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การตรวจหาตัวบ่งชี้ทางพันธุกรรมของเชื้อรา *Aspergillus flavus* ที่สร้างอะฟลาทอกซิน B1 จากตัวอย่างข้าวโพดอาหารสัตว์ด้วยเทคนิค Loop-mediated isothermal amplification (LAMP)

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บทคัดย่อ: การปนเปื้อนอะฟลาทอกซินในข้าวโพดอาหารสัตว์ซึ่งเป็นหนึ่งในผลผลิตทางการเกษตรนับเป็นปัญหาสำคัญที่เกิดขึ้นทั่วโลกโดยเฉพาะในเขตร้อนชื้นเช่นประเทศไทย การศึกษาครั้งนี้นำตัวอย่างข้าวโพดที่มีการติดเชื้อราตามธรรมชาติมาเพาะเลี้ยง และพิสูจน์เชื้อ *Aspergillus flavus* โดยการตรวจหายีน ITS, Beta-tubulin and calmodulin genes ด้วยปฏิกิริยาพอลีมอเรส (Polymerase chain reaction; PCR) ร่วมกับพิสูจน์เชื้อ *A. flavus* ชนิดที่สามารถสร้างอะฟลาทอกซินB1 (AFB1) โดยการตรวจหายีนสี่ชนิดคือ *Ver1, Omt1, Nor1*, *aflR* ซึ่งเป็นยีนที่ควบคุมการสร้าง AFB1 ด้วยวิธี PCR และตรวจยืนยันการสร้าง AFB1 จากเชื้อที่แยกได้โดยตรงด้วยวิธี high performance liquid chromatography (HPLC) จากนั้นนำเทคนิค Loop-mediated isothermal amplification (LAMP) ซึ่งใช้ในการเพิ่มขยายและตรวจหายีนเป้าหมายมาทดสอบในเชื้อราที่แยกได้ โดยใช้ไพรเมอร์ที่จำเพาะต่อยีนทั้งสี่ชนิดดังกล่าว และใช้สารฟลูออเรสเซนต์ calcein เติมก่อนการเพิ่มปริมาณยีน พบว่าสภาวะที่เหมาะสมของปฏิกิริยา LAMP คือ การใช้อุณหภูมิคงที่ 65°C เป็นเวลา 1 ชั่วโมง เมื่อสิ้นสุดปฏิกิริยาสามารถอ่านผลได้ด้วยตาเปล่า มีความแตกต่างของสีอย่างชัดเจนระหว่างผลบวกและผลลบ โดยภายใต้แสงธรรมชาติผลบวกให้สีเหลืองและผลลบให้สีส้ม ส่วนภายใต้แสง UV ผลบวกจะเรืองแสงสีเขียว วิธีนี้มีความไวในการตรวจที่ความเข้มข้นดีเอ็นเอ 10^{-6} (89 pg/µl) ส่วนวิธี PCR มีความไวในการตรวจที่ความเข้มข้นดีเอ็นเอ 10^{-5} (0.0089 ng/µl) สามารถตรวจยืนยันผลผลิตจาก LAMP โดยการทำ agarose gel electrophoresis ที่ความเข้มข้นร้อยละ 1 จากนั้นนำเทคนิค LAMP นี้ไปใช้ในการตรวจข้าวโพดอาหารสัตว์ที่เก็บจากฟาร์มของเกษตรกรจำนวน 50 ตัวอย่าง พบผลบวก 18 ตัวอย่าง (36%) ผลลบ 32 ตัวอย่าง (64%) โดยมีความจำเพาะร้อยละ 100 เมื่อเปรียบเทียบกับวิธี multiplex PCR นอกจากนี้เมื่อวิเคราะห์หาความสัมพันธ์ระหว่างผลของ LAMP กับการตรวจวิเคราะห์ AFB1 ด้วยวิธี HPLC โดยใช้ค่าสัมประสิทธิ์สหสัมพันธ์เพียร์สันพบว่ามีความสัมพันธ์ทางบวกอย่างมีนัยสำคัญทางสถิติ (r = 0.83, P<0.05) แสดงว่าตัวอย่างข้าวโพดที่ให้ผลบวกด้วยเทคนิค LAMP มีความเสี่ยงสูงที่จะมีโอกาสปนเปื้อน AFB1 ดังนั้นเทคนิค LAMP ที่ใช้ในการศึกษาครั้งนี้สามารถตรวจหาตัวบ่งชี้ทางพันธุกรรมของเชื้อ *A. flavus* ที่สร้าง AFB1 จากตัวอย่างข้าวโพด โดยเป็นวิธีที่ง่ายและรวดเร็ว มีความไวและความจำเพาะสูง สามารถนำไปใช้เป็นวิธีการทดสอบความเป็นไปได้และความเสี่ยงของการปนเปื้อนอะฟลาทอกซินจากอาหารในปศุสัตว์ได้

