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Polymerase Chain Reaction for Detection of Aflatoxigenic Strains of *Aspergillus* Species Using Aflatoxin Genes

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ABSTRACT: Humans inhale the *Aspergillus* spores which are present in the environment and they rarely experience complications. However, under special circumstances, *Aspergillus* species can produce a spectrum of diseases involving lungs and later on other organs and tissues. Aflatoxins are potent hepatocarcinogens and mutagens produced as the secondary metabolites by several *Aspergillus* species. A total of 81 samples (39 from patients, 39 from environment and 3 American type culture collection controls) were screened for Aflatoxin regulatory gene by using polymerase chain reaction. Out of 39 *Aspergillus* isolates from patient's sample, 58.97% indicated presence of Aflatoxin regulatory (aflR) gene, whereas in 39 *Aspergillus* isolates from environmental samples, 35.89% showed the presence of aflR gene. Difference of 23.05% was statistically significant (Chi-square = 1.77, df = 1, P value < 0.05). American type culture collection control strains showed 100% presence of aflR genes. Our study concluded that aflR genes are normally present in environmental isolates which are activated in the tissues upon entry in patients. The expression of aflR genes was higher in *Aspergillus niger* (66.66%) followed by *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus brasiliensis*, *Aspergillus terrus*.

Keywords: Aflatoxin genes, *Aspergillus*, Polymerase chain reaction, Health.

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INTRODUCTION

Aspergillus species are aflatoxins producers, these aflatoxins may be potent hepatocarcinogens and mutagens. Among the *Aspergillus* species, *Aspergillus flavus* and *Aspergillus parasiticus* may cause infections in human due to contamination of food; however *Aspergillus niger* and *Aspergillus fumigatus* are more responsible for serious infections, they may cause a severe form of infection in Acquired Immune-Deficient Syndrome (AIDS) patients, due to their diminished immunity (Degola et al., 2007). The aflatoxin M1, a metabolic thermo-stable derivative of aflatoxin B1, may be found in the cow's milk due to aflatoxin-contaminated feed, and it still may be found in its active form in cheese and other dairy products (Degola et al., 2007).

Differentiation of toxigenic and non-toxigenic isolates of the *Aspergillus* species was carried out in culture media and aflatoxins may be extracted by organic solvents by using chromatographic techniques, which are time-consuming techniques. The current method including Polymerase Chain Reaction (PCR) is the method of choice for detection of aflatoxins (Erami et al., 2007).

PCR is an advanced method but it also has some disadvantages i.e. being costly and requiring sophisticated instruments and trained person to operate the instruments (Smith et al., 1994). However simpler and faster immunochemical techniques require following the concept of one substance one assay (smith et al., 1994 and Young and Cousin, 2001). Early detection and speciation of *Aspergillus* species isolated from patients or environment samples is done by PCR method. The usefulness of PCR methods is its early detection of aflR genes from non-toxigenic genes, which may prevent morbidity and mortality of patients. However the multiplex PCR is useful for amplification of multiple targets to detect multiple gene mutations (Uggozzoli et al., 1998) and also for identification of causative agents. Single method for detection of Aspergilli along with aflatoxigenic species

would be of useful in minimizing of morbidity and mortality of patients (Latha et al., 2008).

The purpose of this study was to standardize the PCR based procedure to differentiate between aflR genes from non- aflR genes, which helps to provide early treatment for the patient.

MATERIALS AND METHODS

This was a prospective and experimental study, conducted at Department of Microbiology and Central Research Laboratory, MGM Medical College and Hospital, Navi Mumbai.

Period of study

The study was carried out over a period of one year with effect from January 2015 to December 2015.

Number of samples

Total 81 samples (39 from patients, 39 from environment and 3 ATCC controls) were screened for aflR gene by using PCR.

Inclusion and exclusion criteria

All chronic purulent exudates were studied for bacterial and fungal growth.

Specimens which did not show any pus cells were excluded from further studies.

Sample collection

Clinical samples like sputum, BronchoAlveolar Lavage (BAL), paranasal sinuses aspirates, eye swab, ear swab, blood and pus samples from suspected cases of aspergillosis of different patients were collected in a sterile container by taking all aseptic precautions and properly labeled.

