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# A light-initiated chemiluminescent assay for the detection of children's milk protein-specific IgE with excellent ability to avoid interference of specific IgG

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**Abstract**

The detection of allergen-specific IgE is of value for the diagnosis of children's milk allergy. However, its accuracy will interfere with the presence of high levels of specific IgG in the serum of children with milk allergy. To solve this problem, we established a light-initiated chemiluminescent assay (LICA) based on nanomicrospheres, which neutralized the interference of specific IgG by increasing the amount of antigen coated on the microspheres. The ability of this method to resist IgG interference was confirmed by adding extra specific IgG to the serum of allergic patients. Finally, the positive rate of allergen-specific IgE was increased to 85%, which was better than the indirect ELISA (70%), indicating that this method has certain advantages for the detection of specific IgE in children with milk allergy.

**Keywords**: **Specific IgE; Specific IgG; Interference; LICA; Cow's milk allergy**

## 1. Introduction

Cow's milk allergy (CMA) is one of the most common food allergies during infancy. According to reports, the average incidence rate for infants under 1 year old is 2-7.5%(Høst, 2002; Ho et al., 2014). IgE-mediated milk allergy can cause a variety of adverse reactions, which may involve organs such as the skin, gastrointestinal tract or respiratory system, seriously affecting the healthy growth of children(Koletzko et al., 2012; Luedman et al., 2013). The detection of milk allergen-specific IgE is of great value for diagnosing milk allergy and guiding desensitization treatment(Meglio et al., 2008; Wang et al., 2008; Zapatero et al., 2008). At present, many types of detection methods for in vitro allergen-specific IgE have been developed. The radioallergosorbent test (RAST) is the earliest specific IgE antibody detection method, established by Wide in 1967. It is no longer used clinically since it is considered to be a rather inefficient and expensive method compared with others(Wide et al., 1967). The ImmunoCAP system is considered to be the gold standard for in vitro allergen-specific IgE detection. It can accurately quantify specific IgE, but requires specific equipment and has high detection costs, which limits its clinical application to a certain extent(Gleeson et al., 1996; Boyce et al., 2010; Park et al., 2018). Enzyme-linked immunosorbent assay (ELISA) is low cost, simple to operate, and is widely used in laboratories(Sookrung et al., 2019; Furuta et al., 2020; Zhang et al., 2020).

Studies have shown that the serum of infants and young children with milk allergy contains higher levels of allergen-specific IgG, and the amount of specific IgE is often extremely low compared to specific IgG(Rowntree et al., 1985; Schwarz et al., 2016; Larimore et al., 2019). If indirect ELISA is

used to detect milk allergen-specific IgE, the excess specific IgG in the serum will compete with the specific IgE to bind to the solid-phase antigen, resulting in inaccurate specific IgE detection(Plebani et al., 1986; Lee et al., 1988; Kadooka et al., 2000; Zhang et al., 2019). The interference of specific IgG can be avoided by increasing the coating amount of solid-phase antigen or by using capture ELISA. However, the coating area of the microplate used in an ELISA is limited, and it is difficult to increase the amount of antigen on the wells(Brunnée et al., 1996; Kadooka et al., 2000). In addition, for the capture method, the anti-human IgE antibody is coated with a solid phase material that will capture all IgE antibodies, regardless of their specificity(Sakaguchi et al., 1989; Olivieri et al., 1993). Competition for anti-human IgE occurs between nonspecific IgE antibodies and specific IgE antibodies, which can also lead to erroneous detection of specific IgE antibodies, resulting in misdiagnosis and affecting clinicians' judgment of the condition.

For the indirect method, if the coating area of the solid phase carrier used is large enough, it may be possible to reduce the interference of specific IgG by increasing the antigen coating amount(Johansson, 2004; van Hage et al., 2017). Nanomicrospheres are artificially manufactured microparticles with a size of no more than 100 nm that have important scientific research value. Nanospheres with functional groups easily form chemical coupling with antibodies(antigens) and have a large binding capacity. (Wang et al., 2013; Yang et al., 2020). During the reaction, nanospheres can be uniformly dispersed in the entire reaction solution, and the reaction speed is fast.

