10 to 10/10. Each dilution was measured 10 times repeatedly, and the CV was calculated.

2.7.3. Linearity

Referring to the clinical guideline documents CLSI-EP6-A (CLSI, 2003), serum samples with CM-sIgE concentrations of 20.00 kU_A/L and 0.20 kU_A/L were selected to determine linearity. Eleven samples of different concentration levels were prepared at equal concentration intervals. Each sample was performed in triplicate.

2.7.4. Interference/analytical specificity

To evaluate the interference of common interfering substances in serum on the detection of CM-sIgE by LICA assay, triglycerides (14.28 mmol/L), bilirubin (160 μmol/L), and hemoglobin (10.00 g/L) were added to a high value and a low-value CM-sIgE positive serum respectively. In addition, to evaluate the interference of antibodies that may cross-react with CM-sIgE in the serum to the detection system, total IgE (100.00 kU/L), CM-sIgG (353.09 U/mL), and egg-sIgE (4.00 kU_A/L) were added to the CM-sIgE positive serum samples. Then, the same volume of system buffer was added to the serum as a basic sample. The LICA assay was used to detect the concentration of CM-sIgE in the serum after adding interfering or cross-reactive substances and the basic sample. The interference rate was calculated.

2.8. Comparison of LICA and Foresight® assay for detection of milk allergen-specific IgE

Forty-seven serum samples tested by Foresight® (ACON, China) as positive for CM-sIgE and 50 serum samples tested by Foresight® as negative for CM-sIgE were selected. Then LICA method was used to detect the CM-sIgE of these ninety-seven serum samples. The relative sensitivity, relative specificity, positive predictive value (PPV) and negative predictive value (NPV) of LICA method were calculated.

2.9. Statistical analysis

A schematic diagram of LICA was generated with Biorender (<http://biorender.com>). The Calibration curve was drawn using GraphPad Prism 8.0 (GraphPad, CA, USA). Normal distribution and

homogeneity of variance were analyzed using SPSS 12.0 (IBM, New York, USA) and ELISA Calc 0.2.

3. Results

3.1. Optimization of reaction conditions

We optimized the coating ratio of milk allergens, the working concentration of chemibeads and biotinylated anti-human IgE antibody, the dilution ratio of serum, and the reaction time of the second stage. The results are shown in Fig. 2. The antigen coating ratio of the chemibeads has an impact on the test results. When the mass ratio of the chemibeads to the milk allergens was 10:4, the P/N value of the high and low serum results was the largest. The concentration of the chemibeads determines the level of the background signal of the detection system. When the concentration of the chemibeads was 10 μg/mL, the background signal was low, but too few chemibeads would reduce the sensitivity of detection. When the concentration of the chemibeads was 40 μg/mL, the background signal was too high, which will shorten the linear range of the detection method. When the concentration of chemibeads was 20 μg/mL, the distinction between high and low serum was the best, and the background signal was more appropriate. The concentration of anti-human IgE antibodies is also an important factor affecting antigen-antibody binding. When its concentration was 1.0 μg/mL, the high and low serum P/N value was the largest. As the dilution of serum gradually increased, the P/N value gradually decreased, so 1:10 was the best dilution ratio for serum. In addition, the P/N value of high serum showed an upward trend with the reaction time gradually increasing, the difference between 30 min and 40 min was not significant, while the low-value serum showed a gradual downward trend. Considering the above analysis, 30 min was selected as the best reaction time.

3.2. Calibration curve

Under the optimal reaction conditions, we obtained the calibration curve by serially diluting the high-value serum with a CM-sIgE concentration of 35 kU_A/L. As shown in Fig. 3, the CM-sIgE concentration and luminescence signals were input into the GraphPad Prism software for four-parameter fitting, and the calibration curve was obtained as $y = (A - D) / [1 + (x/C)^B] + D$. ($A = 47,509$, $B = -1.04$, $C = 63$, $D = 571$, $r^2 = 0.9999$).

3.3. Performance assessment

3.3.1. Imprecision

For repeatability, the CV ranged from 3.71% to 8.11%. For intermediate precision, the CV ranged from 4.08% to 14.71%. Both the repeatability and intermediate precision were less than 15%, which indicated that the assay had high precision (Table 2).

SD: standard deviation, CV: coefficient of variation.

