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Original Article

Nanoparticle based bio-bar code technology for trace analysis of aflatoxin B1 in Chinese herbs

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ABSTRACT

A novel and sensitive assay for aflatoxin B1 (AFB1) detection has been developed by using bio-bar code assay (BCA). The method that relies on polyclonal antibodies encoded with DNA modified gold nanoparticle (NP) and monoclonal antibodies modified magnetic microparticle (MMP), and subsequent detection of amplified target in the form of bio-bar code using a fluorescent quantitative polymerase chain reaction (FQ-PCR) detection method. First, NP probes encoded with DNA that was unique to AFB1, MMP probes with monoclonal antibodies that bind AFB1 specifically were prepared. Then, the MMP-AFB1-NP sandwich compounds were acquired, dehybridization of the oligonucleotides on the nanoparticle surface allows the determination of the presence of AFB1 by identifying the oligonucleotide sequence released from the NP through FQ-PCR detection. The bio-bar code techniques system for detecting AFB1 was established, and the sensitivity limit was about 10^{-8} ng/mL, comparable ELISA assays for detecting the same target, it showed that we can detect AFB1 at low attomolar levels with the bio-bar-code amplification approach. This is also the first demonstration of a bio-bar code type assay for the detection of AFB1 in Chinese herbs.

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1. Introduction

Aflatoxins (AFs) which belong to a closely related group of secondary fungal metabolites, are toxic and highly carcinogenic substances [1,2]. AFs types include B1, B2, G1, or G2. The mould phenomenon occurred commonly in the cultivation, processing and storage period of medicinal materials, which may result in production of mycotoxins [3]. Mycotoxin contaminations caused by fungi are major issues related to the quality and safety of herbal medicine. AFs exposure remains an important aspect of herbal medicine safety which needs to be paid great attention. Failure to control such contamination may result in serious, even fatal, consequences for the consumers. Thus, some countries have set a limit for the amount of AFs that herbs may contain. In 2001, China promulgated the "Green Industry Standard for Import and Export of Medicinal Plants and Preparations" (WM2-2001), where AFs content limit was set as 5 μg/kg [4]. In 2008, the Food and Drug Administration of Korea issued a directive stipulating that

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Chinese herbs, including licorice, cassia, peach, Breit, Bozi, areca, Semen, Zhiyuan, and safflower, should have a maximum AFB1 amount of only 10 μg/kg [5]. In Italy a law sets guide-values AFB1 5 μg/kg and total AFs 10 μg/kg [6]. "Chinese Pharmacopoeia" 2015 version set the maximum limit of AFs in Cassiae, Polygalae radix, Platycladi semen, Peach, Ziziphi spinosae semen, etc, to 5 μg/kg [7]. As people are becoming more aware of the hazards of AFs and of the safety and quality of foods and drugs, the government has become stringent in regulating the AFs content, and thus called for the development of technology for the rapid detection of AFs. At present the mainly methods of detecting AFs are spectrometry, chromatography, immunoassay and biosensors [8–15]. However, these techniques produce limited sample throughput because of the time-consuming data extraction and cleansing.

The bio-bar code assay technology (BCA) was first introduced in 2003 by Mirkin, as a promising analytical tool for high sensitivity detection of proteins and nucleic acids [16]. The bio-bar code assay relies on a sandwich structure based on specific biological interaction of a magnetic particles and a nanoparticle with a defined biological molecule in a medium [17]. The magnetic particle allows the separation of reacted target molecules from unreacted ones. The nanoparticles aim at amplifying and detecting the target of interest. It uses magnetic microspheres to coat the target antiproteins; these beads have a diameter of up to a few microns [18]. BCA is a low-cost technology that exhibits high sensitivity for it uses gold nanoparticles for signal amplification. Each gold nanoparticle with a diameter of 30 nm can mark about 400 DNA barcode detection regions. Magnetic field causes the gold nanoparticles to form a complex with its target and magnetic microspheres. Subsequently, the DNA barcode chain is released from the gold nanoparticles, and these chains are detected through a variety of methods [19–23]. BCA exhibits the sensitivity of polymerase chain amplification technique, although it does not require enzymatic amplification reaction. The outstanding feature of this method is its high sensitivity, wherein it can detect antigenic concentrations as low as a few hundred molecules, the detection limit is lower than any existing methods [24,25]. Polymerase chain reaction was introduced in 1985 and has revolutionized biology and molecular biology since then. Its sensitivity allows the detection of 5–10 copies of DNA. However some drawbacks such as its complexity, time consuming procedure and narrow target have motivated the findings of new technologies. BCA technology is currently applied in the detection of nucleic acids and proteins; however, it is less applied in the detection of small molecules compounds [26–29].