The light-initiated chemiluminescent assay (LICA) is a homogeneous chemiluminescence analysis method using nanomicrospheres as a solid phase carrier. The principle of luminescence is based on the energy transfer between sensibeads and chemibeads. The chemibeads containing fluorescent substances were coupled to the antigen through functional groups and dispersed in the liquid phase. In addition, these chemibeads have a large relative surface area, which can significantly increase the amount of antigen coating(Bian et al., 2016; Yan et al., 2018). Table 1 shows the comparison of indirect ELISA and LICA for the detection of allergen-specific IgE.

Table 1. Comparison of indirect ELISA and LICA for the detection of allergen-specific IgE.

| | Solid phase | Reaction mode | Reaction time | Interfering substance |
|---|---|---|---|---|
| Indirect ELISA | Microplate | Heterogeneous | 3 h | Specific IgG |
| Indirect LICA | Nanospheres | Homogeneous | 45 min | Specific IgG* |

* Nanomicrospheres have a large relative surface area that can hold more antigens and significantly reduce the interference of specific IgG.

Based on the above analysis, we will take the major allergen αs1-casein (Bos d 9) from cow's milk as an example(D'Urbano et al., 2010) and coat it on the surface of the chemibeads in different amounts. This will allow us to observe the reaction system's ability to resist specific IgG interference and to establish an indirect chemiluminescence immunoassay method for the detection of children's milk protein-specific IgE, without interference of specific IgG, to improve the accuracy of the tests.

## 2. Materials and Methods

### 2.1 Sera

Sera from children allergic to cow's milk were obtained from Tianjin Children's Hospital. A total of 40 subjects aged between 0.3 and 10 years old were enrolled in the present study. Allergy to milk was determined by a convincing clinical history of symptoms and by a milk protein-specific IgE level of at least 0.35 kUA/L. Eight non-allergic individuals were used as negative controls. All sera samples were stored in aliquots at -20°C. The study was approved by the Ethics Committee of Tianjin Medical University. Informed consent was obtained from each patient.

**2.2 Reagents and Equipment**

One major milk allergen protein (αs1-casein) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). The goat anti-human IgE antibody, horseradish peroxidase (HRP)-labeled mouse anti-human IgE&IgG antibody, and biotinylated mouse anti-human IgE antibody were purchased from Abcam (Cambridge, UK). Sensibeads coated with streptavidin and chemibeads 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 Detection of milk protein-specific IgG**

A 96-well ELISA microplate was coated with milk allergen Bos d 9 (0.5 μg/well, diluted in carbonate buffer, pH 9.6) at 4°C overnight. The plates were then blocked with 1% (w/v) bovine serum albumin (BSA) in PBS for 2 h at 37°C. After washing, the plates were incubated with serum from milk allergic patients for 1 h at 37°C, followed by incubation with HRP-labeled mouse anti-human IgG for 40 min at 37°C. The plates were washed with PBST. Bound HRP-labeled antibody was detected by adding 100 μl of 3,3',5,5'-tetramethylbenzidine (TMB), and the reaction was stopped with 50 μl of $H2SO_4$. The optical density at 450 nm was immediately recorded using a Bio-Teck Microplate Reader. An optical density greater than CD + 2 x S.D. above the negative control (non-allergic individuals) was regarded as a positive result. All tests were performed in duplicate.

**2.4 Interference of specific IgG in the detection of milk allergen-specific IgE**

Bos d 9-specific IgE of 40 milk allergic children was detected by indirect ELISA and capture ELISA. The procedure for the indirect ELISA was as described for the detection of milk Bos d 9-specific IgG, except that after adding serum, the second antibody was HRP-labeled anti-human IgE.

For capture ELISA, the plates were coated with anti-IgE antibody (200 ng/well) overnight at 4°C and then blocked with 1% (w/v) BSA in PBS for 2 h at 37°C. Milk-allergic human serum was subsequently added to the plates and incubated for 1 h at 37°C. The plates were then incubated with biotinylated Bos d 9 (5 μg/ml) for 1 h at 4°C. Finally, streptavidin-HRP was added to the plates for 40 min at 37°C before the addition of TMB. The subsequent processing was the same as the indirect ELISA.

