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A novel AAA ATPase exon1-specific Primer for Detection of *Aspergillus niger* Isolated from Various Food Sources using SYBR® Green Real Time PCR ± Assay

Samiraj Ramesh[1]*, Satish kumar Rajasekharan[1], Vinoth Jothiprakasam[1], Ramaraj Elangomathavan[2] and Subban Patharajan[2]

[1]Department of Microbiology, [2]Department of Biotechnology,
Prist University, Thanjavur - 614 904, India.

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PCR based methods are considered as the most accurate and reliable for early diagnosis of food-borne fungal pathogens when compared to the phenotypic detection methods. *Aspergillus* species included in section Nigri often infects food materials such as grapes, onions cereals, coffee and their derivatives, principally in warm and humid climatic conditions. Here, we propose, a rapid molecular method for detection and discrimination of *A. niger* using Real-Time PCR SYBR green melt curve analysis. The coding region of AAA ATPases gene fragment was amplified using specific primers and the product was separated based on the melting temperature. The measured melting temperature of *A. niger* isolates were in the range between 85.16 °C and 85.31°C.

Key words: *Aspergillus*, Real-Time PCR, AAA ATPase gene, SYBR® green, ± assay.

Food-borne illnesses are usual concern in both developing and developed countries[1]. Foodborne illnesses are caused by the consumption of contaminated food materials infected with microbes secreting toxins and other secondary metabolites[2]. All black-spored *Aspergilli* are grouped into *Aspergillus* species Nigri. Black-spored *Aspergilli* are accounted to be the third most frequently occurring *Aspergillus* infection[3]. *A. niger* is a filamentous ascomycete fungus which is generally present in the environment and have been reported to cause opportunistic infections in humans namely aspergillosis [4]. Aspergillosis is an infectious, non-contagious disease caused by in halation of spores produced by *A. niger*[5]. Invasive aspergillosis initiates in the paranasal sinus and accentuates in the orbital base with bone destruction and vessel infiltration[6]. *Aspergillus* sinusitis often occurs in diabetic and immunocompromised patients[7].

A.niger is frequently known to cause black mould diseases in certain fruits and vegetables[8]. It commonly infects onions, peanuts and grapes, which are often mild infections but can become systemic depending on certain constructive conditions. Ochratoxin A (OTA), a secondary metabolite produced by *A.niger* are frequently detected in contaminated grapes, dried wine fruits, coffee and cocoa[9, 10]. This mycotoxin is a powerful nephrotoxin with immune suppressive, genotoxic and teratogenic functions towards numerous species [11].

A. niger is often misjudged with *Stachybotrys chartarum* and *Stachybotrys chlorohalonata* species which are also regarded as “toxic black molds” in US[12, 13]. Hence it requires accurate diagnostic strategies to detect *A. niger*. Rapid detection of the fungus provides ample time to initiate antifungal therapy and tenable

* To whom all correspondence should be addressed.
Tel.: +91 99761 93870; Fax: 04362 265150;
E-mail: marineramesh2020@gmail.com

conception of contaminated food materials infected with *A. niger*. Early detection also provides sufficient time to encounter against ochratoxins producing *A.niger*. Numerous phenotypic and molecular detection of *Aspergillus* spp. methods have been reported. Real time PCR is a standard and exquisite molecular diagnostic tool for amplifying gene fragments due to its high sensitivity and accuracy in quantification with ease[14-20.] We have designed a species-specific primer for rapid detection and discrimination of *A. niger* based on non-specific fluorescent dye, Sybr green using a coding region of AAA ATPase gene.

MATERIALS AND METHODS

Reference strains and isolates

Reference strain of *A.niger* (MTCC 10180, 4325) and *A. flavus* MTCC (9064, 1884) were procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. 30 non-repetitive *A. niger* were isolated from different contaminated food materials including onions, pomegranate, grapes and green chilly.

Primer design

The nucleotide sequences were retrieved from NCBI GenBank: AJ812008.1.The primers were designed using Primer 3 (v. 0.4.0) online software and primer parameters were analyzed with oligoanalyzer (v.1.0.3).

DNA isolation

The fungal cultures were grown on potato dextrose broth (PDB). After 72 hours of incubation at 25°C, the fungal material (100 mg) was homogenized with mortar and pestle. HipurA ™ fungal DNA purification kit (Himedia) was used for the isolation of the chromosomal DNA. The extracted fungal DNA was dissolved in TE buffer and the purity of the DNA was quantified by UV Spectrophotometer (Model no: UV2310). The samples were run on 1% agarose gel and confirmed on UV transilluminator following ethidium bromide staining. The genomic DNA was further used for Real time PCR assays.

