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Development of TRFIA-kit of Quantitative Analysis for FumonisinB_1

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ABSTRACT

Objective TO establish time-resolved immunofluorescence analysis (TRIFA) kit used to detect fumonisin B_1 (FB_1) in food quickly. **Methods** Ascites was got after intraperitoneal injection of hybridoma cell lines against monoclonal antibody of FB_1 into the mice, and the ascites was purified with protein-A affinity chromatography so as to get a large number of monoclonal antibody. Based on this antibody, the TRFIA-kit was established and the parameters including the detection limit, specificity, stability, recovery, repeatability and reproducibility were optimized. Nineteen corn samples and 1 blind sample of corn were detected with the established kit and the results were verified with the commercially available ELISA-kit. **Results** The detection limit of the established kit was 2 ng/ml, the linear ranger of detection was 2-512 ng/ml, the linear equation was $Y=-0.644X+12.872$ ($R^2=0.9976$), and the 50% concentration of inhibition was 256 ng/ml. The rate of recovery from corn samples ranged from 78.32% to 116.76%. There was no reaction with deoxynivalenol, aflatoxins A and BSA. At room temperature, the kit can be saved for more than 315 days. **Conclusion** The fast and sensitive time-resolved immunofluorescence analysis assay was established to detect FB_1 in food.

KEY WORDS: FumonisinB_1, Monoclonal antibody, Time-resolved immunofluorescence, TRFIA-kit

INTRODUCTION

It is in 1988, Gelderblom et al. first isolated fumonisin [1, 2], and fumonisin is found mainly contaminates maize and maize products and is also found in rice, wheat, barley, sorghum, milk and other types of food and feed at a low concentration. To date, a total of 11 kinds of fumonisin have been discovered, but the main group showing high toxicity is the fumonisin B family (FBs) which including FB_1, FB_2 and FB_3 [3, 4]. Epidemiological data show that FB_1 contamination in the human diet is relevant to the high incidence of esophageal cancer [5, 6]. Experimental results indicate that FB_1 is the main cause of the toxic effect of fumonisin, and its cancer-promoting effect and carcinogenicity have been confirmed in rats [7, 8]. The International Cancer Research Center has classified it as a possible carcinogen for humans [9]. So, recently, the significance of fumonisin to livestock and human health has drawn increasing attention.

Many countries have established a standard limit for FB_1 in food, however, the detection methods of FB_1 are often based on the expensive instruments or high-quality, high-priced standards which limited the application of these technology. On the other hand, FB_1 proved to be carcinogenic agents, ingestion of such FB_1-contaminated food and feed pose to a threat to the people. Thirdly, there is no an internationally accepted detection method to detect the levels of FB_1, so the inter-laboratory comparability of the available data is poor. So as a consequence of the constraints of the existing quantitative analysis methods, the development of new and rapid techniques based on immunochemical principles is imperative.

The immunochemical method might be an ideal technique to detect the level of FB_1 in food samples because of its high sensitivity as well as the specificity of the antibody. In addition, the time-resolved fluorescence immunoassay (TRFIA) is a non-radioactive immunological labeling technology that was developed in the mid-1980s. It is used for the detection of trace substances and was established based on the high specificity of the immune response and high sensitivity of the labeling tracer. Compared with conventional enzyme immunoassays and radioimmuno assays, its detection limit of 10^{-18} mol/L is much higher. The World Health Organization (WHO) has ranked this technology as the most-recommended technology for use in application medicine [10]. Therefore, in this study, a detection method of time-resolved immunofluorescence was established based on the development of anti FB_1 monoclonal antibody with hybridoma technique in our laboratory[11], which could be used for FB_1 detection in food, so as to provide credible monitoring tools for the food safety risk monitoring.

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1. MATERIALS AND METHODS

1.1 Materials

The FB_1 antigen was purchased from Fermentek (Jerusalem, Israel). Monoclonal mouse antihuman IgA was obtained from the Wuhan Institute of Virology (Wuhan, China). A Eu^{3+}-labeled kit and enhancement solution were purchased from PerkinElmer Wallac (Turku, Finland). Bovine serum albumin (BSA) and Keyhole limpet hemocyanin (KLH) was obtained from LKT Lab. (St. Paul, USA). Ninety-six-well polystyrene microtiter plates were purchased from Thermo Labsystems (Milford, MA).The commercial ELISA kits for FB1-IgA detection were purchased from Zhongshan Bio-tech Co. Ltd (Zhongshan, China).