คำสำคัญ: อะฟลาทอกซิน B1 *Aspergillus flavus* ข้าวโพด Loop-mediated isothermal amplification (LAMP) ยีนที่ควบคุมการสร้างอะฟลาทอกซิน B1

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Detection of Aflatoxin B1 Producing *Aspergillus flavus* Genes from Maize Feed using Loop-mediated Isothermal Amplification (LAMP) Technique

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Abstract: Aflatoxin contamination in maize feed, one of several agriculture crops grown for livestock feeding, is still a problem throughout the world mainly under hot and humid weather conditions like Thailand. In this study *Aspergillus flavus* (*A. flavus),* the key fungus for aflatoxin production especially aflatoxin B1 (AFB1), isolated from naturally infected maize were identified and characterized according to colony morphology and PCR detected on ITS, β-tubulin and calmodulin genes. The isolates were analysed for the presence of four aflatoxigenic biosynthesis genes in relation to their capability to produce AFB1, *Ver1, Omt1, Nor1*, and *aflR*. AFB1 production of the isolates was then confirmed using high performance liquid chromatography (HPLC) technique. A loop-mediated isothermal amplification (LAMP) was applied as an innovative technique for rapid detection of target nucleic acid. The reaction condition was optimized at 65°C for 60 min and calcein fluorescent reagent was added before amplification. The LAMP results showed clear differences between positive and negative reactions in end point analysis under daylight and UV light by the naked eye. In daylight, the samples with AFB1 producing *A. flavus* genes developed a yellow color, but those without the genes retained the orange color. When excited with UV light, the positive samples become visible by bright green fluorescence. LAMP reactions were positive after addition of purified target DNA until dilutions of 10^{-6} (89 pg/µl) whereas PCR reactions were positive after addition of purified target DNA until dilutions of 10^{-5} (0.0089 ng/µl). The reaction products were then confirmed and visualized with 1% agarose gel electrophoresis. To test the LAMP utility, 50 maize samples were collected from livestock farms and tested for the presence of four aflatoxigenic biosynthesis genes. The results were positive in 18 samples (36%) but negative in 32 samples (64%). All of the samples were rechecked by multiplex PCR and the results were the same as LAMP, indicating 100% specificity. Additionally, when assessing LAMP results with the HPLC aflatoxin analysis results using correlation coefficient, there was a significant correlation between the two methods (r = 0.83, P<0.05) which suggested that positive maize samples were likely to be a high- risk feed. In conclusion, the LAMP technique in this study can provide a simple and rapid approach for detecting AFB1 producing *A. flavus* genes from maize and appeared to be a promising tool for the prediction of potential aflatoxigenic risk in livestock feedings.

Keywords: Aflatoxin B1, *Aspergillus flavus*, maize, Loop-mediated isothermal amplification (LAMP), Aflatoxigenic biosynthesis genes

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Introduction

Aspergillus flavus (A. flavus) is one of the major producers of the well-known carcinogenic aflatoxins. Based on their fluorescence under UV-light (blue or green), the four major aflatoxins are call aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) (Squire, 1981). Not all strains of *A. flavus* are aflatoxin-producing, several strains are non-toxigenic because aflatoxin synthesis may become unstable in these fungi (Bennett and Christensen, 1983; Rodrigues *et al*., 2007). Aflatoxin-producing strains of *A. flavus* can produce AFB1 and AFB2 (Horn and Corner, 1999). Among them, AFB1 is of great concern because of its toxic effects such as carcinogenicity and immunosuppression in human and animal. Contamination by AFB1 can occur in several agricultural products and commonly found in maize. AFB1 production on maize feed appeared to be greatly influenced by environment under hot and humid weather conditions and it may cause serious consequences to animal health (Hussein and Brasel, 2001). Several reports have shown the impact of AFB1 on animal health including pigs (Harvey *et al*, 1989; Lindemann *et al*., 1993), poultry birds (Espada *et al*., 1992) and dairy cows (Fink-gremmels., 2008).

Analytical methods for detection of aflatoxin contamination in food and feedstuffs have been carried out including thin-layer chromatography (TLC), immunochemical methods and high-performance liquid chromatography (HPLC). One of the major chromatographic methods used for determination of AFB1 is HPLC. The HPLC technique makes use of a stationary phase and a mobile phase which flow through the solid absorbent. While HPLC is considered the gold standard for AFB1 analysis, it requires expertise, extensive sample preparation and expensive equipment (Wacoo *et al*., 2014).