This study established a highly sensitive novel technology for the rapid detection of AFB1. Similar to that of PCR, the sensitivity of BCA allows for the detection of biological molecules at a very low concentration. Thus, BCA may be used in the determination of AFB1, which exists in trace amounts in Chinese herbs. BCA may also be applied in the detection of other small molecule compounds. The linearity, accuracy, method precision, and system precision of BCA has already been validated. This is the first demonstration of a bar code type assay for the detection of AFB1.

2. Experimental

2.1. Materials and equipment

AFB1 polyclonal antibody and monoclonal antibody was prepared in ourselves lab. Chloroauric acid and sodium citrate from Sigma was used without further purification. Barcode DNA chain and complementary probe NP chain were purchased from Sangon Biotech (Shanghai). All reagents were prepared using analytical grade reagents and doubly distilled water. The gold nanoparticles were measured using transmission electron microscope (Hitachi High Tech International Trading Co. Ltd.). The magnetic microparticle MMP probes were measured using scan electron microscope (type JSM-7800F, JEOL Co.Ltd.). The barcode DNA chain was detected by fluorescence quantitative polymerase chain reactor (type H-7900, Biosystems Co., USA).

2.2. Preparation of gold nanoparticles

50 mL of boiling doubly distilled water was added to a 100 mL round bottom flask, and 0.5 mL 1% chloroauric acid solution was added quickly. With the high-speed stirring, 0.9 mL l% sodium citrate solution was added. Continue to boil until the solution turns red, and keep the red unchanged for 15 min. After cooling, the solution was constant volume in 50 mL volumetric flask, the products were stored in a dark place at 4 °C with 1% NaN_3. The gold nanoparticles were diluted to a certain concentration, and a small amount of them were put on the support film. The morphology of the gold nanoparticles was observed under transmission electron microscope (TEM).

2.3. Preparation of NP probe

A total of 10 mL of gold nanoparticles were collected, and their pH was adjusted to the optimum level use 0.1 mol/L K_2CO_3. About 1.3 mg of AFB1 polyclonal antibody was added, and stirring for 10 min. After 1 mL of 11% BSA was added dropwise, the mixture was left to stand for 30 min and then centrifuged at 4 °C. The precipitate was suspended to the original volume with 0.01 mol/L pH 7.6 PBS containing 1% BSA. After balancing overnight and centrifuging twice, the precipitate was suspended in 1 mL of PBS. A total of 1 mL of gold nanoparticles was decorated with AFB1 polyclonal antibody, and 500 μL of complementary probe NP chain with the concentration 2 μmol/L was added. The mixture was left to stand for 16 h at 25 °C, PBS solution was used to adjust the pH to 7.0, and ion concentration was increased use 0.1 mol/L NaCl. The solution was left to stand for 40 h at 25 °C. To obtain gold nanoparticles decorated with complementary probe NP chain after centrifugation for 30 min at 10 000 r/min, the precipitate was washed using 0.1 mol/L NaCl and 0.01 mol/L PBS (pH 7.0) three times, and then the unlabeled gold nanoparticles were deleted. A 500 μL solution of barcode DNA chain (concentration 2 μmol/L) was added to the above-mentioned gold nanoparticles decorated with complementary probe NP chain to hybridize for 4 h at 25 °C, and then centrifugation was performed for 30 min at 14 000 r/min to obtain the NP probe. The precipitate was then dissolved using 0.1 mol/L NaCl and 0.1 mol/L PBS (pH 7.0).

2.4. Preparation of MMP probe

The magnetic beads were lightly shaken for 5 min, and then 200 μL of these beads were collected to prepare a re-suspension with 2 mL of PBS, and 200 μL of PBS re-suspension magnetic beads was saved for later use. A total of 1.6 mL PBS was used to induce vigorous oscillation, and the supernatant was discarded by magnetic separation. The above steps were repeated four times. The solution was vigorously oscillated after adding 0.8 mL of 5% glutaraldehyde and then placed in a centrifugal tube in a nonmagnetic mixer to induce reaction for 3 h. The solution was vigorously oscillated again after adding 1.6 mL of PBS. The above steps were repeated four times after discarding the supernatant through magnetic separation.

