academicJournals

Vol. 7(14), pp. 1179-1183, 2 April, 2013
DOI: 10.5897/AJMR2012.2346
ISSN 1996-0808 © 2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

One-hour loop-mediated isothermal amplification assay for the detection of quarantinable toxigenic *Fusarium garmanirum*

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Accepted 25 March, 2013

Fusarium head blight (FHB) caused by several *Fusarium* species is one of the most serious diseases affecting wheat throughout the world. The *in vitro* production of the toxins deoxynivalenol, zearalenone fumonisin, T-2, and HT-2 was quantitvely evaluated in 8 different isolates of *Fusarium* species collected from feed samples. It was possible to detect zearalenone and the other mycotoxins in 100% and 50% of the isolates, respectively. In the present study, loop-mediated isothermal amplification method (LAMP) was designed for diagnosing *Fusarium garmanirum* infections and testing against feed samples, infested samples and pure cultures. The LAMP amplicon was directly visualized in the reaction tubes by the naked eye following the addition of calcein fluorescence. The LAMP products appeared as DNA marker pattern, with many bands of different sizes from 145 base pairs up to the loading well. Loop-LAMP procedure was used to detect genomic DNA of *F. graminearum* in fungal pure culture and in contaminated feed samples. In the future, this assay will support plant quarantine programs in Saudi Arabia and Gulf Cooperation Council states, to prevent the introduction of foreign FHB species.

Key words: LAMP-PCR, Fusarium head blight, feed samples.

INTRODUCTION

Head blight is caused by several *Fusarium* spp.; distribution and predominance of species significantly vary among climatic conditions, geographical zones, countries, and years (Doohan et al., 2003; Xu, 2003). Morphological identification of *Fusarium* spp. is difficult due to their similarities. For example, *F. avenaceum* are very difficult to separate by the morphological characteristics of their spores (Yli Mattila et al., 2004). Recently, the novel nucleic acid amplification method loop-mediated isothermal amplification (LAMP) has been reported as a simple rapid diagnostic tool for early detection of microorganisms (Parida et al., 2008). LAMP was also developed for rapid detection of pathogenic or allergenic fungal in the environment (Sun et al., 2010). Recently, LAMP-PCR using internal labeled probes have been developed for the major FHB pathogens in different plant materials (Niessen and Vogel, 2010; Abd-Elsalam et al., 2011; Niessen et al., 2012). LAMP depends on using a set of

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Table 1. Production of mycotoxin by *Fusarium* spp. isolated from feed samples.

Isolate	Mycotoxins (µg/kg)				
	Fumonisin	Zearalenone	T-2	DON	HT-2
F. semitectum	0	85	0	0	0
F. semitectum	0	50	0	0	0
F. semitectum	0	60	0	0	0
F. semitectum	0	130	0	0	0
F. verticillioides	1050	245	8.0	640	3.0
F. graminearum	850	354	2.0	550	0
F. graminearum	700	420	4.0	320	0
F. graminearum	450	557	2.0	120	0

six oligonucleotide primers with eight binding sites hybridizing specifically to different regions of a target gene, and a thermophilic DNA polymerase from *F. garmanirum* for DNA amplification (Niessen and Vogel, 2010; Abd-Elsalam et al., 2011). This methods has been used to detect plant pathogenic fungi like *Phytophthora ramorum* (Tomlinson et al., 2007), *Phytophthora sojae* (Dai et al., 2012), *A. flavus* and *A. parasiticus* (Ahmed et al., 2010), toxigenic Fusarium (Denschlag et al., 2012; Niessen et al., 2012). The current study describes the LAMP for the detection *of F. garmanirum* in infested feed sample.

MATERIALS AND METHODS

Feed Samples

Twenty samples of fodder used in camel feeding in Saudi Arabia were collected. Samples including natural feed (14 samples) and compound feed (6 samples), were collected from various animal feed factories, storehouse, and fodder markets from vendor or distributors during 2009.

Isolation and identification of *Fusarium* spp.