Six indirect ELISA-negative but capture ELISA-positive sera were diluted with PBS at a ratio of 1:20 and then mixed with 100 μl Protein G resin for 30 min at room temperature to allow IgG from serum samples to bind to the resins. After removing Protein G resins by centrifugation, 200 μl of

supernatant was collected. As a control, nonallergic serum was treated in the same way. Bos d 9-specific IgE in serum before and after IgG adsorption was detected by indirect ELISA.

Bos d 9- specific IgG positive serum without specific IgE was diluted in different proportions and added to the Bos d 9-sIgE positive serum of milk allergy patients. Here, we used serum without Bos d 9-sIgG and sIgE as diluents. The control sample was prepared without adding any sIgG-positive serum. Bos d 9-specific IgE in serum was detected by indirect ELISA after adding different proportions of specific IgG, and control samples were detected by indirect ELISA.

**2.5 Establishment of a light-initiated chemiluminescent assay for milk protein-specific IgE without interference of specific IgG**

The assay was conducted in 1% BSA dissolved in PBS (pH 8.0). The detection procedures were performed in two steps. First, 25 μl of human serum, biotinylated anti-human IgE antibody, and chemibeads coated by Bos d 9 were added to 8-well plates. The plates were placed in the chemiluminescence analyzer instrument for incubation at 37°C for 30 min. Then, streptavidin-conjugated sensibeads were added for another 15-min incubation in the dark. The results were read on the LICA instrument. Samples were tested in duplicate.

The process of antigen coating beads was performed according to the method reported by our group[30]. The chemibeads were washed with $dd'H_2O$ and mixed with αs1-casein in different mass ratios overnight at 37°C. After reduction and sealing, the beads were resuspended in the storage solution and stored at 4°C in the dark.

The coating ratio and working concentration of the antigen were optimized to obtain the best working conditions. First, two serum pools containing high and low levels of Bos d 9-specific IgE were collected. Sera from nonallergic individuals served as negative controls. Then, chemibeads coated with different Bos d 9 were used as detection reagents. The Bos d 9-specific IgE of each sample was detected according to the LICA process. The positive serum pool and negative serum pool were designated P and N, respectively. Positive to negative (P/N) ratios were calculated, and the optimal antigen coating ratio was obtained.

To obtain the best working concentration of the chemibeads, the beads with the best antigen coating ratio obtained above were diluted at ratios of 1:200, 1:400, and 1:800. The positive sera pool and negative sera pool were tested at the same time, and the P/N ratios were calculated.

To verify the ability of this system to resist IgG interference, Bos d 9- sIgG positive serum without sIgE was diluted in different proportions and added to Bos d 9-sIgE-positive serum from milk allergy. The control sample was prepared without adding any sIgG-positive serum. The established homogeneous chemiluminescence immunoassay method was used to detect the Bos d 9-specific IgE of each sample after adding different proportions of sIgG.

**2.6 Preliminary application of a light-initiated chemiluminescent assay in the detection of specific IgE**

Bos d 9-specific IgE of 40 children with milk allergy and 8 healthy volunteers was detected by LICA and indirect ELISA. The positive rate of Bos d 9-specific IgE was calculated. Receiver operating

characteristic (ROC) curves were used to compare the performances of LICA and indirect ELISA. To evaluate the test's performance in distinguishing between patients with and without milk allergy, sensitivity, specificity, and area under the curve (AUC) were calculated, and the optimal cutoff levels to predict milk allergy were selected.

**2.7 Statistical analysis**

Statistical analysis and plotting of the correlation curves were performed using GraphPad Prism 9.1 (GraphPad, La Jolla, CA, USA). Differences between groups were analyzed using paired Student's *t*-test. $P<0.05$ indicated a statistically significant difference.