A total of 60 μg of AFB1 monoclonal antibody was dissolved in 12 mL of PBS. The solution was vigorously oscillated after adding active magnetic beads and then placed in a mixer for mixing reaction of 20 h. The magnetic beads were re-suspended in 24 mL of PBS and vigorously oscillated after adding 12 mL of quenching agent (0.1 mol/L glycine). The solution was then placed in a mixer to enable reaction for 30 min. After washing three times with PBS, the supernatant was discarded through magnetic separation. Then, 1.6 mL of solution was used to resuspend the magnetic beads, and temperature was maintained at 4 °C.

2.5. Preparation of MMP-AFB1-NP sandwich structure

A total of 10 fg of AFB1 was added to 200 μL MMP probe with a vigorous oscillation at 37 °C for 1 h. Then fix MMP probe with a magnetic field and wash 4 times with PBS buffer solution in order to remove disconnected AFB1 antigen and other impure proteins. A total of 200 μL of gold nanoparticles decorated with double chain DNA and AFB1 polyclonal antibody were added, followed by vigorous oscillation for 30 min to form a sandwich structure. The MMP probe was fixed with magnetic field and washed four times with 400 μL of PBS buffer solution to remove disconnected NP probes.

2.6. Release of barcode DNA

A total of 100 μL of sterilized water was added to the sandwich structure, which was vigorously oscillated at 60 °C for 45 min to remove the hybrid from the barcode DNA chain. The MMP probe was then fixed with magnetic field, and the supernatant containing barcode DNA chain was collected. The bio-bar code assay allows for the amplification of the protein signal. Two binding events create a sandwich molecule. Bar-code DNA is released with the addition of water. The number of oligonucleotides released is proportional to the number of sandwich complexes formed. Here, the AFB1 isolated from the assay is detected, significantly amplifying the signal (Fig. 1).

2.7. FQ-PCR detection of barcode DNA chain

A Super Real Fluorescent quantitative premixed reagent detection kit (Tiangen Company) was used in this paper. The obtained barcode DNA chain was used as the template to enable FQ-PCR detection. FQ-PCR detection was performed using a two-step technique.

Primer 1: 5′-CGC ATT CAG GAT TGC ATG AT-3′
Primer 2: 5′-TAC GAC TTG ACA CCG TTA AG-3′
Barcode DNA chain: 5′-CGC ATT CAG GAT TGC ATG ATT GCC TCG TCT TAA CGG TCT CAA CTC GTA-3′
Complementary probe NP chain: 5′-TAC GAG TTG AGA CCG TTA AGA CGA GGC AAT CAT GCA ATC CTG AAT GCG A10-(CH2)6-SH-3′

2.8. Method validation

The method developed was validated for the detection of AFB1 in Chinese herb samples, including (Casslae semen, Polygalae