Real time PCR assay

The LightCycler real-time polymerase chain reaction (Applied Biosystem's Real-Time PCR system StepOne® model no-271003195) was used for detection and melt curve analysis. The primers forward (5'-AAGATGAATACGATGAG-3') and reverse (5'- ATGTAGGAGATAGTGTTG-3'), were used for PCR amplification of AAA ATPase exon1 fragment. 20 µl reaction volume containing 10 µl of 2X Power SYBR® PCR master mix , 1 µl (10 pmoles) of each forward and reverse primers and 1 µl of the template DNA (30–60 ng) were used for the PCR reaction. The cycling parameters for the two-step PCR reaction were as follows: Holding stage of 10 mins at 95 °C (Initial denaturation), 45 cycles of 00.15 secs at 95 °C (denaturation), 1 min at 60 °C (annealing and extension) followed by melt curve analysis.

RESULTS AND DISCUSSION

Species-specific primers based on ITS regions (internal transcribed spacers of rDNA) are extensively reported [15]. We have designed primers based on coding regions of AAA ATPases,

Table 1. Real time PCR ± assay for species-specific detection of *A.niger*

Strains/Isolates	Food materials	Genus	Species	AAA ATPase exon 1	Melting temperature (T_m)
MTCC10180	*Potato*	*Aspergillus spp.*	*A.niger*	+	85.31°C
MTCC 4325	-	*Aspergillus spp.*	*A.niger*	+	85.31°C
MTCC 9064	-	*Aspergillus spp.*	*A.flavus*	-	-
MTCC 1884	Vegetable waste	*Aspergillus spp.*	*A.flavus*	-	-
Isolate 1	*Onion*	*Aspergillus spp.*	*A.niger*	+	85.16°C
Isolate 2	*Onion*	*Aspergillus spp.*	*A.niger*	+	85.16°C
Isolate 3	*Onion*	*Aspergillus spp.*	*A.niger*	+	85.31°C
Isolate 4	*Pomegranate*	*Aspergillus spp.*	*A.niger*	+	85.30°C
Isolate 5	*Grapes*	*Aspergillus spp.*	*A.niger*	+	85.31°C
Isolate 6	*Green chilly*	*Aspergillus spp.*	*A.niger*	+	85.31°C

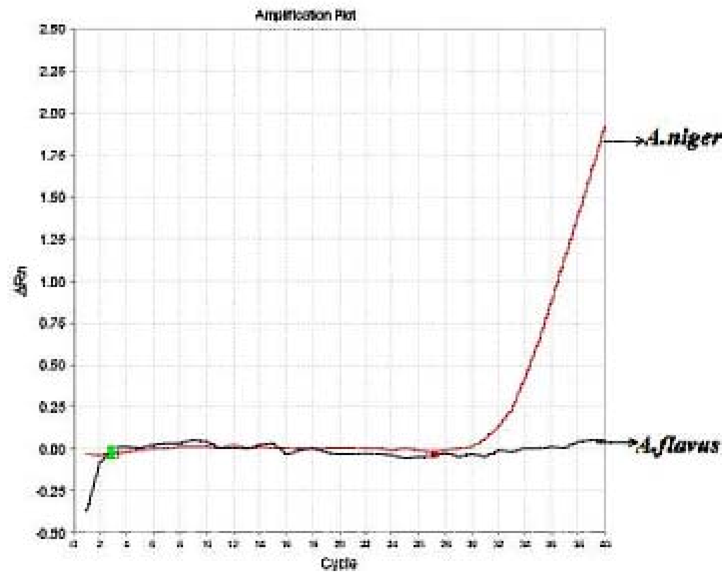

Fig. 1. Amplification plot showing the species-specific discrimination of *A.niger* and *A.flavus* based on RT-PCR ± assay

associated with diverse cellular activities (AAA), which are found in all organisms. Our primers are designed with high specificity and complementarily with *A.niger* genomic DNA. Insilico Analysis of the primers and amplicon using nBLAST (nucleotide Basic Local Alignment Search Tool) showed high degree of similarity with *A.niger* genomic contigs. The Amplification plot ("Rn vs cycles) of the two different molds is dissipated in Figure-1 showing the presence of the AAA ATPase exon 1 sequence in *A.niger* and absence of the sequence in *A.flavus.* The melt temperature (T_m) of the amplicon was calculated to be 82°C using online MBCF oligo calculater (DANA-FARBER Cancer institute). The measured melt temperature of AAA ATPase exon 1 sequence for *A.niger* isolates between 85.16 and 85.31 °C (Table 1). Several molecular techniques are employed in the detection of *Aspergillus* spp. The phenotypic and molecular detection methods, previously reported are based on RFLP (Restriction fragment length polymorphism), SSCP (Single strand conformational polymorphism) and qualitative Polymerase chain reaction based on primers amplifying ITS region [16-20]. In the present study, we have devised a Real-Time PCR based molecular detection system using SYBR green chemistry for species-specific detection of *A.niger.* This procedure offers a rapid, accurate and inexpensive procedure for early detection the food-borne fungal pathogens.