Conventional methods used to distinguish between aflatoxin producing and non-aflatoxin producing strains of *A. flavus* are based on cultural and analytical methods (Bennett and Papa, 1988; Hamed *et al*., 2004). These methods are time-consuming, labor-intensive and need the expertise of mycologists to avoid misidentification. To date, molecular methods have been introduced as a powerful tool for detecting and identifying aflatoxin producing strains of *A. flavus* (Shapira *et al*., 1996). Recently, it has been demonstrated that 25 identified genes clustered in 70 Kb DNA regions in the chromosome of aflatoxin producing strains of *A. flavus* are involved in the biosynthetic pathway of AFB1 and the sequences of these genes appear to be highly conserved in *A. flavus* (Yu *et al*., 2004). Therefore, PCR

detection of aflatoxin biosynthetic gene presence has been used as a diagnostic tool for aflatoxin producing strains of *A. flavus* (Giesen, 1998; Levin, 2012). However, it needs sophisticated instruments like thermal cycler to maintain different temperature and a long detection period. These limitations affect their applications to use in the low-technology laboratory.

Loop-mediated isothermal amplification (LAMP) was originally described as a specific, rapid and cost-effective method by Notomi *et al*. (2000). This novel nucleic acid amplification method relies on auto cycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase. All reactions can be conducted under isothermal conditions ranging from 60 to 65 C°. The specificity of the reaction is very high because it uses four primers recognizing six distinct regions on the target DNA. The amplification products are stem-loop DNA structures with several inverted repeats of the target and cauliflower-like structures with multiple loops and can be detected by visual assessment of turbidity or fluorescence of the reaction mixture (Mori *et al*., 2001).

For these reasons, LAMP has been used as a powerful tool for the detection of bacteria (Iwamoto *et al*., 2003; Zhuang *et al*., 2014), virus (Zhou *et al*., 2011; Zhang *et al*., 2011) and parasites (Kuboki *et al*., 2003; Ikadai *et al*., 2004) but only few application of LAMP have been used for detecting and identifying mycotoxin producing fungi (Niessen *et al*., 2013). The aim of this study was to apply LAMP technique for the detection of AFB1 producing *A. flavus* genes in maize feed samples collected from livestock farms, and compared its sensitivity and specificity with results from PCR. The correlation between LAMP and HPLC aflatoxin analysis results were assessed using correlation coefficient.

Materials and methods

Fungal isolation and identification:

1) Media and culture conditions

A. flavus tested in this study were isolated from five naturally infected samples selected from contaminated stored maize. They were directly cultured on Petri dishes containing Yeast Extract Sucrose (YES) Agar. Petri dishes were incubated at 25° C for 7 days at pH 7. Macromorphological observation of fungal colonies was done based on the study of Varga *et al*. (2011).

2) Aflatoxigenic ability of the isolates

For the determination of AFB1 production in YES agar, aflatoxin extraction was carried out as follows. After incubation, the entire culture was blended and extracted with 100 mL of 80% methanol. The extraction solution was filtered through a glass micro

fiber filter (VICAM, Milford, MA, USA). Two mL of the filtered extraction from YES agar were applied to the immunoaffinity cleanup column AflaTest (VICAM, Milford, MA, USA). Then, the column was washed by 5 mL of distilled water for two times and aflatoxins were eluted with 1 mL of methanol. The eluted extract was dried under gentle air stream and the residue redissolved in 0.25 mL of HPLC mobile phase. AFB1 was determined by HPLC/fluorescence detector set at an excitation wavelength of 365 nm and an emission wavelength of 435 nm (Agilent 100 Series, Agilent Technology, Santa Clara, CA, USA).

3) Multiplex PCR analysis of fungal isolates

The purified fungal genomic DNA was extracted using DNA extraction kit (Biotech rabbit GmbH, Neuendorf str. 24a 16761 Hennigsdorf, Germany) according to the manufacturer's instructions. DNA samples were resuspended in 100 µl of sterile distilled water at a concentration of 5 ng μL^{-1}.

For multiplex PCR analysis of *A. flavus*, amplification of a part of the ITS, β-tubulin and calmodulin genes were performed using the primer sets as shown in Table 1. Each multiplex PCR reaction contained 10 ng of template DNA, 12.5µl of 1X multiplex PCR, 1µM of forward and reverse primers and 25 µl of final volume. Thermal cycling conditions were: initial denaturation temperature of 95°C for 5 minutes, followed by 30 cycles of denaturation temperature of 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72° C for 30 seconds. Final DNA extension was at 72°C for 10 minutes and then 4°C was set at the holding temperature for storage.