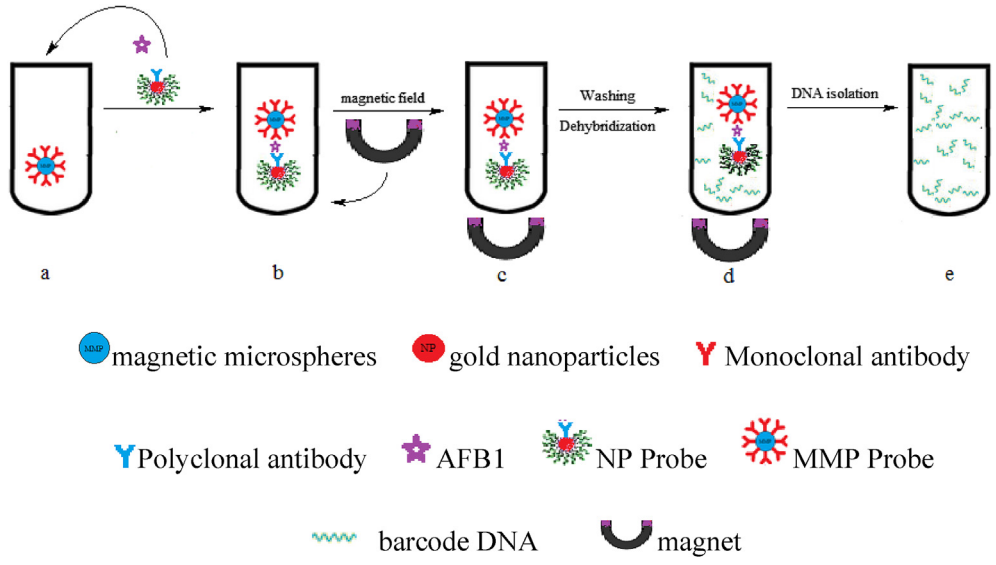

Fig. 1 – a: Interaction between MMP and target. b: Recognition between target and particles in complex biological medium: sandwich MMP-target-NP. c: Magnetic separation of MMP. d: Redispersion of sandwiches in distilled water causes dehybridization of bio-bar codes. e: Removal and analysis of bio-bar codes using FQ-PCR methods.

radix and Platycladi semen). Each sample was accurately weighed after drying into 10 mL centrifuge tubes and 2.5 mL of 75% methanol in water then added to extract AFB1 from the sample. After extraction, the solution was centrifuged 12 000 *g* for 12 min with Beckman Coulter XPN100 centrifuge (USA). The supernatant was filtrated and concentrated to 0.5 mL under a nitrogen steam. The residue was redissolved with 1 mL aqueous methanol solution and ready for FQ-PCR detection.

3. Results and discussion

3.1. *Identification of gold nanoparticles*

The colloidal gold was prepared by citrate sodium reduction method. The prepared gold nanoparticles were diluted to a certain concentration, and took a small amount added to the support film. After air drying, the gold nanoparticles, which were not muddy and had no sediment and flotsam, were observed under a transmission electron microscope. Observation through the transmission electron microscope revealed that the gold nanoparticles were round or oval with the same diameter, about 30 nm. UV–vis scanning was performed to prepare colloidal gold within the range of 200–800 nm. The highest absorption occurred at 520 nm. The results of TEM identification of gold nanoparticles, as shown in Fig. 2a, the morphology of gold nanoparticles are round or oval, the particle size is basically the same, the size is about 30 nm.

3.2. *Purification and identification of NP probe*

Excess electrolyte in the protein solution lowered the zeta potential of the colloid gold particles and affected protein absorption. Therefore, salt should be thoroughly removed before marking. Protein was dialyzed with low concentration saltwater (0.005 mol/L NaCl, pH 7.0) for 3 d, and the dialysate was changed every 6 h. The solution was then placed in a centrifuge at 12 000 r/min for 30 min at 4 °C, to remove the polymer. The unbalanced pH made the gold nanoparticles unstable. The optimal pH range was 7–8. To determine the optimal protein amount, >20% of the lowest stabilized amount obtained in the actual experiment was used because gold nanoparticles cannot be stabilized by an insufficient amount of AFB1 polyclonal antibody. Accordingly, 26 μg of polyclonal antibody was used in this experiment.

UV–vis scanning revealed that the λ_{max} of the NP probe was 528 nm. TEM revealed a gray black corona ring around the gold nanoparticles, showing that polyclonal protein was absorbed onto the particle surfaces (Fig. 2b). Previous studies have shown that the concentration of gold nanoparticles was about 0.106×10^{-11} mol/L.

3.3. *Preparation and determination of MMP probe*

Glutaraldehyde cross-linking method was used to activate the magnetic beads and examine the MMP probe using scan electron microscope (SEM). Before and after coupling reaction of the magnetic beads, the surface transformed from smooth to rough (Fig. 3). Magnetic bead coupling entirely proceeded

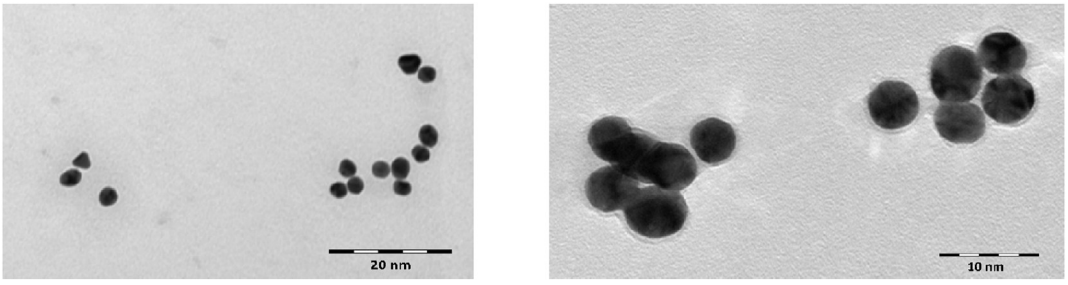

Fig. 2 – The TEM scan spectrum of gold nanoparticles (a) and NP probe (b).