1.2 Animals and Cell Lines

Eight seven-weeks-old, SPF grade female BALB/c mice were supplied by Guangdong Medical Experimental Animal Center (certification No.0059665)； Mouse myeloma cell line sp2/0 was kindly gifted by Dr. Yingsong Wu of Southern Medical University.

1.3 Produce FB_1 antibodies

The hybridoma cells was pipetted up and down and then centrifuged at 1000 r/min for 7 min. After discarding the supernatant, the cells were suspended in complete medium, and the cell number was adjusted to 5×10^6/ml. Each BALB/C mouse was injected with 0.5 ml of hybridoma cells, and an ascites preparation of myeloma cells was injected as the negative control. At 7-10 days before cell injection, the mice were treated via intraperitoneal injection of Freund's complete adjuvant, and the ascites was collected after 10-14 days and purified with protein-A affinity chromatography[11].

1.4 Sample collection and processing

A total of 18 corn samples were collected randomly in a market, which were then ground so that 90% of the sample could pass through a 250-500 μm mesh. In a 5 g corn sample, 1 g of NaCl and 25 ml of methanol in water (V:V, 3:1) were added, with vigorous vortexing for 3 min. After filtering with filter paper, the samples were ready for the test.

1.5 Labeling of the secondary goat anti-mouse monoclonal antibody

Prior to labeling, a 50 ku molecular weight cutoff (MWCO) spin column was used for pretreatment of the goat anti-mouse monoclonal antibody (1 mg) to remove substances that would interfere with the labeling reaction, including phosphate and sodium azide. Labeling was carried out based on a reference in literature[12].

1.6 Preparation of the standard curve and optimization of conditions

The FB_1-BSA antigen was adsorbed onto the solid phase of a 96-well plate, (100 μl in each well, at concentrations of 200, 400, 800, 1000, 1500, 2000, 3000, and 4000 ng/ml, diluted in PBS). After incubation at 37 °C for 2 h, the wells were washed with washing solution twice. Next, blocking buffer (0.1% BSA) was added (100 μl in each well), followed by incubation at 37 °C for 1-2 h, after which the liquid in the wells was discarded. Next, 50 μl of purified antibody (the concentration was 103 μg/ml, and three dilutions of 1:1000, 1:1500, and 1:3000 were set up) and 50 μl of FB_1 antigen (at concentrations of 0, 20, 40, 80, 160, and 320 ng/ml) were added to each well, followed by reaction for 1 h. A 100 μl aliquot of the labeled N^1-benzyl isothiocyanate-DTTA-Eu goat anti-mouse secondary antibody was then diluted with assay buffer to 1:500 and 1:1000. After sealing with tape, the sample was incubated at 37 °C for 30 min and then washed with washing solution 6 times. A 100 μl aliquot of enhancement solution was subsequently added to each well an allowed to react for 5 min, and then detected with Auto DELFIA 1235 TRIFA apparatus (PerkinElmer, MA,USA) . Further exploration of the technical parameters of the experiment was conducted after the optimized conditions were identified using the standard curve, thereby determining the important parameters of the kit[13].

1.7 Optimization of technical parameters

1.7.1. Limit of detection (sensitivity) and specificity

The optimized time-resolved fluorescence immunoassay (TRFIA) standard curve of indirect competition was used to determine the linear range and detection limit of the antibody. The monoclonal antibody produced by the hybridoma cell line could detect the cross-reaction of the anti-FB_1 monoclonal antibody with the toxins aflatoxin (AFB1) and deoxynivalenol (DON) and the carrier protein BSA at different concentrations via the established TRFIA method[14].

1.7.2. Stability

The stability of 96-well plates containing the coating agent and other agents was investigated in a 37 °C destruction experiment[12]. The post-coating kit was placed at 37 °C or 4 °C, and the TRFIA assay was performed

on the 3rd day and the 7th day. Incubation at 37 °C for 1 day was equivalent to being held in a 4 °C refrigerator for 1.5 months.

1.7.3. Recovery

The labeling recovery test was carried out on the corn samples at concentrations of 50%, 100%, and 200%. Five duplicate tests were set up for each labeled sample in parallel (the extraction method was described in 1.4). The recovery was calculated [12, 15] as follows:

Recovery (%) = [measured value of the new sample (labeled) - measured value of the original sample] / known labeling amount × 100%.