3.3.2. Limit of blank, limit of detection, limit of quantitation

For LoB, a total of 60 results obtained from 4 blank serum were analyzed for normality, and the results showed a non-normal distribution. The non-parametric test was used to calculate the LoB of the LICA assay, which was 0.04 kU_A/L (Table S1). For LoD, the test results of each sample showed homogeneity of variance. The parametric statistical method was used to calculate the LoD to be 0.11 kU_A/L (Table S1). For LoQ, when the serum was diluted to a CM-sIgE concentration of 0.22 kU_A/L, the CV was less than 20%. Therefore, the LoQ of this method is 0.22 kU_A/L (Table S2).

3.3.3. Linearity

The linearity was 0.9977 (> 0.9900) for CM-sIgE in the concentration range from 0.20 to 18.20 kU_A/L and the regression equation was $y = 0.8841x - 0.0759$ (Fig. 4).

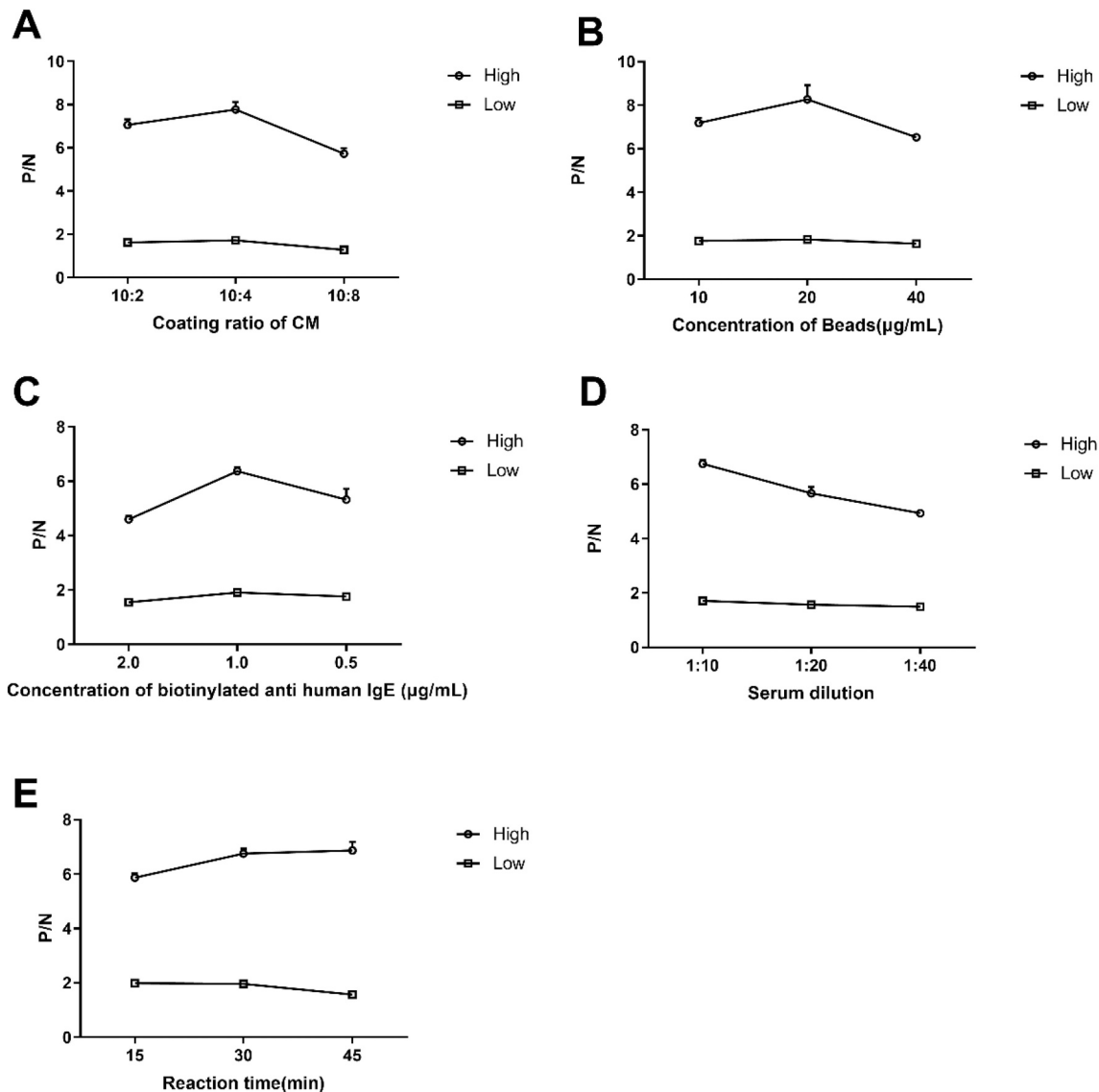


Fig. 2. Optimization of reaction conditions. (A) The effect of different coating ratios of CM. (B) The effect of different concentrations of chemibeads. (C) The effect of different concentrations of biotinylated anti-human IgE. (D) The effect of different dilutions of serum. (E) The effect of different reaction times.