**3. Results**

**3.1 High levels of specific IgG are present in children with milk allergy**

The ELISA results showed (Fig 1) that the positive rate of Bos d 9-specific IgG in 40 milk allergic sera was 100% (OD 450 nm>0.291). By calculating the P/N values of Bos d 9-specific IgG of each patient and healthy controls, it was found that the strongly positive specific IgG samples with a P/N value above 5.57 accounted for 80%, the moderate-strength sera with a P/N between 4.37-5.57 accounted for 10%, and the weakly positive sera with a P/N below 4.37 accounted for 10% (Fig 2). High levels of specific IgG are generally present in the serum of children with milk allergy.

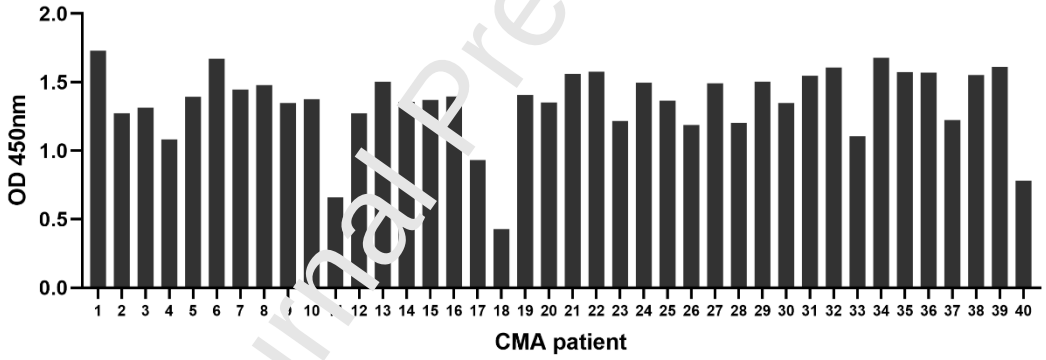



Fig 1 Specific IgG level to Bos d 9 in CMA patients.

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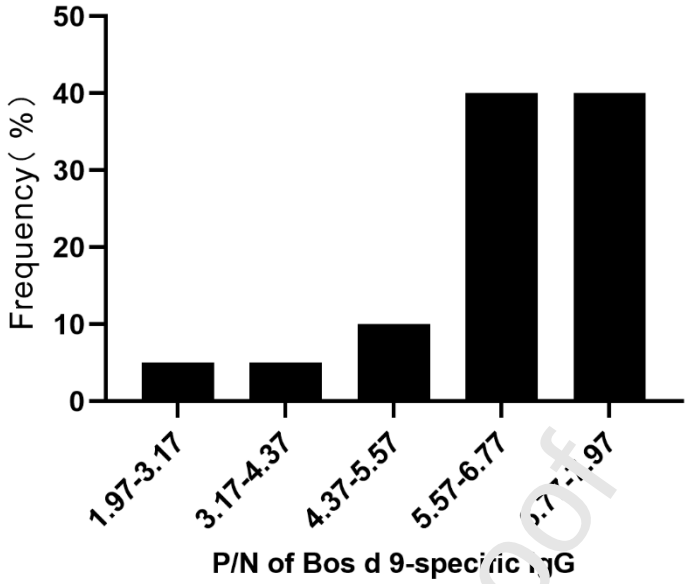



Fig 2 Frequency distribution histogram of specific IgG to Bos d 9. P, positive sera; N, negative sera

**3.2 Interference of specific IgG in the detection of specific IgE in children with milk allergy**

Among the 40 children with milk allergy, 28 tested positive by indirect ELISA, but 32 tested positive by capture ELISA. Six indirect ELISA-negative but capture ELISA-positive sera were observed. The six sera all contained a high level of Bos d 9-specific IgG (Fig 3).