1.8 Validation of the kit and the ELISA method

Detection was performed for the 19 corn samples and their products as well as the blind FB_1 corn sample provided by Trilogy Lab (Washington, MO USA) using the kit developed in this study, with verification by ELISA.

2. RESULTS

2.1 Preparation of the standard curve

2.1.1 Optimization of the parameters of the standard curve

The performance of the obtained monoclonal antibody at a 1:3000 dilution showed no significant difference from that at a 1:1500 dilution (Figure 1). Finally, according to previous experimental experience, the proportion of europium-labeled secondary antibody was determined to be 1:1500, and the dilution of the monoclonal antibody was therefore set to 1:1500. As shown in Figure 1, the dilution of the europium-labeled secondary antibody varied with the dilution of the monoclonal antibody. The coating concentrations of the antigen for the tests were 1, 2, 3, 4, 0.2, 0.4, and 0.8 ng/ml, as shown in Figure 2. No significant difference was found among them, and the coating concentration was finally set to 0.2 ng/ml.

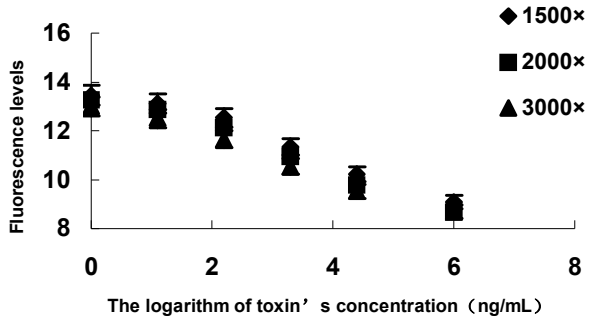

Fig.1 The competitive inhibition curve of MAb with different concentration

Dilution factor：◆1500×、■2000×、▲3000×

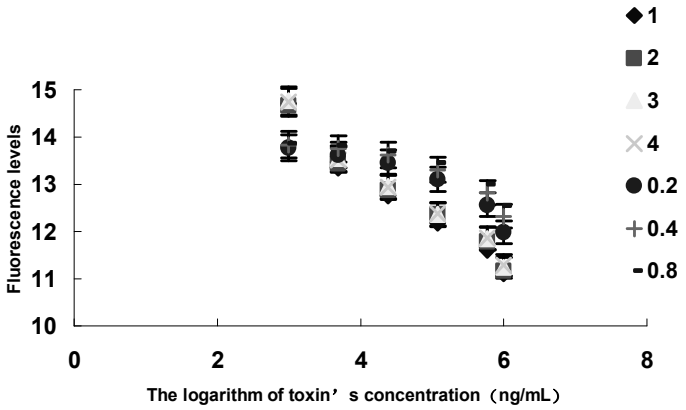

Fig.2 The competitive inhibition curve of coating antigen liquid with different concentration

coating antigen concentration：1、2、3、4、0.2、0.4、0.8 ng/ml

2.1.2 Preparation of the standard curve

The FB_1 stock solution was diluted with 10% methanol-PBS to different concentrations. At a monoclonal antibody dilution of 1:1500, a stable standard curve was prepared through indirect competitive TRFIA (Figure 3). The linear range of the calibration curve was 2-512 ng/ml, and the linear equation was Y = -0.644X + 12.872 (R^2 = 0.9976), with a minimum detectable concentration of FB_1 of 2 ng/ml and a 50% inhibitory concentration of 10.07 ng/ml.

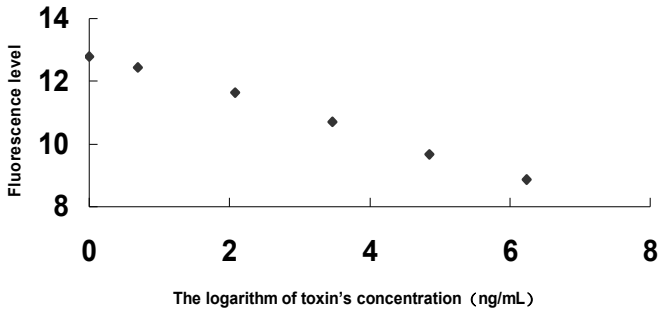

Fig.3 The competitive inhibition curve of Antibody and FB_1
X:The logarithm of FB_1's concentration Y: The Ln of Fluorescence levels

2.2 Characteristics of the kit

2.2.1 Sensitivity and specificity

The minimum detectable concentration for the resulting kit was 2 ng/ml, and no cross-reaction with vomitoxin (also known as deoxynivalenol, DON), aflatoxin (AFB1) or the carrier protein BSA was observed, as shown in Table 1. Accordingly, the fluorescence values of the two kinds of toxins at several concentrations were close to the fluorescence value in the blank control well with a concentration of 0 in the standard curve, indicating no inhibition of FB_1; that is, the two toxins exhibit no cross-reactivity.

