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Development of loop-mediated isothermal amplification assay for rapid screening of fungal contamination in pepper and paprika powder

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RESEARCH ARTICLE

Abstract

Fungi cause hazards such as mildew, spoilage, and mycotoxins, so the Chinese government has formulated maximum level limits (MLs) for fungi in spices. However, the current standard culture-based detection method is time-consuming (5-7 days) and unsuitable for the rapid on-site screening of fungal contamination to facilitate immediate control measures. Therefore, we developed a loop-mediated isothermal amplification (LAMP) assay for the rapid (<4 h) screening of fungi at levels greater than or equal to those of Chinese MLs (10^4 cfu/g) in spices, without requiring any expensive instruments. Contaminated samples could be identified based on colour change that was visible to the naked eye. The specificity based on spiked sample experiments and comparisons with the traditional culture-based method demonstrated that this LAMP method could be applicable to the on-site testing of pepper and paprika powder samples, and it could also be used by laymen. Furthermore, this method could be applied to the rapid screening of fungal contamination in other spices, foods and feeds. In addition,Tween-20 was included in the assay to enhance the collection of fungus from sample suspensions.

Keywords: maximum level limits, 18S rRNA, colour change, cut-off

1. Introduction

Fungi have worldwide distributions and grow in a wide range of habitats. They can also cause mildew and spoilage of spices, grains, feeds and fruits (Li *et al.*, 2004; Xie *et al.*, 2001). Several fungi are toxigenic and produce mycotoxins with a variety of toxic effects including carcinogenicity and teratogenicity, which could directly harm the health of people (Frisvad *et al.*, 2006). Thus, the level of fungal contamination is an important indicator of the hygienic quality of foods and feeds. Spices, such as pepper and paprika, are popular condiments because of their distinctive flavours, colours and aromas. The fungal contamination of spices cannot be ignored in most countries where spice production and consumption occur. Previous investigations have shown that the level of fungal contamination in Arabic red pepper and ginger exceeded 10^3 cfu/g (Hashem and Alamri, 2010), whereas that in chilli powder reached 10^4-10^6 cfu/g in Kunming (Zhang *et al.*, 1998). Our own investigation and research by Liu detected fungal contamination levels of 10^3-10^4 cfu/g in pepper and paprika (Liu and Xu, 1998). These levels of fungal contamination have prompted the Chinese Ministry of Agriculture (MOA) to establish the maximum level limits (MLs) of <10^4 cfu/g for fungi in spices (MOA, 2006). Therefore, the rapid screening and analysis of fungal contamination is required, so that immediate control measures can be taken.

The rapid screening and analysis of fungal contamination demands a reliable detection method. At present, standard detection methods, such as the bacteriological analytical manual of the U.S. Food and Drug Administration (Tourans *et al.*, 2001), GB/T 4789.15 (MOH, 2010) and ISO 21527-1 (ISO, 2008), are based on the traditional culture-based

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method, which has the advantages of simple operation and the isolation of strains via cultivation. However, the detection period (5-7 days) required by the traditional culture-based method may be unsuitable for rapid screening. Over the course of 5-7 days, the level of fungal contamination in foodstuffs changes dramatically, which means that timely information cannot be obtained to guide the control of fungal contamination (Zhao *et al.*, 2010). Molecular biology methods such as PCR can allow rapid detection, but they require sophisticated instrumentation, as well as elaborate and complicated assay procedures. These requirements may not be suitable for spice factories. Thus, there is a growing demand for a simple and economical form of molecular testing that could be applied in the field.

Loop-mediated isothermal amplification (LAMP) is a novel DNA amplification method developed by Notomi *et al.* (2000). This method is based on an autocyling and strand displacement DNA synthesis, which uses the large fragment of *Bst* DNA polymerase and a set of four specially designed primers. LAMP assays have been demonstrated to be sufficiently robust for field applications to detect pathogenic bacteria and viruses. Sun *et al.* (2010), Luo (2008) as well as Niessen and Vogel (2010) have developed LAMP assays for pathogenic fungi, aflatoxigenic strains and *Fusarium graminearum*, respectively. The amplification principle employed by LAMP allows the synthesis of large quantities of target DNA with a visible by-product. Thus, there are two ways of evaluating LAMP reactions: agarose gel electrophoresis and the visual observation of a white precipitate or colour change. Visual inspection is particularly convenient for field applications.