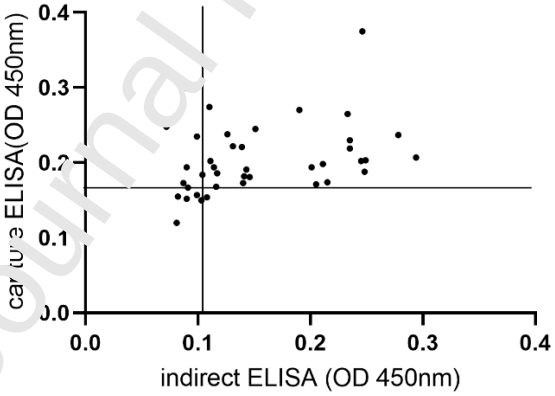



Fig 3 Correlation between indirect ELISA and capture ELISA results. The results are expressed as the optical density value at OD 450 nm of CM-specific IgE. Cutoff values (2 SD above the mean value of the 8 negative controls) are represented by the horizontal line for capture ELISA and by the vertical line for indirect ELISA.

Six indirect ELISA-negative but capture ELISA-positive sera were assessed by indirect ELISA for Bos d 9-specific IgG and IgE before and after adsorption with protein G(Fig 4). The results showed that the P/N value of Bos d 9-specific IgG of each sample had a significant decrease (P <0.001) after IgG adsorption. This indicated that we successfully removed the specific IgG in the serum. In contrast, the comparative analysis of the P/N of Bos d 9-specific IgE showed a significant increase ($P < 0.001$) after IgG adsorption. The presence of specific IgG in the serum interferes with the detection of specific IgE.

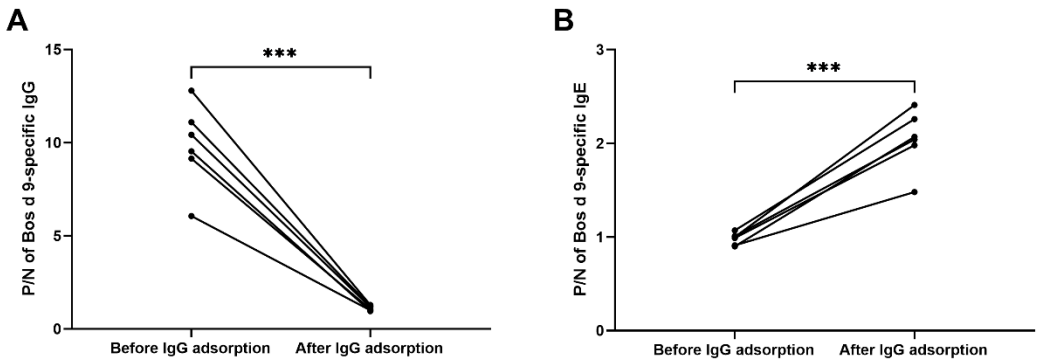



Fig 4 Comparison of the changes in specific IgG and specific IgE to Bos d 9 before and after IgG adsorption. (A) P/N of specific IgG and (B) P/N of specific IgE. Abbreviation: P, positive sera; N, negative sera. The difference before and after IgG adsorption was analyzed using paired Student's t-test; *** represents $P < 0.001$.

To determine whether the interference depends on the concentration of specific IgG, 3 dilutions of Bos d 9-specific IgG from Bos d 9-specific IgE-negative patients were prepared (1:10, 1:20, and 1:40) and added to the serum of three CMA patients. The control samples were prepared without adding any sIgG-positive serum. The results showed that as the concentration of Bos d 9-specific IgG increased, the P/N of Bos d 9-specific IgE in patient serum gradually decreased. When the specific IgG-positive sample was diluted at 1:20, the P/N of specific IgE decreased to approximately 1.0. Under this condition, the Bos d 9-specific IgE-positive sample was negative(Fig 5).