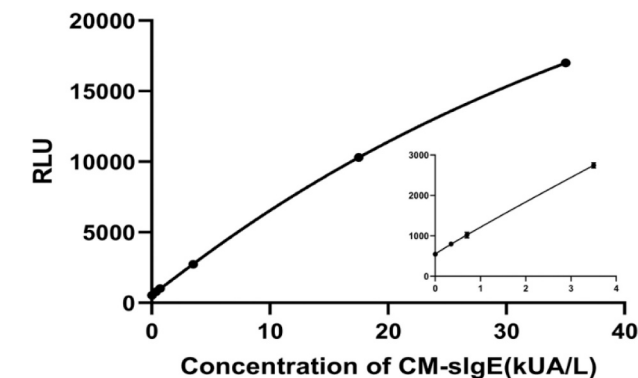


Fig. 3. Calibration curve of LICA for detecting CM-sIgE. Different concentrations of sIgE (0, 0.35, 0.7, 3.5, 17.5, 35 kUA/L) serially diluted from a cow's milk allergy serum (35 kUA/L) were used to generate a calibration curve.

Table 2
Imprecision of LICA for CM-sIgE.

	Sample	Mean(kUA/L)	SD	CV(%)
Repeatability	Sample 1	16.73	0.62	3.71
	Sample 2	0.37	0.03	8.11
Intermediate precision	Sample 3	10.58	0.43	4.08
	Sample 4	0.68	0.10	14.71

3.3.4. Interference/analytical specificity

The allergen-sIgE antibody detection system should have good anti-interference ability. We observed that the clinically considered high levels of 14.28 mmol/L triglycerides, 160.00 μmol/L bilirubin, and 10.00 g/L hemoglobin didn't significantly interfere with this method, and the interference rate was less than 8% (Table 3). To verify whether the excessive non-specific IgE in the serum will neutralize the biotinylated anti-human IgE, the patient's serum containing 100.00 kUA/L IgE was added to the CM-sIgE positive serum. The results showed that the interference rates for high serum and low serum were 6.92% and 6.80% (Table 3). It showed that non-sIgE has little neutralizing effect against human IgE. In addition, we evaluated the interference of CM-

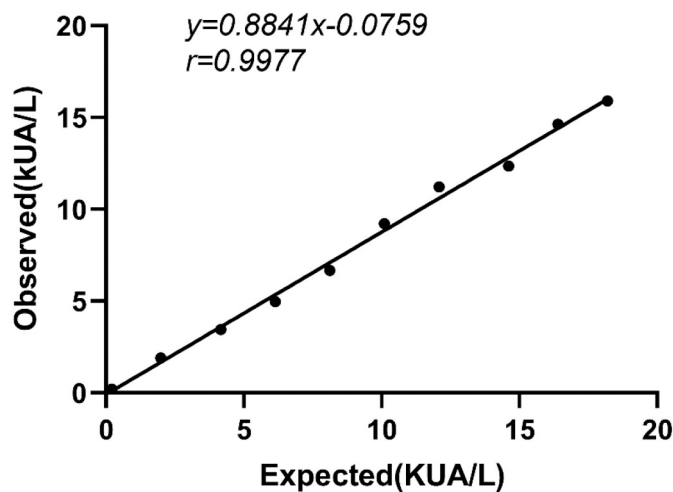


Fig. 4. Linearity of LICA for CM-sIgE. Serum with different CM-sIgE concentrations (0.20, 1.98, 4.16, 6.14, 8.12, 10.10, 12.08, 14.60, 16.40, 18.20 kU_A/L) prepared at equal intervals were used for linearity evaluation.

Table 3
Interference test of LICA for CM-sIgE.