In the present study, we developed a simple LAMP assay for detecting fungal contamination with a specific cut-off (10^4 cfu/g). This assay allowed the amplification of genomic DNA from fungi after a 60-min reaction at 65 °C in a water bath. Positive amplification products could be identified based on a colour change from violet to sky blue using the naked eye. This LAMP method was successfully applied to the rapid screening of paprika and pepper powder samples based on the MLs of 10^4 cfu/g for fungi. Furthermore, this method could be applied to other spices, foods and feeds.

2. Materials and methods

Strains and media

Table 1 shows the eleven reference strains or isolates of bacteria, and the 89 reference stains or isolates of fungi used in this study. With the exception of *Candida albicans*, the fungal cultures were maintained as working cultures on CDM at 28±1 °C for 5 days. The bacterial cultures were grown on Luria-Bertan agar, MacConkey agar, or nutrient agar at 37±1 °C for 18 h.

DNA preparation

Three millilitre of 0.85% sterile normal saline (NS) was added to agar slants of fungal stains and homogenised using a vortex mixer (XW 90A; Luxi, Shanghai, China P.R) for 1 min. The concentration of the fungal suspension was adjusted using a haemocytometer (Improved Neubauer; Yuhuan Qiujing Corporation, Zhejiang, China P.R.) and 1 ml of this suspension was transferred to a 1.5 ml Eppendorf tube containing 100 µl sterile 0.1% Tween-20. This mixture was vortexed for 1 min and centrifuged for 5 min at 21,000×*g* in ambient temperature conditions (MicroCL 21; Thermo Scientific, Waltham, MA, USA). The precipitate was used to obtain fungal genomic DNA with a fungal genomic DNA extraction kit (BSC14M1; Bioflux, Tokyo, Japan). The DNA was extracted from bacterial strains using a bacterial genomic DNA extraction kit (Tiangen, Beijing, China P.R.).

Primer design and LAMP amplification

The primers were designed based on conserved sequences at the fungal kingdom level by blasting the 18S rRNA genes of fungi (data not shown). A 274-bp sequence was selected as the target. All of the primers were designed using the Primer Explorer V.4 software tool (http://primerexplorer.jp/elamp4.0.0/index.html; Eiken Chemical Co. Ltd., Tokyo, Japan). The sequences of the primers were listed as follows:

F3: 5'CAAAGTCTTTGGGTTCTGG3'
B3: 5'CCAACTAAGAACGGCCAT3'
FIP: 5'CAAATTAAGCCGCAGGCTCCTATGGTCGCA AGGCTGAA3'
BIP: 5'CTCAACACGGGGAAACTCACCCACCATCCA AAAGATCAAGAA3'
LF: 5'GCCCTTCCGTCAATTTCTTTAAGT3'

LAMP was performed using a 25 µl reaction volume, which contained 0.2 µM each of F3 and B3 primers (Life Technologies, Shanghai, China P.R.), 1.6 µM each of FIP and BIP (Invitrogen), 1.2 µM of LF (Life Technologies), 1.8 mM dNTPs (Roche, Basel, Switzerland), 1× ThermoPol Buffer (New England Biolabs, Ipswich, MA, USA), 0.9 M betaine (Sigma, Hamburg, Germany), 2 mM $MgSO_4$ (New England Biolabs), 12 U of the *Bst* DNA polymerase large fragment (New England Biolabs), 80 µM HNB (Spectrum, Gardena, CA, USA) and 2 µl of the DNA extract as the template. The LAMP reaction was incubated at 65 °C in a conventional water bath (HH.S11-2; Beijing ChangAn Company Scientific Instruments, Beijing, China P.R.) for 60 min, followed by heating at 85 °C for 2 min to terminate the reaction.

Table 1. Strains and cultures used in the present and the loop-mediated isothermal amplification (LAMP) amplification results.