Feed sample were surface-sterilized for 1 min with a 1% sodium hypochlorite solution, rinsed twice in sterile distilled water and dried in a laminar flow cabinet. One growth media, Potato Dextrose Agar (PDA) was used for fungal isolations. The plates were incubated at 28°C in the dark for 5-7 days. All *Fusarium* isolates were subcultured on PDA, Spezieller Nahrstoffarmer Agar (SNA) using a single spore technique (Leslie and Summerell, 2006). The fungal cultures were maintained at 10°C on SNA medium (0.5 g l^{-1} $MgSO_4$ × 7 H2O, 1 g l^{-1} KNO_3 (Roth, Karlsruhe, Germany), 0.2 g l^{-1} sucrose, 0.2 g l-1 glucose, 0.5 g l-1 KCl, 1 gl^{-1} KH2PO4 and 15 g l^{-1} agar (Merck, Darmstadt, Germany). Fungal and *Fusarium* isolates used in the current study are listed in Table 1.

Feed samples infestation

Plant Pathology Research Institute (PPathRI) strain of *F. graminearum* were inoculated on PDA slants and incubated for 2–4 weeks and spores were harvested as previously reported by Hilton et al. (1999). Feed samples were infested by placing 100 µL of *F. graminearum* conidial suspension (containing 106 conidia) on each gram feed sample. Control sample were treated with 100 µL of sterile distilled water. Inoculated sample were incubated in a moist chamber for 72 h and returned to the growth chamber at 21°C (day)/16°C (night) temperatures with day/night regime of 16 h/8 h.

Fusarium mycotoxins

Fusarium mycotoxins (Fumonisin, HT-2, Zearalenone, T-2, Neosolaniol and DON) content was determined using the VICAM (1998) method. The method was similar with all former toxins except the dilution buffer, developer and immunoaffinity column. Each isolate was grown in elmnyer flask 100 ml on SMKY media. The incubation period was 7 days at 25±2°C. After blending on high speed for 1 min with 5 g of sodium chloride. Twenty milliliter of culture filtrate was added to 80 ml of methanol (HPLC grad) and filtered through a fluted filter paper. The extract (10 ml) was diluted with 40 ml of phosphate buffered saline (PBS)/0.1% Tween-20 wash buffer and filtered through a 1.0-Am microfiber filter. The diluted extract was passed through the column, which was washed with 10 ml of PBS/0.1% Tween-20 wash buffer followed by 10 ml of PBS. Fumonisins were eluted from the column with 1 ml HPLC grade methanol. A mixture of developer A and developer B (1 ml) was added to the elute collected in a cuvette that was placed in a fluorometer (VICAM Fluorometer Series 4, Watertown, USA) for fumonisin measurement.

Fungal mats

The *Fusarium* spp. isolates and fungal cultures obtained from Saudi MycoBank for DNA isolation were grown for 8 days in 100 ml Potato Dextrose Broth (PDB, 24 g l^{-1}; Scharlau, Barcelona, Spain). Fungal mycelium mats (100 mg) from 3-day-old colonies grown on duplex agar medium was homogenized by an electric grinder prior to DNA extraction. One hundred milligram mycelium was scraped off the agar using a spatula.

DNA extraction

A hand-operated or electric grinder (Retsch, Germany) was used to homogenize fresh mycelium in a 1.5-mL microfuge tube. To prevent cross-contamination, the pestle tip was changed and sterilized between isolations (the pestle tip was immersed in 70% EtOH and the grinder was turned on for 3 s and subsequently the pestle tip was rinsed in sterile distilled water and dried with sterilized filter paper. Pre-warmed (at 65°C) 600 µL of DNA extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, 1% PVP) was added to homogenized samples. Six microliter of RNase A (20 µg/ml) was added and microtubes were incubated at 65°C in hot water bath for 15 min with irregular shaking and swirling for every 5 min. For protein precipitation 160 µl was added (3 M so-

Table 2. LAMP primers list used in the current study.