Environmental samples were collected from Medical Intensive Care Unit (MICU), Surgical Intensive Care Unit (SICU), Intensive Care Unit (ICU), Pediatric Intensive Care Unit (PICU), Chest Tuberculosis (TB) ward, Ear Nose Throat (ENT) ward, Skin Outpatient Department (OPD), Surgery Ward, Burns Ward, Male Orthopedics Ward (MOW) and Ophthalmology Ward.

ATCC control strain of *Aspergillus oryzae* (ATCC10124, Lot No. 177-15-5, Expiry Date: 2014-09), *Aspergillus niger* (ATCC 6275, Lot No. 500-19-13, Expiry Date: 2014-11) and *Aspergillus* brasiliensis (ATCC16404, Lot No. 392-210-1, Expiry Date: 2015-02) were obtained from Microbiologics Inc, USA.

PCR amplifications were performed in accordance to a procedure as followed by Chao-Zong Lee et al. According to the procedure master mix "GoTaq Green Master Mix" (Promega BioSciences, LLC., USA), 5µl DNA, 20 pmol of primers were added and mixed to obtain 50µl final volume of the PCR mix. PCR primers were obtained from Sigma (USA) (Table 1 & 2).

Statistical analysis

Statistical analysis was done using Chi Square test, statistical package for the social sciences (SPSS) (version 17.0) was used for testing the hypothesis.

RESULTS AND DISCUSSION

Aflatoxins are potent hepatocarcinogenes and mutagens produced by various *Aspergillus* species. Production of aflatoxins is under control of aflR gene.

The present study was conducted for detection of aflR gene in patients and environmental *Aspergillus* species. The study was conducted over a period of six months from July to December 2015. Total 81 samples (39 from patients, 39 from environment and 3 controls) were screened for aflR gene by using polymerase chain reaction.

Out of 39 *Aspergillus* isolates from patient's sample, 58.97% showed presence of aflR gene, whereas in 39 *Aspergillus* isolates from environmental samples, 35.89% showed presence of aflR gene. A difference of 23.05% is statistically significant (Chi-square = 1.77, df = 1, P value = < 0.05). However ATCC control strains showed 100% presence of aflR genes. (Table 3, Graph 1&2)

Comparison of patients and environment isolates showed overall higher prevalence of aflR genes in various species in patient samples

Species wise aflR was detected more in *A. niger* as compared to other species. This finding corresponds to higher percentage of other virulence factors in *A. niger*. (Table 4 & 5, Graph 3&4)

Our study showed the presence of aflR genes in patient isolates as 58.97% where as Dehghan et al. found 17.4% and Dheeb et al. found aflR gene expression in patients with *Aspergillus* isolates. This difference could be due to geographical and climatic changes in various countries which may favour the aflR gene producers *Aspergillus* species.

Study of aflR gene in *Aspergillus* isolates from environment was done by various workers. Our values were the lowest at 35.89%, other researchers reported higher values ranging from 45.45% (Latha R et al., from India) to 85.71% (Erami et al., from Iran).

In our study out of 39 *Aspergillus* isolates from patients samples. (58.97%) showed presence of aflR gene, whereas in 39 *Aspergillus* isolates from environmental samples. (35.89%) showed presence of aflR gene. Various *Aspergillus* species isolated from patients showed higher positivity for aflR gene as compared to those from environment. It also means that aflR genes are normally present in environmental isolates which are activated in the tissues upon entry in patients. Difference is statistically significant (P<0.05). Difference in aflR gene prevalence or expression in *Aspergillus* isolates could be due to different environmental and climatic conditions in various countries. The aflR genes were detected more in *A. niger* (66.66%) followed by *A. fumigatus*, *A. flavus*, *A. brasiliensis*, *A. terrus*.

Table 1. Primers used to amplify aflR gene fragments from isolated DNA of patients and environment samples according to Lee et al. 2006.

Gene	Primer*	Sequence	Position D
aflR	F1	TCGGTACGTAAACAAGGAAC	2232 to 2206 (20-mer)
aflR	R1	AGTTGAGCCGCTGGTAGTCT	245–226 (20-mer)
aflR	F2	CCGATTTCTTGGCTGAGT	581–598 (18-mer)
aflR	R2	CCTAACACACCTACTCCT	1110–1083 (18-mer)
aflR	F3	GCTGTCTGACGGAAGAGCG	1034–1052 (19-mer)
aflR	R3	CCTAGGCAGAAACAGTACCA	1527–1508 (20-mer)
aflR	F4	GCAATCCGCGCGCTCCCAGT	197–216 (20-mer)
aflR	R4	GCTGGGCAAGGAGCTCAGCC	667–648 (20-mer)

Table 2. Cycling conditions of first- and nested-step PCR reactions.