Genus Species	Strains	Media	LAMP	Genus Species	Strains	Media	LAMP
Enterococcus				*Penicillium*			
faecalis	ATCC 29212[a]	LB[i]	–	*funiculosum*	MIG3.104[f]	CDM	+
Staphylococcus				*viridicatum*	CGMCC3.4517	CDM	+
aureus	ATCC 6538	nutrient agar	–	*citrinum*	As 3.2788[g]	CDM	+
Salmonella				*citrinum* (4 isolates)	PHLTSI 511001-4	CDM	+
enteritidis	CMCC 50335[b]	MAC[j]	–	*roqueforti*	PHLTSI 511101	CDM	+
Shigella				*islandicum*	PHLTSI 511201	CDM	+
flexneri	CMCC 51061	MAC	–	*simplicissimum*	PHLTSI 511301	CDM	+
Escherichia				*resedanum*	PHLTSI 511401	CDM	+
coli	ATCC 8099	LB	–	*pinophilum*	PHLTSI 511501	CDM	+
coli (O157:H7)	ATCC 882364	MAC	–	*Fusarium*			
coli (EAEC)	PHLTCI 5501[c]	MAC	–	*verticillioides*	PHLTFI 5512[h]	CDM	+
coli (EHEC)	PHLTCI 5502	MAC	–	*verticillioides*	PHLTSI 511601	CDM	+
coli (ETEC)	PHLTCI 5502	MAC	–	*tricinctum*	PHLTSI 512001	CDM	+
coli (EPTC)	PHLTCI 5502	MAC	–	*graminearum*	PHLTFI 5513	CDM	+
coli (EIEC)	PHLTCI 5502	MAC	–	*Trichoderma*			
Aspergillus				sp. (4 isolates)	PHLTSI 513001-4	CDM	+
parasiticus	CGMCC3.0124[d]	CDM[l]	+	*Alternaria*			
versicolor	CGMCC3.4416	CDM	+	sp. (5 isolates)	PHLTSI 514001-5	CDM	+
niger	ATCC 16404	CDM	+	*Rhizopus*			
niger (17 isolates)	PHLTSI 510001-17[e]	CDM	+	sp. (5 isolates)	PHLTSI 515001-5	CDM	+
flavus	ATCC 28539	CDM	+	*Mucor*			
flavus (19 isolates)	PHLTSI 510101-19	CDM	+	sp.	PHLTSI 516001	CDM	+
fumigatus (6 isolates)	PHLTSI 510201-6	CDM	+	*Ceotruchum*			
terricola	PHLTSI 510301	CDM	+	sp.	PHLTSI 517001	CDM	+
albicans (2 isolates)	PHLTSI 510401-2	CDM	+	*Candida*			
nidulans	PHLTSI 510501	CDM	+	*albicans*	ATCC 10231	SDA[k]	+
oryzae (2 isolates)	PHLTSI 510601-2	CDM	+				
clavatus (3 isolates)	PHLTSI 510701-3	CDM	+				
ochraceus	PHLTSI 510801	CDM	+				

[a] ATCC = American Type Culture Collection, Manassas, VA, USA.
[b] CMCC = National Center for Medical Culture Collections, Beijing, China P.R.
[c] PHLTCI = Clinical Isolates of Department of Public Health Laboratory Technology, Sichuan University, Chengdu, China P.R.
[d] CGMCC = China General Microbiological Culture Collection Center, Beijing, China P.R.
[e] PHLTSI = Spice Isolates of Department of Public Health Laboratory Technology, Sichuan University, Chengdu, China P.R.
[f] MIG = Guangdong Institute of Microbiology, Guangdong, China P.R.
[g] As = Guangdong Academy of Sciences, Guangdong, China P.R.
[h] PHLTFI = Food Isolates of Department of Public Health Laboratory Technology, Sichuan University, Chengdu, China P.R.
[i] LB = Luria-Bertan Agar, Beijing Land Bridge Technology Co., Ltd., Beijing, China P.R.
[j] MAC = MacConkey Agar, Beijing Land Bridge Technology Co., Ltd., Beijing, China P.R.
[k] SDA = Sabouraud Dextrose Agar, Beijing Land Bridge Technology Co., Ltd., Beijing, China P.R.
[l] CDM = Czapek Dox Medium, Beijing Land Bridge Technology Co., Ltd., Beijing, China P.R.

Observation of LAMP amplification products

The colour changes in the LAMP amplification products were observed using the naked eye. The LAMP amplification products were also analysed by 3% agarose (GenView, Houston, TX, USA) gel electrophoresis (PowerPac Basi, Biorad, Hercules, CA, USA) and by measuring the absorbance of the LAMP reaction solution at 650 nm (λ_{max} of HNB = 650 nm) (Goto *et al.*, 2009). The validities of the LAMP amplification products were confirmed using the restriction enzyme *Fnu4HI* (New England Biolabs). The enzyme reaction mixture comprised a total volume of 20 µl, which

contained 2 µl 10×NEB buffer, 0.5 µl LAMP amplification products and 0.5 µl *Fnu4HI* (5 U/µl). The reaction mixture was incubated in a conventional water bath at 37 °C for 18 h.