Tab.1 Cross reaction with competitive inhibition method

	conc (ng/ml)	value	DON's concentration(ng/ml)	value	AFB_1's concentration(ng/ml)	value
1	0	960210	0	1127334	0	962335
2	2	909105	25	1064384	30	105473
3	8	636984	50	1034907	60	984851
4	32	223000	100	1137155		
5	128	83401	250	1089665		
6	512	32421				

2.2.2 Stability

On the 3rd day and the 7th day, TRFIA detection was carried out for the kit at 37 °C (Figure 4 and Figure 5). The performance after incubation at 37 °C for 24 h was equivalent to that after remaining at room temperature for 45 d. The kit is valid for more than 315 d at room temperature (10.5 months).

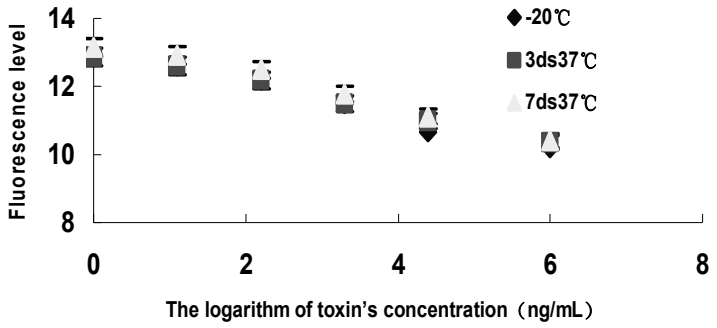

Fig.4 The competitive inhibition curve of MAb, after 3 days and 7 days on 37℃

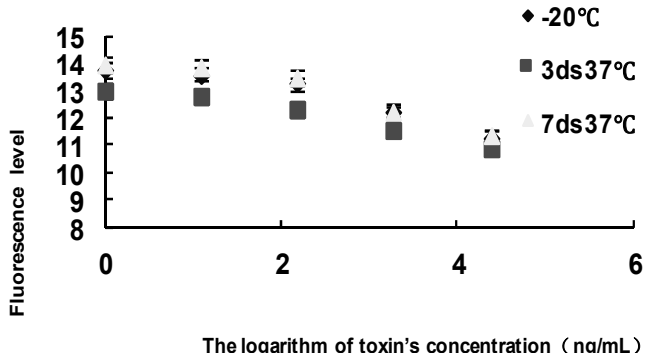

Fig.5 The competitive inhibition curve of standard toxin, after 3 days and 7 days on 37℃

2.2.3 Determination of the recovery rate

The recovery experiment was performed with three labeling concentrations (50%, 100%, and 200%), and the results are shown in Table 2. The recovery rate was 78.32%-116.76%, with an average recovery rate of 95.67% and an average coefficient of variation of 8.86%.

Tab.2 The recovery of standard addition with 3 levels

No.	50%	100%	200%
1	85.67%	90.45%	97.78%
2	107.69%	116.76%	104.43%
3	90.32%	89.29%	93.49%
4	95.41%	97.94%	90.49%
5	98.56%	94.76%	93.28%
6	80.65%	78.32%	92.19%
7	95.32%	103.56%	102.39%
8	97.21%	95.23%	105.17%
X	0.94%	0.96%	0.97%
SD	0.0831	0.1123	0.0588
CV	8.84%	11.69%	6.06%

X: average, SD: standard deviation, CV: coefficient of variation.

2.4 Validation through ELISA

Blind samples were tested using the kit (TRFIA) and ELISA methods. A Beacon kit was used to measure total FB_1, FB_2 and FB_3, while TRFIA was used for the specific detection of FB_1. The test results are shown in Table 3. For the quality control standards for the blind samples, the following results were obtained (mg/kg): FB_S: 2.7±0.06, FB_1: 2.0, FB_2: 0.5, and FB_3: 0.2. The Spearman correlation analysis of the data obtained through the two methods showed a correlation coefficient = 0.707 and P = 0.001.