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REFERENCES

1. WHO. Removing obstacles to healthy development. World Health Organization Geneva: 1999.
2. Jones K.E., Patel N.G., Levy M.A., Storeygard A., Balk D., Gittleman J.L. Global trends in emerging infectious diseases. *Nature.* 2008; **451**: 990-993.
3. Gams W., Christensen M., Onions A. H. S., Pitt J. I., Samson R. A. Infrageneric taxa of *Aspergillus.* Advances in *Penicillium* and *Aspergillus* systematics. , *In*: R. A. Samson and

J. I. Pitt ed., Plenum Press, New York, NY.1985; pp. 55–61.
4. Perfect J.R., Cox G.M and Lee J.Y. The impact of culture isolation of *Aspergillus* species: a hospital-based survey of aspergillosis. *Clin. Infect. Dis*., 2001; **33**: 1824-1833.
5. Atkinson R., Brojer C. Unusual presentations of aspergillosis in wild birds. *Proc Assoc Avian. Vet*., 1998; 177-181.
6. Chakrabarti A., Sharma S.C. Paranasal sinus mycoses. *Indian. J. Chest. Dis. Allied.Sci*., 2000; **42**: 293-304.
7. Erbrecht R., Denning D.W., Atterson T.F. Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. *N. Eng. J. Med.,* 2002; **347**: 408-15.
8. Hayden N.J., Maude R.B. The role of seed borne *Aspergillus niger* in transmission of black mould of onion. *Plant. Pathol.* 1992; **41**: 573-581.
9. Abarca M.L., Bragulat M.R., Castella G., Cabanes F.J. Ochratoxin A production by strains of *Aspergillus niger var. niger*. *Appl. Environ. Microbiol.,* 1994; **60**: 2650–2652.
10. Cabanes F.J., Accensi F, Bragulat M.R., Abarca M.L., Castella G, Minguez S, Pons A. What is the source of ochratoxin A in wine? Int. *J. Food Microbio.,* 2002; **79**: 213-215.
11. Samson R.A., Houbraken J.A.M.P., Kuijpers A.F.A., Frank J.M., Frisvad J.C. New ochratoxin or sclerotium producing species in *Aspergillus* section Nigri. *Studies in Mycology* 2004; **50**: 45-61.
12. Baggerman W.I., Samson R.A. Heat resistance of fungal spores. *In*: Samson R.A., Hoekstra E.S. Introduction to Food-Borne Fungi. Centralbureau Voor Schimmelcultures, Baarn, 3rd Ed. The Netherlands, 1988.
13. Fujikawa H., Itoh T. Tailing of thermal inactivation curve of *Aspergillus niger* spores. *Appl. Environ. Microbiol*., 1996; **10**: 3745-3749.
14. Jimenez L, Bosko Y, Smalls S, Ignar R, English D. molecular detection and identification of *Aspergillus niger* contamination in cosmetic/ pharmaceutical raw materials and finished products. *J. Rapid. Metho. Automa. Microbio.,* 1999; **7**(1): 39-46.
15. Salgado G.A., Patino B., Vazquez C., Gonzalez-Jaen M.T. Discrimination of *Aspergillus niger* and other *Aspergillus* species belonging to section Nigri by PCR assays. *FEMS. Microbio. Lett*., 2005; **245**: 353-361.
16. Someren K.V. M.A., Samson R.A., Visser J. The use of RFLP analysis in classification of the black Aspergilli: reinterpretation of the *Aspergillus niger* aggregate. *Curr. Genet*., 1991; 19:21-26.
17. Varga J., Juhasz A., Ferenc K., Kozakiewick Z. Molecular diversity of agricultural important *Aspergillus* species. *Eur. J. Plant Pathol*., 2004; 110: 627-640.
18. Rath P.M., Ansorg, R. Identification of medically important *Aspergillus* species by a single strand conformational polymorphism (SSCP) of the PCR amplified intergenic spacer region. *Mycoses.,* 2000; **43**: 381-386.
19. Accensi F., Cano J., Figuera L., Abarca M.L., Cabanes F.J. New PCR method to differentiate species in the *Aspergillus niger* aggregate. *FEMS Microbiol. Lett*., 1999; **180**: 191-196.
20. Varga J., Kevei E., Fekete C., Coenen A., Kozakiewicz Z., Croft J.H. Restriction fragment length polymorphisms in the mitochondrial DNAs of the *Aspergillus niger* aggregate. *Mycol. Res.,* 1993; **97**: 1207-1212.