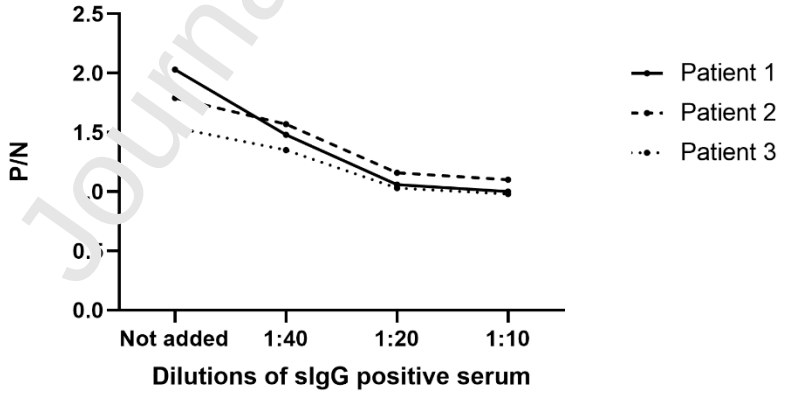



Fig 5 Comparison of Bos d 9-sIgE in 3 samples after adding IgG positive serum of different dilutions (not added, 1:40, 1:20 and 1:10).

**3.3 Establishment of a light-initiated chemiluminescent assay for the detection of milk protein-specific IgE without interference of specific IgG**

Our research found that there was a large amount of specific IgG in the serum of children with milk allergy that will compete with specific IgE to bind to the antigen and affect the accuracy of the test results. The interference of specific IgG with the detection of specific IgE can be reduced by increasing the amount of antigen coating. We prepared 3 antigen-labeled chemibeads with different

coating ratios. The results showed (Fig 6A) that when the mass ratio of the chemibeads to the antigen was 10:2, the P/N value of the sample was the largest. However, when the coating ratio was 10:4, the P/N decreased, which may be related to the hook effect. Therefore, we used 10:2 as the best antigen coating ratio.

The antigen-antibody reaction is proportional. Excessive chemibeads will increase the background signal. Similarly, too low a concentration of chemibeads will reduce the sensitivity of immunoassays. We diluted the antigen-labeled chemibeads at ratios of 1:200, 1:400, and 1:800 for determination. The results showed (see Fig 6B) that when the dilution ratio was 1:800, the P/N value was the largest. However, due to the low concentration of chemibeads, the sensitivity to low-value serum was not significantly improved. Therefore, we chose 1:400 as the best antigen dilution ratio.

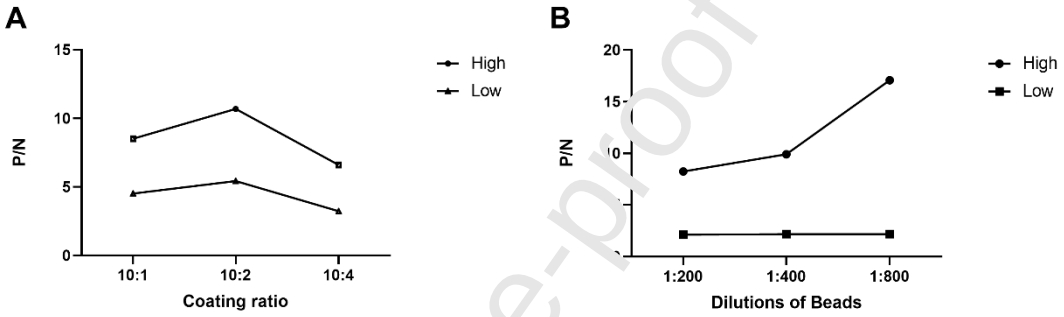



Fig 6 Optimization of LICA-sIgE reacting conditions. (A) The effect of different coating ratios of beads and antigen. (B) The effect of different dilutions of beads.

To verify the ability of the reaction system to resist the interference of specific IgG, different concentrations of specific IgG were added to the serum of 3 milk allergic children with different specific IgE levels. Fig 7 shows that when the dilution ratio of specific IgG added was less than 1:10, the P/N value of serum-specific IgE did not change significantly; when the dilution ratio of specific IgG added was less than 1:5, the P/N of serum specific IgE decreased. Our results indicated that specific IgG would affect the detection of specific IgE only when the concentration of specific IgG is particularly high in this method. Therefore, the LICA established in this study has good resistance to specific IgG interference under certain conditions.