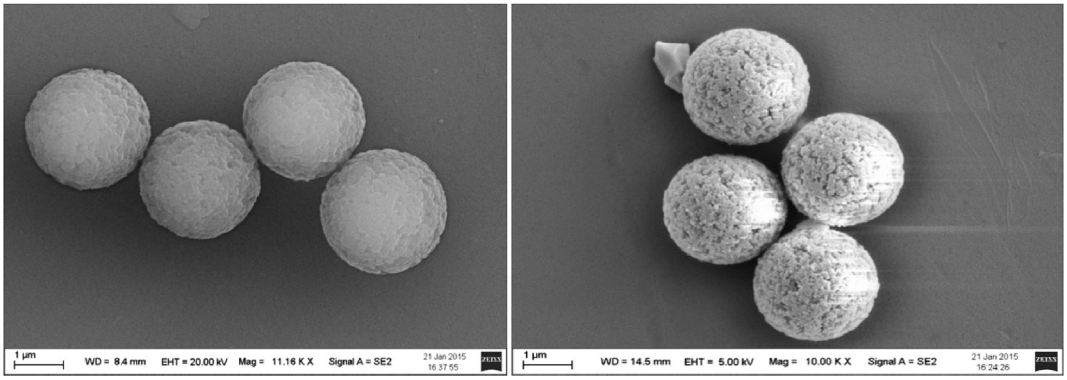

Fig. 3 – The SEM scan spectrum of magnetic beads and MMP probe. Note: Left—magnetic beads; Right—MMP probe.

in a mild environment. Changes in the surface indicated that coupling occurred between magnetic beads and AFB1 monoclonal antibodies. The entire process required a high magnetism for the beads. Therefore, washing was very critical to the process of activating and marking the beads, as well as to the formation of a sandwich structure and DNA release. Contaminated magnetic beads in any step can crucially affect the results. Only under high-temperature and low-salt conditions that double-chain DNA can be released. Therefore, the temperature should be controlled to obtain the DNA chain.

3.4. *Establishment of BCA detection system*

FQ-PCR amplification was conducted for the control sample diluted in a 10-fold gradient (10^{15}–10^{0} copies/μL). The standard amplification plot of FQ-PCR was showed on Fig. 4a. After the reaction was complete, the linear correlation between the Ct value and the copy number of the corresponding starting template was based to screen out five points (10^{15}–10^{11} copies/μL) within the linear range. At the same time, a better linear relationship was observed between them, and the standard curve was $y = -2.9054x + 54.581$ ($r = 0.9991$).