Specificity and sensitivity (cut-off) of the LAMP assay

DNA extracts from eleven bacterial strains and 89 fungal strains were used as templates. The positive control was DNA from *Aspergillus niger* (ATCC 16404). A reaction mixture without fungal DNA was used as the negative control. The sensitivity (cut-off) was tested using two methods. First method was using a dilution series of spiked samples in paprika matrices, which ranged from 1.1×10^1 to 1.1×10^6 cfu/ml. The fungal genomic DNA was obtained from spiked samples using the extraction kit mentioned in section 2.2. Second, a ten-fold dilution series of PGM-T recombinant plasmid (Tiangen) was produced to yield 10^6 to 10^0 copies per reaction. The insert fragment of the target DNA used in PGM-T recombinant plasmid was the PCR amplification product of *A. niger* (ATCC 16404) DNA, which was generated by the F3 and B3 primers. The LAMP analysis was conducted using the method described in section 2.3. The LAMP reactions were repeated thrice with each template concentration.

Application to spice samples from an agricultural products market

Fifty pepper and 48 paprika powder samples were randomly collected from an agricultural products market in Chengdu during April 2012 and January 2013. Samples weighing 25 g were added to 225 ml sterile 0.85% NS in sterilised homogeneous bags (Beijing Land Bridge Technology Co. Ltd., Beijing, China P.R.) and homogenised thoroughly using homogenisers (BA-1S; Benang Company, Shanghai, China P.R.) for 2 min. One millilitre of the homogeneous mixture was transferred to a 1.5-ml Eppendorf tube, which contained 100 µl sterile 0.1% Tween-20. The mixture was vortexed for 1 min and centrifuged for 5 min at 21,000×*g* in ambient temperature. The precipitate was washed one time by the mixture of 1 ml 0.85% NS and 100 µl 0.1% Tween-20, before it was used to obtain fungal DNA from samples, as described in section 2.2. The positive control spice samples were inoculated with strains of *A. niger* (ATCC 16404), *Penicillium citrinum* (As 3.2788), *Fusarium verticillioides* (PHLTFI 5512) and *C. albicans* (ATCC 10231), and incubated at 28±1 °C for 5 days. The negative controls were the sterile spice samples without fungal DNA. The LAMP analysis of samples was conducted as described in section 2.3. One millilitre of the homogeneous mixture was added to 15 ml Rose Bengal Medium (Beijing Land Bridge Technology Co. Ltd.) and incubated at 28 °C for 5 days, followed by colony counting by traditional culture-based method (MOH, 2010).

3. Results

The LAMP assay required 60 min in a water bath at 65 °C, and the LAMP products were visualised using the naked eye. Positive reactions produced a sky blue colour, whereas negative reactions remained violet. In the verification tests of the LAMP amplification products, the 3% agarose gel electrophoresis produced ladder-like patterns of positive LAMP products (Gel Logic200; Kodak, Rochester, NY, USA) (Figure 1A). The ladder-like patterns of positive reactions were digested to yield three DNA fragments of 74 bp, 133 bp and 214 bp using *Fnu4HI* enzyme (Figure 2). These enzyme digestion results were consistent with the expectations and demonstrated the validity of LAMP amplification. The specificity assay showed that positive amplification occurred with fungal strains but not with bacteria (Table 1). The sensitivity (cut-off) was 1.1×10^3 cfu/ml (spiked sample) (Figure 1A) and 100 copies/reaction (data not shown). The corresponding absorbance values for the LAMP reaction solution at 650 nm also indicated that the sensitivity (cut-off) was 1.1×10^3 cfu/ml (spiked sample) (Figure 1B) and 100 copies/reaction (data not shown). A comparison of the LAMP assay and colony counting using the traditional culture-based method showed that there was 90.0% agreement for the 50 pepper powder samples and 89.6% agreement for the 48 paprika powder samples (Table 2).