Substance	Basic sample (kU _A /L)	Sample after adding interference (kU _A /L)	Interference rate (%)
Triglycerides (mmol/L)	9.50	10.24	7.79
14.28	0.68	0.64	-6.62
Bilirubin(μmol/L)	9.50	9.37	-1.42
160.00	0.68	0.71	4.41
Hemoglobin(g/L)	9.50	9.90	4.21
10.00	0.68	0.68	-0.74
Total IgE(kU/L)	10.27	9.56	-6.92
100.00	0.52	0.55	6.80
CM-sIgG(μmL)	10.27	10.27	0.00
353.09	0.52	0.52	0.00
Egg-sIgE(kUA/L)	10.27	10.06	-2.05
4.00	0.52	0.56	7.77

sIgG and non-CM-sIgE, such as egg-sIgE, which may cross-react with CM-sIgE. The results showed that the interference rate was less than 8%, indicating that there was no obvious cross-reaction (Table 3).

Interference rate = (CM-sIgE concentration after adding interference - CM-sIgE concentration of Basic sample)/CM-sIgE concentration of Basic sample × 100%).

3.4. Comparison of LICA and Foresight® assay for detection of CM-sIgE

A total of 97 samples were included in the comparison, among which 38 samples gave double-positive CM-sIgE results in the two assays, and 44 samples gave double-negative results. According to Table 4, the relative sensitivity of LICA is 80.9%, the relative specificity is 88.0%, the PPV and NPV are 86.4% and 83.0%, respectively.

Table 4
Comparison of results in LICA and Foresight® assay for the detection of milk allergen-specific IgE (n = 97).

LICA	Foresight®		Total(n)
	Positive(n)	Negative(n)	
Positive(n)	38(a)	6(b)	44
Negative(n)	9(c)	44(d)	53
Total(n)	47	50	97

4. Discussion

The detection of milk allergen sIgE is of great significance to the diagnosis of cow's milk allergy. ImmunoCAP (Thermo Fischer Scientific) is perceived as the reference method for the in vitro detection of allergen-specific IgE. It provides IgE testing against allergen extracts or molecules (Component) and usually used to verify new sIgE laboratory tests (van Hage et al., 2017; Di Fraia et al., 2019; Quintero-Campos et al., 2020; Potapova et al., 2021).

In this study, we have established a homogeneous and rapid quantitative detection method for milk sIgE in serum based on LICA. As a quantitative method, a calibration curve was established firstly. Due to the lack of CM-sIgE calibrator and the serum level of sIgE in milk allergy patients is much lower than that of inhalation allergy patients, a sample with a CM-sIgE concentration of 35.0 kU_A/L was selected as the highest concentration calibrator for LICA. Then we adjusted the concentration of the remaining calibrators to 0, 0.35, 0.7, 3.5, and 17.5 kU_A/L according to the calibration points of the ImmunoCAP to obtain the calibration curve of LICA.

Under the best working conditions, we evaluated the performance of LICA for the detection of CM-sIgE according to the CLSI standard. The validations of repeatability and intermediate precision were 3.71%–8.11% and 4.08%–14.71%, respectively, which showed that the LICA method has good precision. It has high analytical sensitivity for its LoD and LoQ were 0.11 kU_A/L and 0.22 kU_A/L, respectively. It is linear within 0.20–18.20 kU_A/L, covering the concentration range of common samples in clinical laboratories, and could meet the needs of clinical laboratories.

Since this method does not require washing, it may be more sensitive to some interfering substances in the serum. Usually, the interfering substances in the serum include hemoglobin, bilirubin, and triglycerides. In this study, it was found that the common concentrations of hemoglobin, bilirubin, and triglycerides in clinical laboratories interfere with this method were within acceptable ranges. In addition, the presence of excessive IgE in the serum may neutralize the biotinylated anti-human IgE and affect the accuracy of the test results (Olivieri et al., 1993). The results showed that the total IgE of 100 kU_A/L did not cause significant interference to the test results, and this neutralization effect can also be offset by adding sufficient secondary antibodies. Since sIgG and sIgE recognize the same or similar epitopes, excess sIgG in the serum may compete with sIgE for binding to the solid-phase antigen (Kadooka et al., 2000; Larimore et al., 2019; Sereme et al., 2021). In this study, it was found that high levels of CM-sIgG will not cause significant interference to the detection of CM-sIgE, which is consistent with the results of another study on the ability of LICA to resist IgG interference (Zhang et al., 2021). By increasing the coating amount of the surface antigen of the chemibeads, the interference of sIgG could be resisted. We also conducted interference experiments on substances that may have cross-reactions with CM-sIgE in serum, such as egg-sIgE, and found that there was no significant cross-reaction.