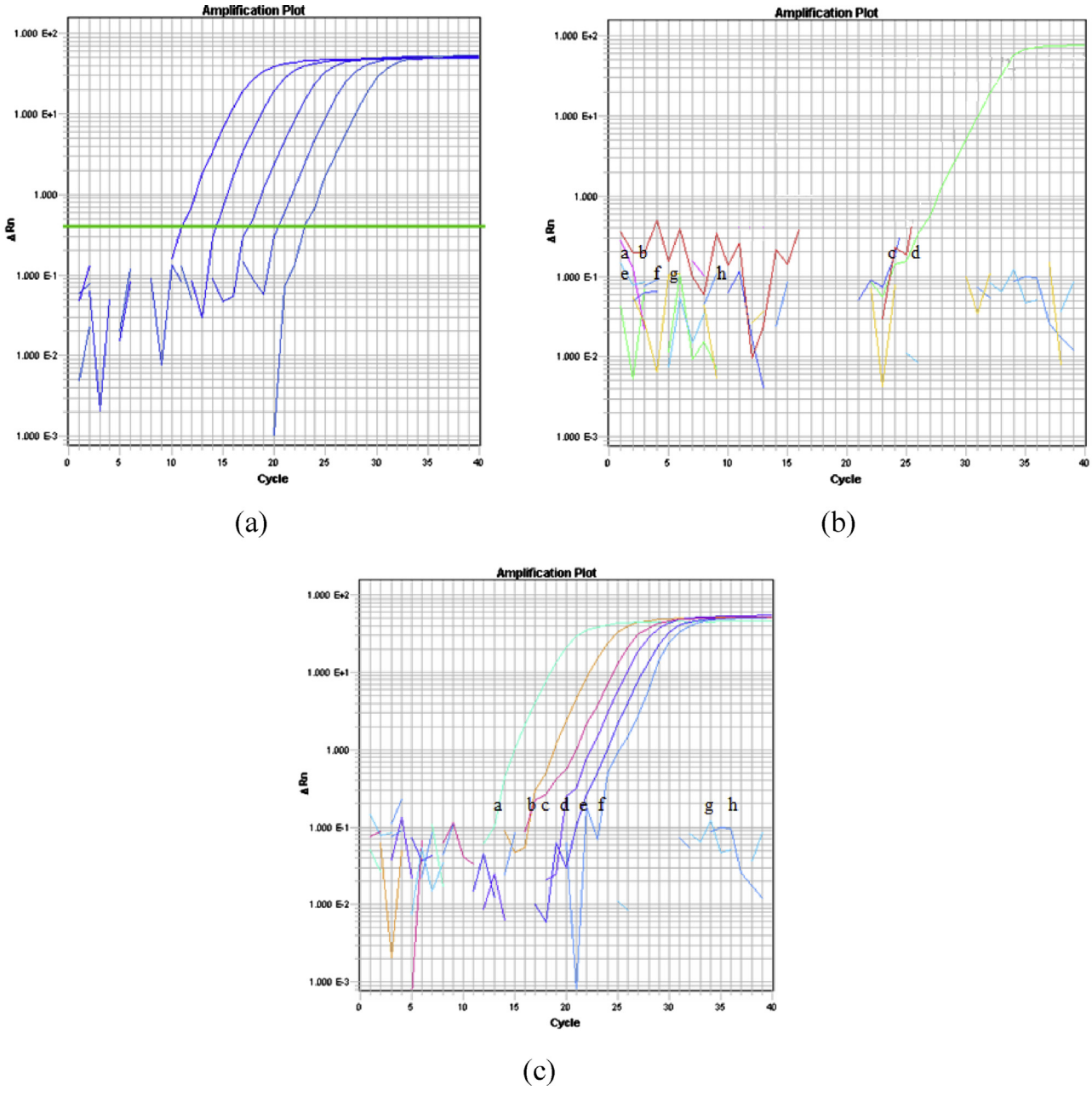

Fig. 4 – (a) The standard amplification plot of FQ-PCR. From left to right the copy numbers are 10^{15}, 10^{14}, 10^{13}, 10^{12} and 10^{11}. (b) The specificity test of FQ-PCR. a to h are AFB2, AFG1, AFG2, AFB1, Zearalenone, Vomitoxin, Fumonisins and Ochratoxin A respectively. (c) The sensitivity of FQ-PCR. The concentration of AFB1 are 10^{-3} ng/mL, 10^{-4} ng/mL, 10^{-5} ng/mL, 10^{-6} ng/mL, 10^{-7} ng/mL, 10^{-8} ng/mL, 10^{-9} ng/mL and 0 (a to h).

Table 1 – Determination of AFB1 spiked into Chinese herb samples.

Samples	Spiked concentration (ng/mL)	Mean recovery (%)	Intra-day repeatability[a]	Inter-day repeatability[b]
Casslae semen	2	94.3	3.26	3.28
	1	107.5	4.17	5.65
	0.2	93.2	5.84	7.26
Polygalae radix	2	92.5	2.90	3.45
	1	96.7	4.86	5.01
	0.2	104.5	4.34	6.52
Platycladi semen	2	90.6	2.69	4.33
	1	92.8	3.57	5.28
	0.2	95.1	5.38	8.63

[a] Intra-day repeatability was estimated by analysis of six replicate samples at two concentration level on the same day.
[b] Intra-day repeatability was estimated by analysis of six replicate samples at two concentration level in three day.

Table 2 – Comparison of the available methods for the detection of AFB1.