Reaction	Cycling condition
Initial denaturation	94°C for 2 minutes followed by 30 cycles
Denaturation	94°C for 10 seconds
Annealing	60°C for 30 seconds
Extension	72°C for 2 minutes
Final extension	72°C for 5 minutes

[a]GoTaq Green Master Mix (2X) (Promega BioSciences, LLC., USA) was used. The 2X master mix contains chemically-modified Taq DNA Polymerase, MgCl2, ultrapure nucleotides with an optimized dUTP:dTTP ratio and Uracil-DNA Glycosylase (UDG)

Table 3. Showing comparison of patients and environmental *Aspergillus* isolates for aflR genes using PCR method

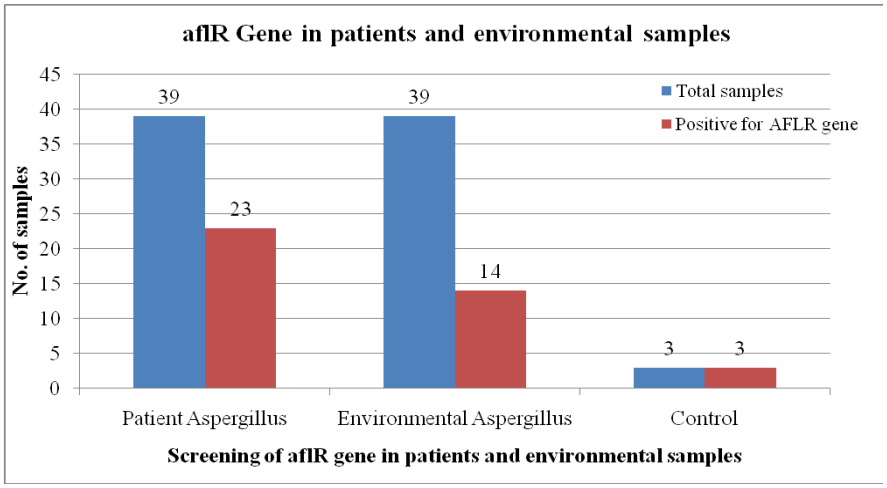
Isolates	Total samples	Positive for aflR gene	Percentages (%)
Patient *Aspergillus*	39	23	58.97%
Environmental *Aspergillus*	39	14	35.89%
Control	03	03	100%
Chi-square = 1.77, df = 1, P value = < 0.05, Significant			

Table 4. Showing aflR gene species in patient samples to detect Aflatoxin strains

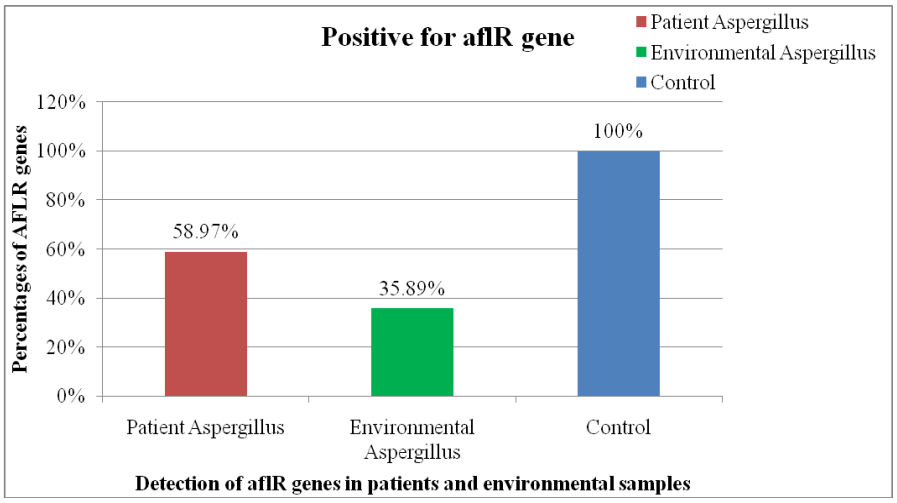
***Aspergillus* species isolated from patient sample**	**Total**	**Positive for aflR**	**Percentages**
Aspergillus niger	18	12	66.66%
Aspergillus fumigatus	9	5	55.55%
Aspergillus flavus	7	4	57.14%
Aspergillus brasiliensis	3	1	33.33%
Aspergillus terrus	2	1	50%
Total	39	23	58.97%