By comparing with Foresight® based on immuno-dot blot assay, the relative sensitivity of this method is 80.9%, and the relative specificity is 88%. The low sensitivity has the problem of missed detection. This may be related to the types and methods of coating antigens between different detection methods, or related to the antigen heterogeneity in the serum of different patients. We also compared the quantitative results of LICA and Foresight®. It showed that there was no obvious correlation between the two methods. This may be related to the difference in the coating antigen components used in the two methods. Regarding this point, it needs to be confirmed in future research.

In recent years, it has been reported that light-initiated chemiluminescence assay detection methods of HCV antibodies (Yang et al., 2019), estradiol (Li et al., 2020), human cytomegalovirus IgG antibody (Liu et al., 2021), and interferon-γ (Wu et al., 2016) have been established. However, LICA was less used in the field of allergen detection and might be unstandardized. This may be related to factors such as the

difficulty of standardization of allergen components and the inability to trace the source. There was also a study on the establishment of a LICA method for the detection of sIgE in eggs based on component-resolved diagnosis, with good analytical performance characteristics, which was similar to the results obtained in this study (Bian et al., 2018).

As a homogeneous immunoassay method, LICA does not require separation and washing steps in the entire reaction process. The detection could usually be completed within 1 h, which could greatly save the detection time. Through optimization of reaction conditions, we have obtained that the serum cost of this method was only 10 µL/test, which could greatly reduce the serum cost compared with other conventional allergen-sIgE detection methods. In addition, LICA has only two incubation steps, which is simple to operate and easy to automate. Currently, the LICA 500 instrument has a very high throughput, processing close to 200 samples per hour. It not only greatly reduces the workload of experimenters, but also shortens the time for patients to wait for the test report, which is conducive to doctors to make faster diagnosis and treatment.

In summary, we have developed a new quantitative method for the detection of CM-sIgE, and its performance characteristics was good enough to meet the needs of clinical laboratories. We believe that this new method will promote the immunological detection of CM-sIgE and has a good clinical application value.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jim.2021.113209>.

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Supplementary materials

Table S1 The results of 4 blank samples and 4 low samples used to calculate LoB and LoD

	LoB (kUA/L)				LoD (kUA/L)			
	Blank 1	Blank 2	Blank 3	Blank 4	Low 1	Low 2	Low 3	Low 4
Day1	0.05	0.03	0.04	0.04	0.11	0.14	0.12	0.11
	0.00	0.01	0.00	0.00	0.11	0.19	0.14	0.17
	0.00	0.01	0.00	0.00	0.08	0.11	0.13	0.18
	0.01	0.05	0.00	0.00	0.07	0.08	0.13	0.13
	0.00	0.02	0.03	0.00	0.12	0.16	0.14	0.22
Day 2	0.00	0.00	0.00	0.03	0.12	0.07	0.12	0.10
	0.00	0.03	0.00	0.00	0.07	0.12	0.10	0.09
	0.02	0.01	0.00	0.00	0.06	0.14	0.20	0.12
	0.00	0.00	0.00	0.00	0.04	0.11	0.13	0.15
	0.00	0.00	0.00	0.00	0.14	0.18	0.10	0.14
Day 3	0.00	0.02	0.00	0.00	0.08	0.04	0.14	0.15
	0.00	0.00	0.00	0.00	0.12	0.12	0.11	0.10
	0.02	0.00	0.00	0.00	0.03	0.21	0.20	0.21
	0.00	0.00	0.00	0.00	0.04	0.18	0.11	0.17
	0.00	0.00	0.00	0.00	0.08	0.10	0.09	0.17

Table S2 High serum serial dilution test results used to calculate LoQ

Dilution	Expected value (kUA/L)	SD	CV (%)
1/10	0.06	0.05	32.0
2/10	0.11	0.07	38.0
3/10	0.17	0.07	23.0
4/10	0.22	0.04	15.0
5/10	0.28	0.06	17.0
6/10	0.34	0.06	16.0
7/10	0.39	0.08	15.0
8/10	0.45	0.05	11.0
9/10	0.5	0.09	17.0
10/10	0.56	0.07	11.0