Method	Detection limit (ng/mL)	Sample Mean[a] ± SD[b] (ng/mL)			Reference
		Cassiae	Polygalae radix	Platycladi semen	
FQ-BCA	10^{-8}	0.302 ± 0.024	0.240 ± 0.017	0.151 ± 0.004	This work
HPLC	0.12	0.355 ± 0.047	0.297 ± 0.081	0.204 ± 0.053	[c]
ELISA	1.98	Not detected	Not detected	Not detected	[d]

[a] The mean of three experiments.
[b] SD = standard deviation.
[c] The results were obtained by HPLC using Pharmacopoeia of China method with modifications.
[d] The results were obtained using ELISA method with Wang TY, 2017.

3.4.1. *Repeatability*

To evaluate the practical applicability and accuracy of this method, it was validated for detection of AFB1 in Chinese herbs samples, which were spiked with AFB1 at 2 ng/mL, 1 ng/mL and 0.2 ng/mL concentrations levels. There recoveries were in the range of 90.6–107.5%. To calculate intra-assay and inter-assay repeatability, all measurements were done in triplicate. FQ-BCA detection results for different copy numbers showed that both of intra-assay and inter-assay CV values were <10%. The RSDs of the intra-day were in the range of 2.69–5.84%. Inter-day precision for each compound was also investigated with RSDs in the range of 3.45–8.63%. All these data revealed that the established method had an acceptable precision (Table 1).

3.4.2. *Specificity*

Seven mycotoxins were selected as interferences to determine the specificity of this method. 1 μg/mL of other six mycotoxins including zearalenone, vomitoxin, ochratoxin A, AFB2, AFG1, and AFG2 and 0.1 μg/mL AFB1 were examined respectively. Depends on the specificity of the monoclonal and polyclonal antibodies, AFB1 had no cross-reaction with zearalenone, vomitoxin, or ochratoxin A, AFB2, AFG1, and AFG2. The results indicated that the probes would not interact with other mycotoxins besides AFB1 (Fig. 4b).

3.4.3. *Sensitivity*

Following extensive experimentation with the FQ-PCR detection method, we were able to detect AFB1concentrations down to 10^{-8} ng/mL using the bio-bar code assay (Fig. 4c). FQ-PCR detection was performed for the barcode DNA chain obtained from release. This technique had high sensitivity for polymerase chain reaction, did not require enzyme amplification, and was able to detect antigenic substances with a wide concentration range, which have not been achieved by any existing quantitative immunoassay.

3.5. *Sample determination*

This method can be used for the trace determination of AFB1 in medicinal materials. FQ-PCR detection was performed for DNA released from the preparation of sandwich by using Chinese herb samples. As shown in Table 1, Casslae semen, Polygalae radix, Platycladi semen had cycles of 21.23, 21.62, and 21.11, respectively. Using the standard curve, the obtained copy numbers were $10^{11.48}$, $10^{11.38}$, and $10^{11.18}$, respectively, equal to AFB1 containing 0.302 ng/mL, 0.240 ng/mL, and 0.151 ng/mL. Accurate dropping of each reagent was very important; any tiny error can cause a large deviation through PCR magnification. To the best of our knowledge, this assay presents no serious hazards, though caution should be taken to avoid skin and eye contact with the AFB1 solution. In addition, when the assays are used in conjunction with unknown biological samples or known AFs samples, all proper government safety protocols should be followed. A highly sensitive method for detecting AFB1 through a nonenzymatic nanomaterials-based amplification method, the bio barcode assay is established. Compared with the currently available

instrumental and rapid screening methods, this method is more sensitive than ELISA [14,28] and HPLC [29] (Table 2). The experimental results demonstrate that the bio-bar code method can be used for rapid AFB1 detection.

4. Conclusions

A rapid and sensitive bio-bar code method for determination of AFB1 in Chinese herbs was well developed and validated, with the LOD at 10^{-8} ng/mL. This new method has been successfully applied to three different Chinese herbs. It is comparable to many PCR-based approaches without the need for enzymatic amplification. Because this method approach is a pseudohomogeneous system with both MMP and NP in solution, the probes can be used to very efficiently bind target, thereby reducing the time required for high sensitivity detection experiments. The results have laid important foundation for further improvement of detection standards for AFs in Chinese herbs and provided a new vision for the trace determination of other small molecular compounds. Indeed, an advantage of the method approach over conventional microarray sandwich assays is that the entire assay can be carried out in 3–4 h, regardless of target concentration. In consequence, the suggested technique could be a powerful method for AFB1 detection in the future.

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