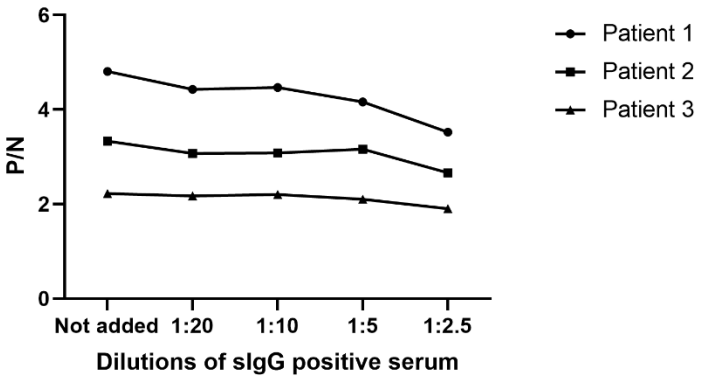



Fig 7 Comparison of Bos d 9-sIgE in 3 samples after the addition of different dilutions (not

added,1:20,1:10 ,1:5 and 1:2.5 ) of IgG-positive serum.

### 3.4 Preliminary application of a light-initiated chemiluminescent assay in the detection of milk allergen-specific IgE

Indirect ELISA and LICA were used to detect Bos d 9-specific IgE in 40 milk allergic children and 8 healthy volunteers. According to Table 2, compared with the indirect ELISA (positive rate of 70%), the LICA method increases the positive rate to 85% by detecting the same serum Bos d 9-specific IgE in 40 milk allergic infants. Six indirect ELISA-negative but capture ELISA-positive sera were also positive by LICA. ROC analysis was applied to compare the performances of indirect LICA and ELISA. The areas under the curves (AUCs) for indirect LICA and ELISA were 0.969 and 0.922, respectively (Fig 8). For LICA, we chose a cutoff value of ≥ 1133 (signal value) for our test, achieving a sensitivity of 85.0% and specificity of 100.0%. For indirect ELISA, an optimal cutoff value ≥ 0.106 (OD value) achieved a sensitivity of 80.0% and specificity of 100.0%. Therefore, it is believed that the indirect LICA method established in this study has certain advantages for the detection of children's milk allergen-specific IgE.

Table 2. Positive rate of Bos d 9-specific IgE for sera from patients with CMA as determined using indirect-ELISA and LICA.

| Methods | Positive rate |
|---|---|
| Indirect ELISA | 28/40 (70%) |
| Indirect LICA | 34/40 (85%) |

Abbreviations: ELISA, enzyme-linked immunosorbent assay; LICA, light-initiated chemiluminescent assay.

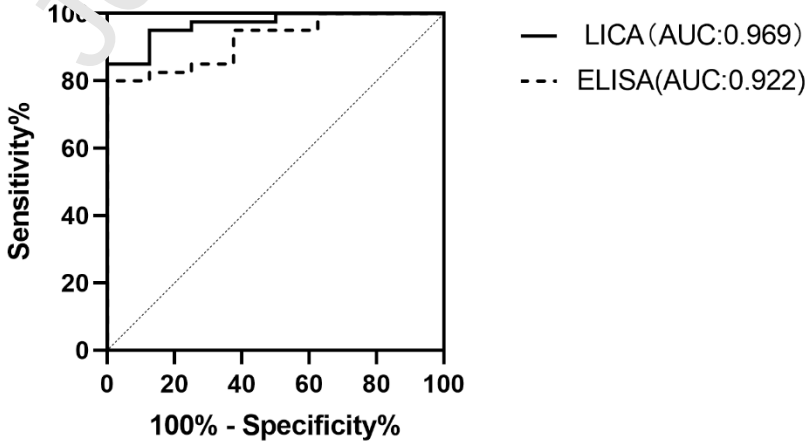



Fig 8 ROC curves of indirect LICA and ELISA in predicting milk allergy in the overall population (n = 48).

**4. Discussion**

The early and accurate quantification of allergen-specific IgE is of great significance for the diagnosis of milk allergy in infants and young children(Linhart et al., 2019). Traditional allergen-specific IgE detection often uses an indirect ELISA method. However, studies have shown that allergen-specific IgG in the serum of children with milk allergy is significantly increased. Excessive IgG will compete with the low concentration of allergen-specific IgE in the serum to bind to the solid-phase antigen, reducing the ability of indirect ELISA to accurately detect allergen-specific IgE(Sereme et al., 2021).