Primer code	Primer Sequence	Concentration
FIP-gaoA ID4	5′-CGC AAG TGA CGG CCC AGT TGC TTC GAG CCT CAG CAC CTA-3′	1.6 mM
BIP-gaoA ID4	5′-TGC AAC AAG GCC ATT GAT GGC CGT TGG CGC CAT AGA ATG T-3′	1.6 mM
F3-gaoA ID4	5′-AGG GAG TCT TCA GTT CCT GA-3′	0.2 m M
B3-gaoA ID4	5′-GTG AGG GGG CTT TGG ATC-3′	0.2 m M
LoopF-gaoA ID4	5′-GTT GCG AGA AAT GGC GCT TCC G-3′	0.8 mM
LoopB-gaoA ID4	5′-ACA AGG ATA CCT TTT GGC AC-3′	0.8 mM

Niessen and Vogel (2010). All nucleotides were purchased from Eurofins MWG Operon, Ebersberg, Germany.

dium acetate, pH 5.3) and mixed thoroughly by inverting the tube. The mixture was centrifuged at 10,000 rpm for 8 min at room temperature. The supernatant was carefully decanted and transferred to a fresh tube and was precipitated with equal volumes of cold lsopropanol (molecular biolgy grade), and quietly mixed to generate fibrous DNA and incubated at room temperature for 10 min. Genomic DNA was collected by centrifuging at 10000 rpm for 10 min. The resulting pellet was washed twice with 80% ethanol and then was centrifuged at 8000 rpm for 2 min. The pellet so obtained has been dried under vacuum using Vacufuge Concentrators 5301 (Eppendorf) at 37°C for 5 min. In the final step, DNA was eluted in 100 µL of sterile TE buffer (10 mM Tris–HCl, 1 mM EDTA pH 8.0) and kept at -20°C until used as template for PCR amplification (Abd-Elsalam et al. , 2011).

LAMP reaction

We used six primers designed by Niessen and Vogel (2010) with nucleic acid sequences and molarities concentration as shown in Table 2. The LAMP assay was carried out in a total of 25 µl reaction mixture containing 10× Bst-DNA polymerase buffer (2.5 mM each), betaine (10 µM each), deoxynucleotide triphosphates (2.5 mM each), $MgSO_4$ (10 mM each), FIP and BIP (1.6 mM each), F3 and B3 primers (0.2 mM each), loop-F and loop-B (0.8 mM each) (Table 2), Bst DNA polymerase (8 U) (New England BioLabs), dd H_2O, and template DNA (1 µl). Before incubation 25 µM calcein and 0.5 mM $MnCl_2$ were added to each 25 µl reaction mix. The template DNA was omitted in one reaction for negative control. LAMP reactions were carried out at 63°C for 60 min using Swift Spectrum 48 Thermal Cycler (ESCO, Singapore). Visual inspection of the LAMP amplicons in the reaction tube were performed by adding fluorescent detection reagent before the incubation of the reaction tubes, the fluorescent signals of the solutions were observed under UV light. Direct visualization of amplified product was achieved by separation of the LAMP reactions on 1.5% agarose gels following incubation. Gels were run at 60 V, 55 mA for 45 min and stained with ethidium bromide solution (1 g/mL). PCR amplicons were documented using a Biorad Gel Doc System (Biorad, NSW, Australia).

RESULTS

Screening of *Fusarium* spp. for mycotoxin production

Toxin-producing isolates varied in the type and concentrations of toxin produced. *F. verticillioides* was the highest producer of fumonisin, zearalenone and DON (1050, 245, and 640 µg/kg, respectively). *F. semitectum* only produces zearalenone toxin (Table 1). *F. graminearum* isolates were not able to produce HT-2. Fumonisins were produced by a number of *Fusarium* species, notably *F. verticillioides* and *F. graminearum.*