Tab.3 The level of samples with different methods

No.	ELISA（ug/ml）	TRFIA（ug/ml）
1	0.114	0.0186
2	0.17	0.0263
3	0.141	0.0391
4	0.432	0.4304

5	0.205	0.4022
6	1.948	1.0718
7	0.091	0.0328
8	0.168	0.0369
9	0.077	0.0335
10	0.075	0.037
11	0.082	0.0921
12	0.106	0.0319
13	0.136	0.1779
14	0.252	0.5926
15	0.166	0.1863
16	0.155	0.1455
17	0.203	0.1273
18	6.648	3.19
19	0.034	0.0013

R=0.707，P=0.001

3 DISCUSSION

On the basis of the obtained anti-FB_1 monoclonal antibody, this study developed a detection kit for FB_1 through indirect competitive inhibition of TRFIA. The calibration curve was repeated three consecutive times, showing good stability, with a linear range of 2-512 ng/ml, a linear equation of Y = - 0.644X + 12.872 (R^2 = 0.9976), a minimum detectable concentration of 2 ng/ml, and a 50% inhibitory concentration of 10.07 ng/ml. The working concentration of the monoclonal antibody and the coating concentration for antigen detection were optimized. For stability, an accelerated destruction experiment was carried out at 37 °C for three days and seven days, and the results showed that the kit was valid for at least six months at 4 °C. A recovery experiment with three labeling concentrations was performed for the extracted samples, with a recovery of 78.32 %-116.76 % being obtained.

This TRIFA method was more sensitive than the traditional commercial ELISA kit and the newly-publicized method[16] , and was less time-consuming. As a new strategy for the detection of fumonisin B_1 in corn related samples, the TRIFA method is sensitive, accurate, and quick, which makes it suitable for the rapid detection of FB_1 in the field for agricultural and food-safety research.

Because pure subclasses of FBs are not available, experiments to examine the cross-reactions among FB_1, FB_2 and FB_3 et al. are not performed. However, this research work indicated that no cross-reaction with deoxynivalenol (DON) or aflatoxin (AFB1) was found. Using this kit and the Beacon ELISA kit (USA), 19 samples were tested, among which sample No. 18 was the standard blind sample for quality control (mg/kg): FB_S: 2.7±0.06, FB_1: 2.0, FB_2: 0.5, and FB_3: 0.2. To verify that no false positive reactions were produced using the kit, sample No. 19 was collected from organic corn, with all production and processing procedures being strictly controlled for the generation of mycotoxin, and the detection results obtained using the ELISA kit showed higher values compared with the TRFIA method, possibly because of the detection of total FB_1, FB_2 and FB_3. However, in the assessment of the standard blind sample for QC, the result obtained through TRFIA was closer to the quality control standards. The Spearman correlation analysis of the data obtained through two the methods revealed a correlation coefficient of R = 0.707 and p = 0.001, indicating some relevance of the test results using the two kits. As a future research direction, in order to fortify the comparability, specificity and correlation between the results of ELISA kit with TRFIA kit, monoclonal antibodies against FB_2 and FB_3 may be prepared, and detection kits based on the TRIFA principle as well as on ELISA mechanism might be established to test the level of FB_1, FB_2 and FB_3 respectively in the samples. Further, the test results can be integrated and compared with the results of the Beacon ELISA kit from the USA through correlation analysis. Alternatively, we could attempt to assess the detection methods and test kits for FB_1 from other authoritative laboratories and research institutions, to compare their test results with those generated by the kit developed in this study, thereby evaluating the practicality of the results of this study.

In contrast to the commercially available test kits for fumonisin, the method and kit established in this study are specific for the detection of FB_1 alone and accurately reflect the content of FB_1 in a sample. Based on the data obtained after the detection of a large number of samples, quality control measures, such as comparison of the results from different laboratories, can be applied for the detection of FB_1 contents in a sample. Hence, based on the detection results, combined with the toxicity data for FB_1, a national standard for the FB_1 content in grain and cereal products will be developed. In addition, all of the data obtained in this study were a result of the detection of FB_1 contents in grain products. Thus, this method will mainly be used to determine FB_1 contents in these products. After obtaining the content data and the results of analyses for other types of products, such as peanut oil, corn oil, and red wine, our method could also be used for the evaluation of these products.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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