In this study, we used Bos d 9, the major allergen component of milk, as an example and used ELISA to detect the specific IgG level in children with milk allergy. The results showed that the positive rate of Bos d 9-specific IgG reached 100%. Compared with specific IgE, more specific IgG was found in patient sera. Our results verified that there was a large amount of specific IgG in the serum of children with milk allergy(Huang et al., 2018). The specific IgG in indirect ELISA Bos d 9-specific IgE-negative but capture ELISA-positive sera was removed by protein G, and the specific IgE value was significantly increased by indirect ELISA. On the other hand, by adding a certain concentration of specific IgG to 3 Bos d 9-specific IgE-positive serum samples, the results showed that as the concentration of added specific IgG gradually increased, the value of specific IgE in each sample decreased. This proved that specific IgG does interfere with the detection of specific IgE to a certain extent.

To solve this problem, the coating amount of solid-phase antigen can be increased to provide more antibody binding sites. The ImmunoCAP system is regarded as the gold standard for sIgE antibody detection. It coats a mixture of allergen extracts or component allergens on a pool of a cellulose sponge matrix. This solid-phase material has a strong protein adsorption function, so it can hold enough allergen antigens and keep the IgE binding areas even in the presence of high concentrations of specific IgG(Jeon et al., 2018). However, for the ELISA method, microplates do not immobilize allergens as densely as the allergen CAP. Moreover, the bottom area of the microplate used in ELISA was insufficient, and the coating amount was limited compared with the microsphere carrier. It is difficult to fully resist the interference of specific IgG.

LICA is an immunoassay method based on nanomicrospheres. The coating area of chemibeads is larger than that of microplates. Increasing the coating amount of antigen can help resist the interference of IgG to a certain extent and improve the detection accuracy of specific IgE. This study verified the detection performance of chemibeads with different amounts of antigen coating and selected the best antigen-labeled microspheres. Under the optimal reaction conditions, specific IgG was added to serum with different specific IgE concentrations. The results showed that the ability of this method to resist specific IgG interference is superior to the indirect ELISA method. In addition, using this method to detect the serum of the same batch of milk allergic children can increase the specific IgE positive rate from 70% to 85%, which improves the accuracy of detection. In addition, we compared the analytical performance of the two methods. The AUC (0.969) of the LICA was higher than that of the indirect

ELISA (0.922), which is more advantageous for predicting milk allergy.

Of course, as we did in step 2.4, using protein G reagent to preadsorb IgG in serum samples can also significantly reduce the interference caused by sIgG in the detection of allergen-sIgE, but the protein G used in this assay is expensive and will increase the cost of detection. Moreover, pretreatment may lead to loss of part of the sample, and it also increases the operation steps and prolongs the detection time. In addition, there is also a way to add anti-human IgG antibody to the reaction system to neutralize the excessive IgG in the sample to reduce the interference effect of sIgG(Shey et al., 2018). However, this method will also increase the cost of testing. The method we have established only needs to coat enough antigen on the chemibeads, without extra steps and detection costs, and can play a significant role in resisting the interference of sIgG. This method has better clinical practical value than the sample pretreatment method.

The interference of biotin is an important factor affecting the accuracy of immunoassay results. For LICA to detect the allergen sIgE, diluting the serum sample can reduce the biotin contained in the sample to a certain extent In addition, the LICA common reagent used is coupled with a sufficient amount of streptavidin, which can also neutralize the excess biotin in the serum. For patients receiving high-dose biotin therapy, we recommend that relevant indicators should be tested after stopping the drug. All of the above measures can reduce the interference of biotin on the LICA system and improve the accuracy of detection.

In summary, this study has established a highly accurate method for the detection of specific IgE in children's milk allergens that can resist IgG interference, which helps to accurately detect specific IgE in early childhood milk allergy.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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