4) Multiplex PCR analysis of Aflatoxin B1 producing strain of fungal isolate

Multiplex PCR assay for direct identification of pure culture of aflatoxin-producing *A. flavus* was carried out for four genes (Ver1, Omt1, Nor1 and aflR) as these genes are considered as indicators of AFB1 production (Shapira *et al*., 1996, Farber *et al*., 1997 and Rahimi *et al*., 2008). The primers used for multiplex PCR detection of the four genes are shown in Table 2.

Multiplex PCR reaction conditions were optimized with a 10 µL reaction mixture containing 1 unit of Taq polymerase, 0.6 µM of each published forward and reverse primers, 0.2 µM dNTP solution mix, 1 µL 10x buffer with 20 mM $MgCl_2$, and 1 µL of DNA template of aflatoxin-producing *A. flavus* (DNA template from AFB1 nonproducing strain of *A. flavus* which was isolated from this study and water were used as negative control). Thermal cycling conditions were: initial denaturation temperature of 95°C for 5

minutes, followed by 30 cycles of denaturation temperature of 95°C for 30 seconds, annealing at 65 °C for 30 seconds and extension at 72 °C for 30 seconds. Final DNA extension was at 72°C for 10 minutes, then 4 °C was set at the holding temperature for storage. PCR products were viewed using agarose gel electrophoresis at 1% gel concentration.

5) LAMP reaction solution and optimization

LAMP assay for direct identification of pure culture of AFB1 producing *A. flavus* was tested. We used primer sets designed by Nagamine *et al*. (2002) with the nucleic acid sequences as shown in Table 3.

The LAMP assay was carried out in a total of 25 µl reaction mixture. The reaction solution was modified from the protocol outlined by Niessen and Vogel (2010). It contained 2.5 µl of 10X Thermopol buffer, 2 µl of primer mix (10µM of FIP, BIP, F3, B3, LoopF and LoopB primer), 6mM $MgSO_4$, 0.4mM Betaine, 1mM of dNTP, 1.5 µl of calcein, 8U *Bst* DNA polymerase and DNA template of AFB1 producing *A. flavus.* DNA template from AFB1 non-producing strain of *A. flavus* and water were used as negative control. The reaction mixture was incubated in a Thermoblock at 60 °C - 65°C for 1 h.

Assay sensitivity (limit of detection) was determined by using 10-fold serial dilutions of the total genomic DNA of AFB1 producing strain of *A. flavus* ranging from 10^{-1} to 10^{-7} were prepared and amplified with the four gene sets of primers as described above. The experiments were repeated three times. Detection limits were defined as the lowest concentrations that tested positive in all three repeats.

Table 1 The primer sets for detection of *Aspergillus flavus*

Genes	Primer pair	Primer sequence (5'>3')	References
Internal Transcribe spacer	ITS1	TCC GTA GGT GAA CCT GCG G	White *et al*., 1990
	ITS2	TCC TCC GCT TAT TGA TAT GC	White *et al*., 1990
	V9G	TTA CGT CCC TGC CCT TTG TA	Masclaux *et al*., 1995
	LS266	GCA TTC CCA AAC AAC TGC ACT C	Masclaux *et al*., 1995
β-tubulin (Ben A)	Bt2a	GGT AAC CAA ATC GGT GCT GCT TTC	Glass & Donaldson., 1995
	Bt2b	ACC CTC AGT GTA GTG ACC CTT GGC	Glass & Donaldson., 1995
Calmodulin (CaM)	CMD5	CCG AGT ACA AGG ARG CCT TC	Hong *et al*., 2005
	CMD6	CCG ATR GAG GTC ATR ACG TGG	Hong *et al*., 2005
	CF1	GCC GAC TCT TTG ACY GAR GAR	Peterson *et al*., 2005
	CF4	TTT YTG CAT CAT RAG YTG GAC	Peterson *et al*., 2005

Table 2 PCR primers used for the four genes involved in aflatoxin B1 biosynthesis