LAMP for detecting *F. graminearum* contamination in feeds

The LAMP-PCR technique uses four primers that identified six regions on the target nucleic acid, so that the specificity is particularly high. For this reason, in the LAMP method, detection can be done without inspection of the amplification product during or after the amplification. Visualization of DNA amplification during LAMP was achieved by inspection of the reaction vessels under UV 366 nm trans-illumination after adding manganese quenched calcein to the master mix. Intensity of the fluorescence signal was independent from DNA concentrations in positive samples. Samples with water added instead of DNA showed no fluorescence under these conditions. The size of the LAMP reaction products ranged from 145 bp up to the loading well on the agarose gel, indicating specific amplification of the target sequence. Positive results were achieved with *F. graminearum* isolates, while all reactions with Fusarium and fungal species included for comparison remained negative, with a response identical to the negative control. Among the strains of *Fusarium* spp., a fluorescent signal was obtained from four tested *F. graminearum* isolates. Testing of other *Fusarium* spp. and species from other genera revealed no fluorescent signal in the LAMP assay. No cross reactivity was observed with other fungal DNA *F. semitectum* (lane 11), 12= *F. verticillioides* (lane 12), *Aspergillius* (lane 13), *Pencillium* (lane 14) (Figures 1 and 2).

DISCUSSION

Conventional diagnostic methods for the detection and identification of *F. graminearum* in culture or in infected grains are based on micro and macro morphological

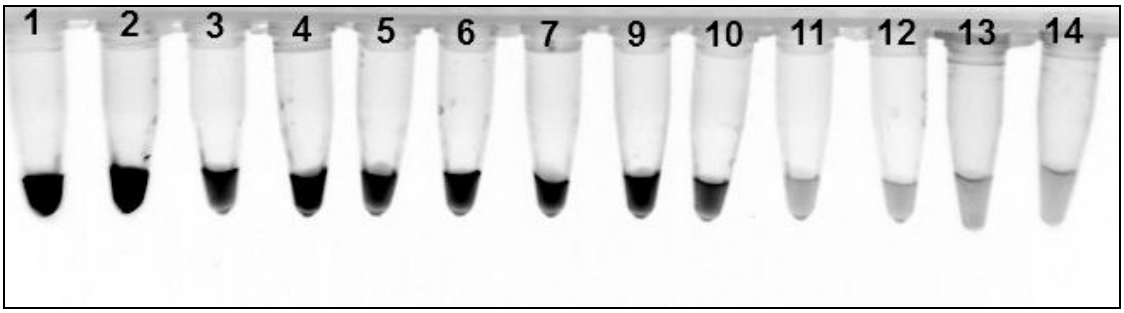

Figure 1. Visual appearance of LAMP-PCR products by using primer set gaoA ID4 and Calcein fluorescence under UV 366 nm light. The reactions were incubated in a water bath for 60 min at 63 ˚C. 1=*F. graminearum*, 2=*F. graminearum*, 3=*F. graminearum,* Infested feed samples with *F. graminearum* (PPathRI 0555) were included; 4=Wheat bran, 5=Wheat bran, 6=Barley grain, 7= fresh barely, 8= Crushed corn, 9= Sorghum, 10=Millet Grain. 11= *F. semitectum,* 12= *F. verticillioides,* 13=*Aspergillius*, 14= *Pencillium*. PPathRI = Plant Pathology Research Institute.