4. Discussion

The aim of the present study was to develop a LAMP assay for the rapid screening of fungal contamination in spices. Thus, a conserved sequence was required at the fungal kingdom level. The identification of fungi based on nucleic acid amplification usually targets ribosomal genes because of the high copy numbers of rRNA genes. The rRNA genes of fungi include 18S rDNA, 5.8S rDNA, 28S rDNA, 5S rDNA and internal transcribed spacer (ITS). Of these, 18S rDNA, 5.8S rDNA and 28S rDNA are highly conserved and can be used to identify members of the kingdom fungi and different genera (Zhao *et al.*, 2010). However, ITS sequences have a high degree of variation even between closely related species, so ITS sequences are used widely for determining the taxonomy and molecular phylogeny of fungal species. Thus, we blasted 18S rDNA, 5.8S rDNA and 28S rDNA sequences from fungi, and selected a 274-bp sequence of 18s rDNA as the target for the LAMP primers. The blast results using GenBank (http://www.ncbi.nlm.nih.gov/genbank) and the specificity experiments showed that a set of five primers yielded satisfactory specificity and amplification efficiency.

In this study, we added Tween-20 to facilitate the more effective collection of fungal cells from fungal suspensions and from homogeneous mixtures of spice samples. The spores and hyphae of some dry fungi, such as *Aspergillus*

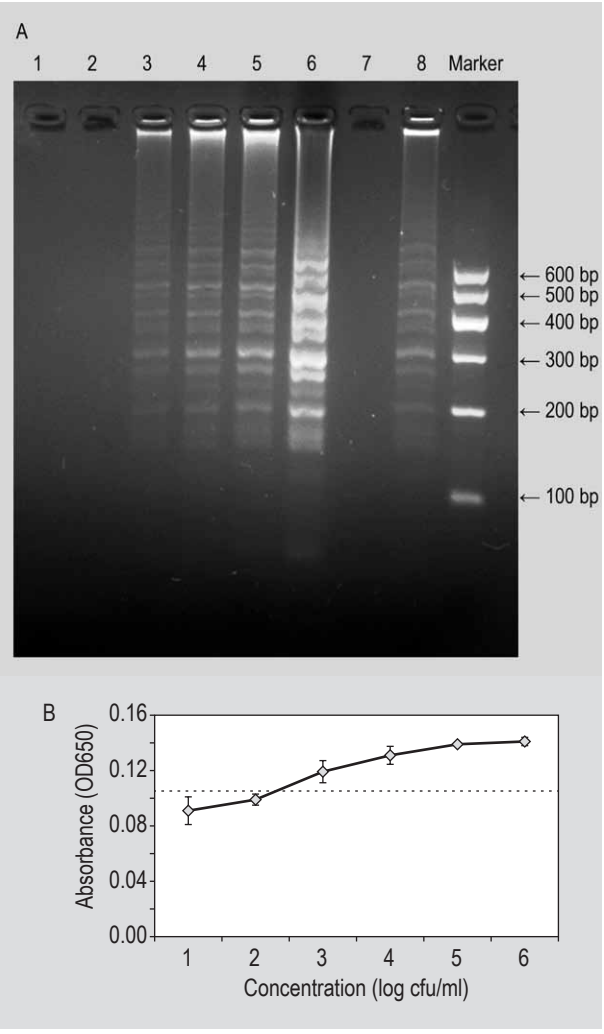

Figure 1. Sensitivity (cut-off) analysis of loop-mediated isothermal amplification (LAMP) for spiked samples. (A) 3% agarose gel electrophoresis of LAMP products. (B) optical density (OD) values measured at 650 nm by ultraviolet spectrophotometer (Shimadzu, Tokyo, Japan). The average result for each concentration of spiked sample was evaluated from three independent tests and represented as the mean ± standard deviation. The horizontal line indicates the OD650 threshold value (0.126) that was calculated by adding two standard deviations to the mean of the negative reaction. 1: 11 cfu/ml; 2: 1.1×10^2 cfu/ml; 3: 1.1×10^3 cfu/ml; 4: 1.1×10^4 cfu/ml; 5: 1.1×10^5 cfu/ml; 6: 1.1×10^6 cfu/ml; 7: positive control; 8: negative control.

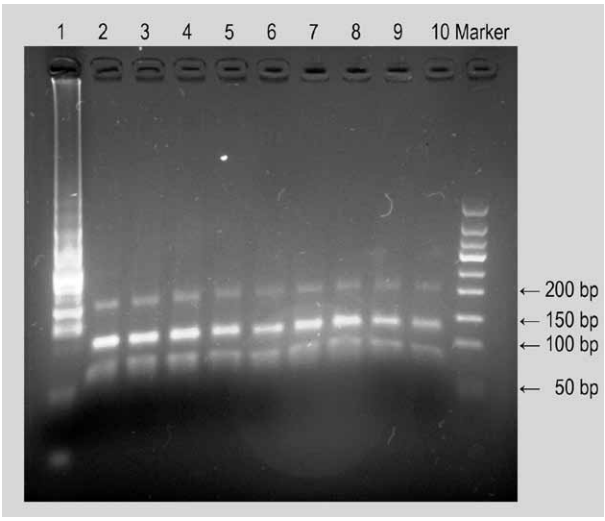

Figure 2. 3% agarose gel electrophoresis of loop-mediated isothermal amplification (LAMP) products after digestion with *Fnu4HI* restriction enzyme. Line 1: LAMP products; 2-10: digestion products of LAMP products by *Fnu4HI* with three DNA fragments of 74 bp, 133 bp and 214 bp.