Primer pair	Gene	Primer sequences (5'>3')	Optimal Annealing Temp. (°C)	PCR product size (bp)	References
VER-F	Ver-1	ATGTCGGATAATCACCGTTTAGATGGC	65	895	Shapira *et al*., 1996
VER-R		CGAAAAGCGCCACCATCCACCCCAATG			
OMT-F	Omt-1	GGCCCGGTTCCTTGGCTCCTAAGC	65	1024	Shapira *et al*., 1996
OMT-R		CGCCCCAGTGAGACCCTTCCTCG			
Nor-F	Nor-1	ACCCGTACGCCGGCACTCTCGGCAC	65	400	Farber *et al*., 1997
Nor-R		GTTGGCCGCCAGCTTCGACACTCCG			
aflR-F	aflR	CGCGCTCCCAGTCCCCTTGATT	65	630	Rahimi *et al*., 2008
aflR-R		CTTGTTCCCCGAGATGACCA			

Table 3 Loop-mediated isothermal amplification primers for the detection of Aflatoxin B1 used in this study

Primer sets	Primer sequences (5'>3')	Reference
FIP-Afla	TAGACCTGCTTGAGCACGCCATGAGGGAGGCTGGTATCC	Nagamine *et al*., 2002
BIP-Afla	AGGTCAGCAAGGGCAACATCCGGCCCAGGAGTAGTCGATAG	
F3-Afla	ACCGCTGTTGCTAAGAACAA	
B3-Afla	TTACGGACGAGACCGAGC	
LoopF-Afla	ATGTCCTCAAAGGTCTCGGG	
LoopB-Afla	GAGCCTGTTCCCCCTAAGAT	

6) Observation of LAMP products

Lamp amplified products were analyzed by electrophoresis on 1% agarose gels and photography.

Maize sample analysis:

1) LAMP assay in spiked maize and maize samples

Fifty samples of maize feed were collected from various livestock farms. Spike maize samples were inoculated by immerge in suspension of AFB1 producing *A. flavus* for 3 days and then used as ~~a~~ positive controls whereas maize samples without AFB1 producing *A. flavus* as well as water were used as negative controls). DNA extraction was performed by the same method as above.

LAMP reactions for the presence of four aflatoxigenic biosynthesis genes also were performed by the same method described above.

2) Multiplex PCR analysis

DNA extraction from the same maize samples used for LAMP assay, with and without inoculation with AFB1 producing *A. flavus* were performed by multiplex PCR using the same method as described above.

3) HPLC analytical method for detection of AFB1

Maize samples were finely ground using a household electric grinder and the ground samples were subjected to solvent extraction with 100 mL of 70% methanol. The extraction solution was filtered through a glass micro fiber filter (VICAM, Milford, MA, USA). Two mL of the filtered extraction were applied to the immunoaffinity clean up column AflaTest (VICAM, Milford, MA, USA). The column was then washed with 5 mL of distilled water for two times and aflatoxins were eluted with 1 mL of methanol. The eluted extract was dried under gentle air stream and the residue re-dissolved in 0.25 mL of HPLC mobile phase. AFB1 was determined by HPLC/fluorescent detector set at an excitation wavelength of 365 nm and the emission wavelength of 435 nm (Agilent 100 Series, Agilent Technology, Santa Clara, CA, USA).

4) Statistical analysis:

Statistical analysis of the relationship between LAMP and HPLC results in 50 maize samples was done using IBM SPSS Statistic (ver. 22, NIDA, Thailand).

Results

Cultural and morphological observations:

The fungal colonies on YES agar were grown well, appeared after 2-4 days and mostly reached all plate within 7 days as shown in Fig. 1. The colonies were first white and rapidly became typical yellow-green colonies of *A. flavus* (Varga *et al*., 2011).

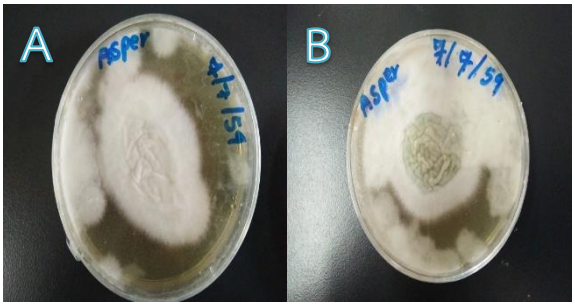

Fig. 1 Characteristic white and yellow-green color change by fungal colonies on Yeast Extract Sucrose (YES) agar

Aflatoxin B1 production of the fungal colonies

The chemical analysis by HPLC revealed that the fungal colonies in this study were able to produce AFB1.

Multiplex PCR identification of Aflatoxin B1 producing strain of fungal isolate

The yellow-green colony was identified as AFB1 producing *A. flavus*

whereas the white colony was identified as non-AFB1 producing *A. flavus*.

PCR identification of the fungal isolates

Multiplex PCR identification of Aflatoxin B1 producing strain of fungal isolate

The yellow-green colony was identified as AFB1 producing *A. flavus* whereas the white colony was identified as non-AFB1 producing *A. flavus*.