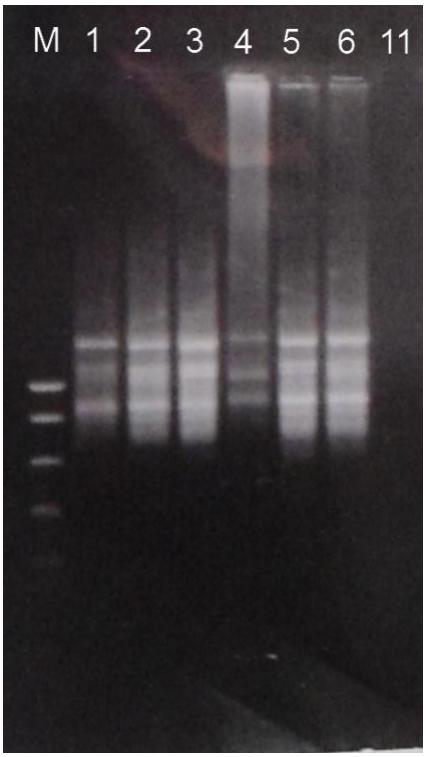

Figure 2. Agarose gel electrophoresis of loop-mediated isothermal amplification products amplified from genomic DNA extacted during the current study. 1=*F. graminearum*, 2=*F. graminearum*, 3=*F. graminearum,* Infested feed samples with *F. graminearum* (PPathRI 0555) were included; 4=Wheat bran, 5=Wheat bran, 6=Barley grain, 11= *F. semitectum.*

features. These methods are time consuming, require experience, and are difficult to distinguish between similar species.

Toxin-producing isolates varied in the type and concentrations of toxin produced. *F. verticillioides* was the highest producer of fumonisin, Zearalenone and DON. The very high levels of *F. verticillioides* and *F. semitectum* in feed samples are known to infect crops in the field and propagate at moisture contents. This fungus is known for the production of fumonisins, of which fumonisin B1 is the most important.

LAMP-PCR using the gaoA gene was used to detect *F. graminearum* in contaminated feed products. The method developed by Niessen and Vogel (2010) is cost-effective, high throughput quantification of *F. graminearum* in wheat grains. The LAMP assay is virtually equipment-free, requiring only a simple device such as a water bath to maintain a constant isothermal temperature. It is therefore much less technically demanding than nested PCR. Detectable copies of DNA were obtained after only 1 h of incubation under isothermal condition of 63°C, and visual evaluation of the outcome could be done. Further-more, LAMP products can be detected by direct fluorescence by adding Loopamp fluorescent detection reagent (FD) (Eiken Chemical Co. Ltd., Japan) at the start of the reaction (Tomita et al., 2008). Other fluorescent dyes such as ethidium bromide, SYBR green and Calcein (Boehme et al., 2007; Abd-Elsalam et al., 2011; Niessen and Vogel, 2010). Evagreen have also been used for visualization of LAMP products under UV light (Poon et al., 2006; Qiao et al., 2007).

Tomlinson et al. (2007) reported on using LAMP for the detection of *P. ramorum*. LAMP assay for the detection of *Fusarium* spp. is capable of producing the gushing inducing hydrophobin Hyd5p set up (Denschlag et al., 2012). LAMP was used to amplify a 167 bp portion of the acl1 gene in *F. tricinctum* (Niessen et al., 2012). LAMP primers were able to amplify AMF DNA from colonized carrot roots (*Glomus intraradices*), and genomic DNA from several taxa of AMF (Gadkar and Rillig, 2008). Dai et al. (2012) development of a LAMP assay targeting the A3aPro element for visual detection of *P. sojae*. LAMP

amplified 10 times much lower diluted DNA samples of *A. flavus* and *A. parasiticus* than PCR amplification.

A robustness of the specific PCR assay for *F. graminearum* was high according to the positive PCR amplification obtained with a wide range of template concentration (10 pg-10 ng) using either DNA from pure cultures or from contaminated sample. This method can be used to prepare DNA from numerous wheat grains for molecular analysis with LAMP-PCR method for detecting toxigenic *Fusarium* collected from cereals.

Conclusion

The LAMP assay is inexpensive, easy to perform and shows rapid reaction, wherein the amplification can be obtained in 60 min under isothermal conditions of 63°C by employing a set of four species-specific primer mixtures and results can be checked through naked-eye visualization. LAMP reaction can be run using simple lab equipment such as a heating block, water bath, hybridization oven or household equipment, example a microwave oven. In future, we can use these assays for quality control of wheat and other cereals at selected points of entry into Saudi Arabia Gulf cooperation Council states (ports, land borders).

ACKNOWLEDGMENT

This project was supported by King Saud University, Deanship of scientific research, College of Science Research Centre. We gratefully thank Hans-Peter Klink DSMZ, Germany for providing *Fusarium* reference strains.

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