Table 5. Showing aflR gene species in environmental samples using PCR method

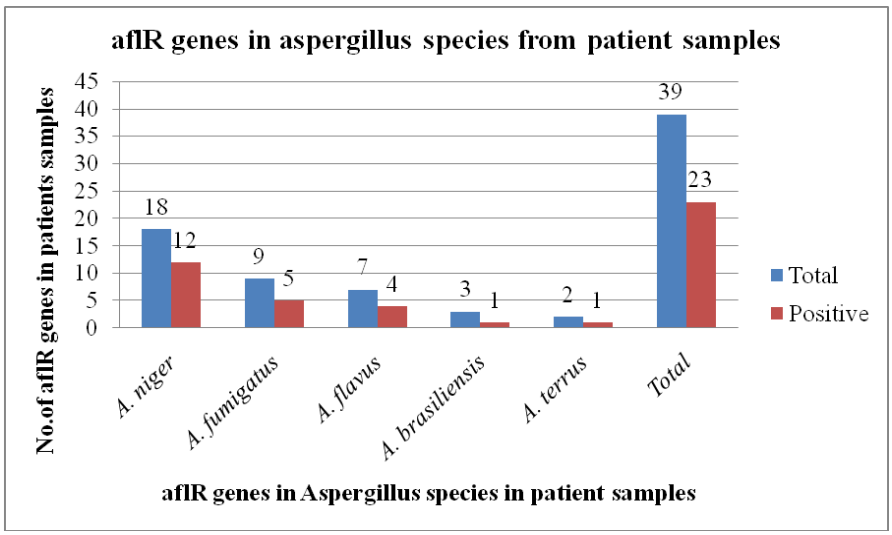
***Aspergillus* species isolated from environmental samples**	**Total**	**Positive for aflR**	**Percentages**
Aspergillus niger	20	8	40%
Aspergillus fumigatus	9	3	33.33%
Aspergillus flavus	5	2	40%
Aspergillus brasiliensis	3	1	33.33%
Aspergillus terrus	2	0	0%
Total	39	14	35.90%



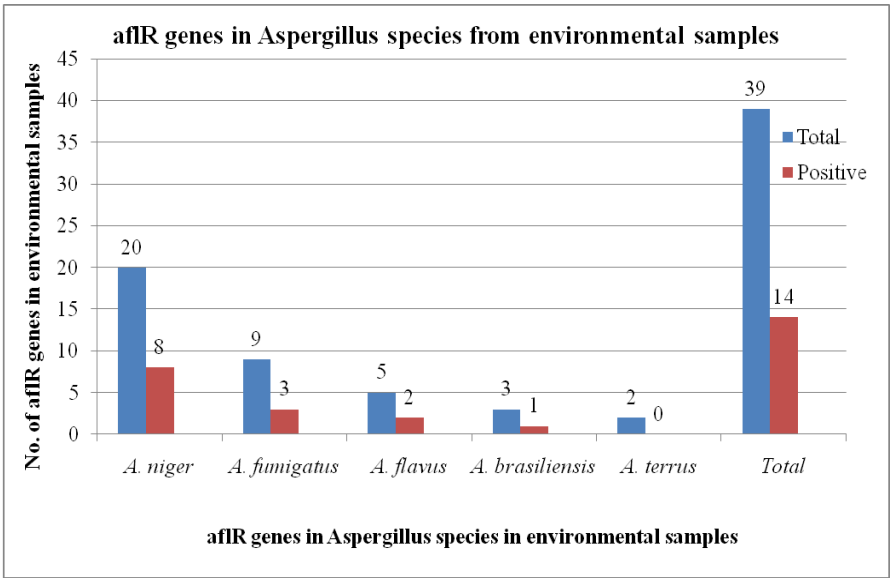
Graph 1. **Showing screening of aflR gene in patients and environmental samples**



Graph 2. **Showing detection of aflR genes in patients and environmental samples**



Graph 3. **Showing aflR genes in *Aspergillus* species in patient samples**



Graph 4. **Showing aflR genes in *Aspergillus* species in environmental samples**

Competing interests

The authors have stated that there are no conflicts of interest in connection with this article.

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