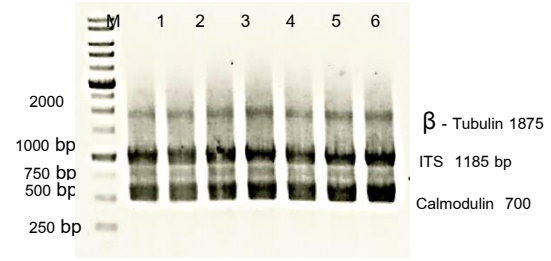

Fig. 2 PCR product of ITS, β-tubulin and calmodulin genes of *A. flavus* were amplified. M: DNA size marker; Lane1-2; The white colony of fungal isolates; Lane 3-7; the yellow-green colony of fungal isolates.

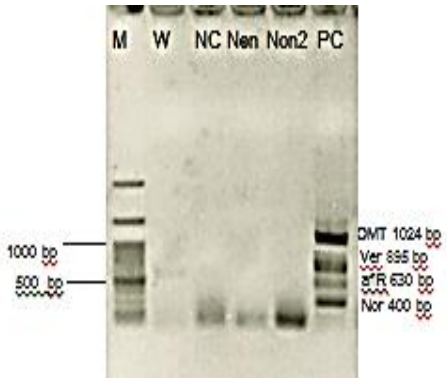

Fig. 3 PCR products of *Omt1, Ver1*, *aflR* and *Nor1* were amplified. M: DNA size marker; W: water; NC: buffer and water; Non1, Non2: the white colony of fungal isolate; PC: the green colony of fungal isolates.

Multiplex PCR identification of the fungal isolates

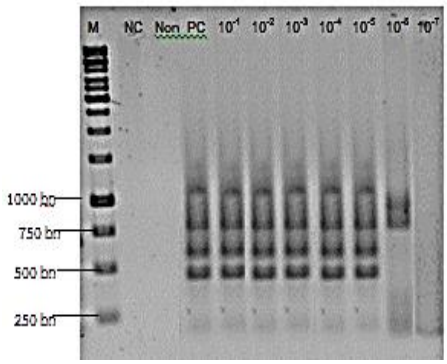

Fig. 4 Multiplex PCR reactions were positive after addition of purified target DNA until dilutions of 10^{-5} (0.0089 ng/µl)

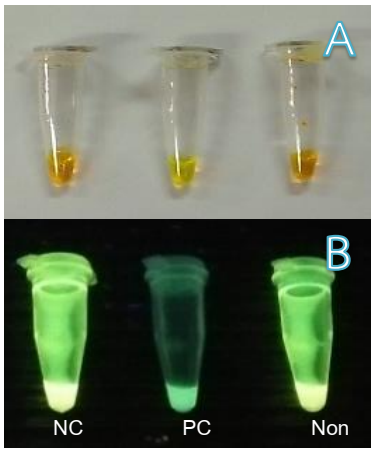

Fig. 5 (A) The LAMP positive result showed yellow color and the negative result showed orange color in daylight. (B) The LAMP positive result showed bright green color under UV light. Negative control (NC) = the tube without DNA template, Positive control (PC) = tube with AFB1 producing *A. flavus*, Non = tube with non AFB1 producing *A. flavus.*

LAMP results of pure culture

The LAMP results showed clear differences between positive and negative reactions in end point analysis under daylight and UV light by the naked eye as shown in Fig. 5

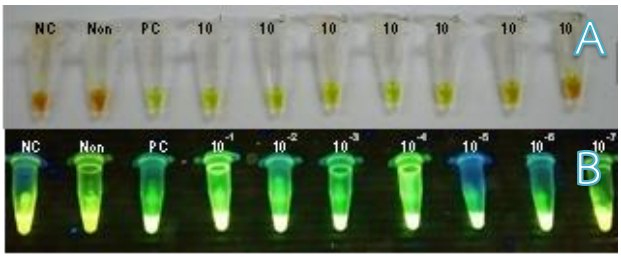

Fig. 6 LAMP reactions were positive after addition of purified target DNA until dilutions of 10^{-6} (89 pg/µl). A = under daylight, B= under UV light.