Table 2. Comparison of the loop-mediated isothermal amplification (LAMP) assay and colony counting using the traditional culture-based method with pepper and paprika powder samples.[1]

	Traditional culture-based method (cfu/g)					
	Pepper powder			Paprika powder		
LAMP	≥10^4	<10^4	Total	≥10^4	<10^4	Total
+	21	4	25	22	3	25
–	1	24	25	2	21	23
Total	22	28	50	24	24	48

[1] 90.0% [(21+24)/50×100%] agreement for pepper samples and 89.6% [(22+21)/48×100%] agreement for paprika samples.

spp. *and Penicillium* spp., are quite light and readily blow away in the wind. This is also a reason why fungi are characterised by worldwide distributions. We found that these light spores and hyphae floated on the surface of the supernatant or adhered to the walls of Eppendorf tubes after high-speed centrifugation. After removing the supernatant, therefore, the quantity of fungal mass available for extracting fungal DNA was decreased. Moreover, the adherence of spores and hyphae to the walls of tubes did not facilitate the effects of the fungal lysis buffer. To avoid reductions in the fungal DNA extraction efficiency, it was necessary to develop a method to ensure that the light spores and hyphae precipitated at the bottom of tubes after centrifugation. Therefore, we tested several reagents to improve the precipitation efficiency, including ethanol absolute, lacto phenol fuchsin, Tween-20 and liquid paraffin. Tween-20 was the most efficient reagent for enhancing the precipitation of light spores and hyphae, while it did not inhibit the DNA extraction and LAMP amplification procedures. Tween-20 is a polysorbate surfactant with good stability and very low toxicity, which allows it to be used as a detergent and emulsifier in a number of foods, cosmetics and biotechnical applications. Tween-20 can be

dissolved or dispersed in water, alcohol and other polar organic solvents. The effective concentration range for Tween-20 in this LAMP method was 0.01-0.1%.

It is necessary to explain the cut-off value applied to solid spice samples. In section 2.6, 25 g samples were homogenised with 225 ml sterile 0.85% NS and 1 ml of the homogeneous mixture were used for LAMP detection. Thus, the pre-treatment of the solid spice samples caused a tenfold decrease in the fungal concentration. Therefore, if the level of fungal contamination was 10^4 cfu/g in solid spice samples, the actual level of fungus analysed using LAMP was only 10^3 cfu/g or 10^3 cfu/ml. Therefore, the cut-off value (1.1×10^3 cfu/ml) used for spiked samples actually represented 1.1×10^4 cfu/g for the solid spice samples. The cut-off value in this study was 1.1×10^4 cfu/g for spices, but the cut-off value could be modified for specific requirements by selecting different LAMP reaction conditions of LAMP. During the development of the LAMP technique, researchers found that the amplification time required to reach a specified degree of turbidity had a linear relationship with the quantity of the initial template (Mori *et al.*, 2004). Thus, a lower mass of fungal template requires a longer reaction time to yield a positive result. We increased the reaction time from 60 min to 70 min and the cut-off value was increased from 10^4 cfu/g to 10^3 cfu/g for solid spice samples. This demonstrated that it may be possible to establish different cut-offs by specifying different reaction conditions for particular requirements. In addition, the fungal DNA extraction efficiency needs to be demonstrated with different spices before this LAMP method can be applied widely to other spices, foods and feeds.

5. Conclusions

This LAMP assay has high potential as a simply, rapid, cost-effective and efficiently method for screening paprika and pepper powders to detect fungal contamination with MLs $\geq10^4$ cfu/g. The LAMP reaction was completed within 60 min in a water bath at 65 °C and the results could be assessed based on a visually inspection of a colour change. Compared with the traditional culture-based method, this LAMP method could be more practical for the rapid on-site screening of fungal contamination. In addition, it has the potential to be applied to other spices, foods and feeds.

Acknowledgements

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