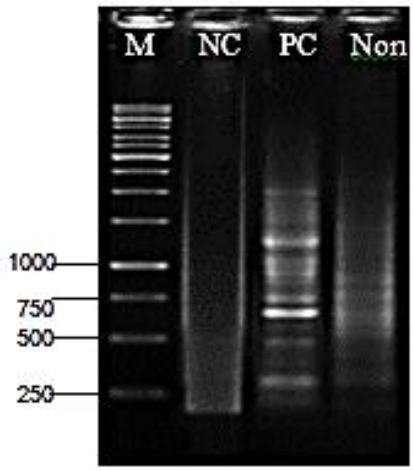

Fig. 7 Electrophoresis analysis of LAMP products of pure culture. Left to right: lane M, DNA size marker; lane NC, negative control (water); lane PC, positive sample; lane Non, non-AFB1 producing *A. flavus*

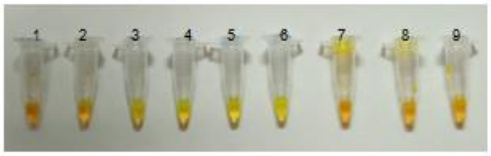

Fig. 8 LAMP results of maize samples: No. 1= water; No 2= negative control maize sample No.3 = spiked maize sample; No. 4, 5 and 6: positive results (yellow color) and No. 7, 8 and 9: negative results (orange color) under daylight

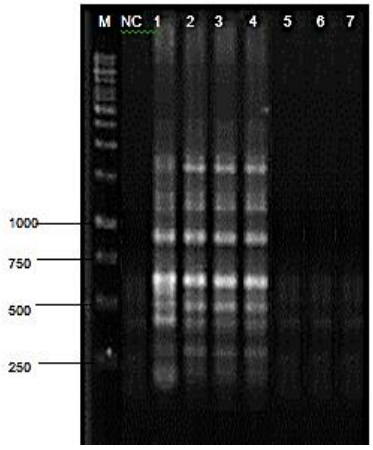

Fig. 9 Electrophoresis analysis of LAMP products of maize samples. Left to right: lane M = DNA size marker; lane NC= water, lane 1= Spiked maize samples 2, 3, 4 = positive sample; lane5, 6, 7=negative maize sample

Assessment of the results obtained with LAMP assays and HPLC analysis using Pearson's correlation coefficient showed that the correlation coefficient (r) equals 0.83, indicating a strong relationship between the two methods (r = 0.83, P<0.05).

Table 4 LAMP and multiplex PCR detection of four aflatoxin B1 biosynthesis genes and HPLC detection of aflatoxin B1 in 50 maize samples

No.	Samples	LAMP	PCR	HPLC
1	A1	-	-	-
2	A2	-	-	-
3	A3	-	-	-
4	A4	+	+	+
5	B1	+	+	+
6	B2	-	-	-
7	B3	-	-	-
8	B4	-	-	-
9	B5	+	+	+
10	C1	-	-	-
11	C2	-	-	-
12	C3	-	-	-
13	C4	-	-	-
14	D1	-	-	-
15	D2	+	+	+
16	D3	+	+	+
17	D4	+	+	-
18	E1	+	+	-
19	E2	+	+	+
20	E3	+	+	+
21	E4	-	-	-
22	F1	-	-	-
23	F2	-	-	-
24	F3	+	+	+
25	F4	+	+	+
26	G1	+	+	-
27	G2	-	-	-
28	G3	-	-	-
29	G4	-	-	-
30	H1	-	-	-
31	H2	-	-	-
32	H3	-	-	-
33	H4	+	+	+
34	I1	+	+	+
35	I2	-	-	-
36	I3	-	-	-
37	I4	-	-	-
38	J1	+	+	+
39	J2	-	-	-
40	J3	-	-	-
41	J4	-	-	-
42	K1	-	-	-
43	K2	-	-	-
44	K3	-	-	-
45	K4	+	+	+
46	L1	+	+	+
47	L2	+	+	-
48	L3	-	-	-
49	L4	-	-	-
50	L5	-	-	-

+: positive; -: negative.

A, B, C, D, E, F, G, H, I, J, K and L represented different livestock farms.

Discussion

In this study, a range of methods was used to detect aflatoxigenic *A. flavus* including cultural, analytical and molecular methods. The identification of *A. flavus* is complicated because of the similarities of morphological characters with *A. parasiticus* and other closely related species (Klich and Pitt, 1988). According to Gqaleni *et al*. (1996) YES agar is a great medium for the biosynthesis of AFB1. For this reason, YES agar was selected to culture the aflatoxigenic potential of *A. flavus.* To identify the toxin production, the culture was directly extracted and AFB1 was identified by HPLC. Thus, the result of this study suggested that the use of YES agar at 25 ºC, at pH of 7 and with an incubation period time of 7 days was suitable

to detect the aflatoxigenic potential of *A. flavus*. However, differentiation of aflatoxin producing and non-aflatoxin producing strains of *A. flavus* is very important because conventional methods like cultural and analytical methods are not entirely reliable (Criseo *et al*., 2001). Furthermore, these methods are time-consuming, require facilities and sometimes they fail to detect some aflatoxin producing strains because instability of aflatoxin production may occur in certain toxigenic strains growing in culture media (Abarca *et al*., 1988). Therefore, multiplex PCR method using three genes (Table 1) ITS, β-tubulin and calmodulin for different target regions is an alternative approach to confirm *A. flavus* (Shapira *et al*., 1996; Haugland *et al*., 2002).

The successful amplification of the three genes (Fig. 2) can be used for species identification but do not confirm aflatoxin production. For this reason, multiplex PCR has been used for distinguishing aflatoxin producing and non-aflatoxin producing *A. flavus* by targeting one or several genes in AFB1 biosynthesis (Levin, 2012; Yin *et al*., 2009). In the present study, the use of multiplex PCR with four sets of primers (Table 2) to amplify AFB1 biosynthetic genes, Ver1, Omt1, Nor1 and aflR was performed in pure culture of aflatoxin producing and non-aflatoxin producing *A. flavus*. The result demonstrated that multiplex PCR assay with aflatoxin producing isolate exhibited the complete sets of genes whereas the non-aflatoxin producing isolate lacked the PCR products (Fig. 3). Here LAMP assay for direct identification of pure cultures of aflatoxin producing *A. flavus* were tested and accomplished using the primer set given in Table 3. The reaction condition was optimized at 65°C for 60 min. The addition of calcein fluorescent reagent before the LAMP reaction enhanced the determination of a positive result. Calcein is a colorimetric indicator which the color gradually changes from orange to yellow or green color. Thus, instead of inspection the white turbidity resulted from magnesium pyrophosphate accumulation, the clear difference color between positive and negative reactions to the LAMP results in end point analysis could successfully make a distinction between infected samples and negative ones. Interestingly, LAMP can produce reliable product even under lower DNA concentration (10^{-6} dilution or more).

Maize is prone to infection by *A. flavus* and contamination with aflatoxins (Medina, 2004). The samples of naturally contaminated maize feed were analyzed during the current study. All samples were collected from various livestock farms and tested for the presence of the four AFB1 biosynthesis genes using LAMP

and multiplex PCR together with data on presence or absence of AFB1 by HPLC in corresponding samples. Results given in Table 4 showed aflatoxin producing *A. flavus* was presented in 18 out of 50 samples (36%). Thirty-two of the samples (64%) were found to be uninfected. The samples that showed positive by LAMP also showed positive by multiplex PCR, indicating 100% specificity when compare LAMP to multiplex PCR. The results from this study showed that LAMP tested in both pure cultures and in naturally contaminated maize samples can be complete within 60 min with about 0.0089 ng/µl DNA concentration while multiplex PCR needed at least 3 hr with about 89 pg/µl DNA concentration to complete the reaction. In Addition, when assessing LAMP results with the HPLC–based aflatoxin analysis results using Pearson's correlation coefficient, there was a significant correlation between the two methods (r= 0.83, P<0.05) which suggested that positive maize samples were likely to be a high- risk feed. The study by Abdel-Hadi *et al*. (2010) also showed that there was a good correlation between the expression of aflatoxin biosynthetic genes and AFB1 production and the study by Priyanka *et al*. (2012) showed that the aflatoxin biosynthetic gene (Nor1) detection matched with the HPLC analysis unequivocally. However, it was observed that four maize samples (D4, E1, G1, L2) with the presence of the complete set of four genes did not produce AFB1. It is likely that environmental factors including temperature, pH and water stress condition have a significant impact on AFB1 biosynthetic gene expression and aflatoxin production in maize (Medina *et al*., 2014).

The two assays, LAMP and multiplex PCR, had enough potential to make differentiation and detect infected samples. Other recent studies using LAMP also showed it to be more effective than multiplex PCR in detecting fungi in contamination of agricultural crops (Jiufeng *et al*., 2010; Adb-Elsalam *et al*., 2011; Ghosh *et al*., 2015). The results of this study suggested that LAMP technique, without the dedicated equipment like multiplex PCR and the need to confirm amplification products, proved to be much more useful as some factors including time, safety, cost and sensitivity were taken into account. It also suggested that LAMP can be used as an alternative method for the detection of AFB1 producing *A. flavus* in maize feed. It may become a promising tool in the prediction of a potential aflatoxin risk at an early stage in various types of food and feed sample.

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