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Original Article

Genetic Characterization of *Aspergillus flavus* and *Saccharomyces cerevisae* in Tracheobronchial Phlegm of HIV-infected Patients on Antiretroviral Therapy in Jalingo, Nigeria

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Abstract

Fungal infections are among the diverse respiratory tract pathogens and account for a proportion of community acquired and nosocomial pneumonias thereby generating concerns particularly in immunocompromised patients. This study aims to genomically extract and sequence fungal DNA using the Basic Local Alignment Search Tool (BLAST) technology for their definitive and confirmatory identification thereby easing off the obstacles in their diagnosis due to similar appearance in their colony and morphology and ultimately determining their infection rates in persons receiving antiretoviral therapy against HIV. Sputa of 100 HIV infected out-patients of a Medical Centre in Jalingo, on anti-retroviral therapy were cultured on Sabouraud Dextrose Agar to isolate fungi species and assess their prevalence and distribution. Fungal colonies characterized culturally and biochemically as Aspergillus flavus and Aspergillus niger were subjected to the BLAST, and the similarities with the biological sequences in the National Center for Biotechnology Information (NCBI) database queried. There were observational variances in the colonial and microscopic appearances of A. flavus and A. niger on Sabouraud Dextrose Agar (SDA) plates and the microscope respectively. The similarities between the queried and biological sequences in the NCBI database, was almost all (99.7%) thus confirming their identity as Aspergillus flavus and Saccharomyces cerevisiae. The overall prevalence of fungi infection was 79.0%. More males (80.0%) than females (78.3%) were infected. In both sexes, fungi were most (92.9%) frequently isolated in patients that were between 30 and 39 years and least (64.7%) in those between 15 and 29 years. There was no established pattern (sex- and age- relatedness) of non-concomitant A. flavus and S. cerevisiae in fifty-seven male and female patients in six age categories even though A. flavus occurred more (48.1%) than S. cerevisae (24.1%) and the prevalence was higher (61.4%) in females than in males (38.6%). The high prevalence of these fungi in the study population, with or without symptoms of cough or fungal disease, mandates an early screening of such infected persons so as to reduce further complications and improve treatment.

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INTRODUCTION

The human immunodeficiency virus (HIV) is a lentivirus that causes acquired immunodeficiency syndrome (AIDS)[1]. Fungi may colonize human body sites with no manifestation of disease.[2,3] Respiratory infection occurs when spores or conidia are inhaled or a latent infection is reactivated.[2] Endemic or opportunistic infections in persons with HIV increase disease morbidity.[4,5,6]

Aspergillus, the aetiological agent of aspergillosis, is commonly transmitted to humans through inhalation of spores.[2] The incubation period is unclear since the effects of spore inhalation mostly depend on an individual's immunological state.[2] Pulmonary aspergillosis is a global healthcare concern because, if left untreated and undiagnosed in immunocompromised individuals, could rapidly progress to other organs and result in lethal invasive illnesses.[2,7] *A. fumigatus* stands out for its frequency in humans (about 90% of cases) and for being substantially responsible for the rise in invasive pulmonary aspergillosis (IPA) among immunocompromised individuals.[8,9,10,11]

Saccharomyces cerevisae ascomycetous yeast mainly used in the brewing and baking industries, can colonize the human respiratory, urinary and gastrointestinal tract. Infections due to *S. cerevisae* have been reported in patients with underlying chronic diseases and immunosuppression and the yeast infection can present as pneumonia, fungemia, peritonitis, endocarditis, vaginitis, oropharyngeal, urinary tract and skin infections.[2,12]

The difficulty in the isolation and cultural identification of most fungi are obstacles in their diagnosis due to similarities in their colonial and morphological appearances.[13] Consequently, genomic DNA extraction and sequencing technologies such as BLAST are used in their definitive and confirmatory identification. Fungal isolates can be characterized by sequencing the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA) with universal primers, ITS-1 and ITS-4, to amplify the ITS target region.[14,15]

Cases of opportunistic pneumoaspergillosis occurring alone, and in combination with other infections excluding *S. cerevisae*, have been recorded even in developing countries like Nigeria, in those immunosuppressed as a result of HIV or AIDS.[5,16,17]

MATERIALS AND METHODS

Study area

The Jalingo area of Taraba State, Nigeria was selected as the study area. According to 2006 census record of the National Population Commission, the State has population figures of 2,300,736 people while Jalingo has an estimated population of 139,845 people with over eighty ethnic groups. Agrarian in nature, farming is the predominant occupation in the city while other occupations complement its economic activities.[18]

Ethical clearance and study population

This cross-sectional hospital-based study was approved by the Institutional Review Board of the Department of Microbiology, Federal University Wukari. Ethical clearance was sought for, and obtained from the Ethical Committee of the Federal Medical Centre (FMC), Jalingo.

Inclusion criteria were HIV-infected out-patients of both sexes with or without cough symptoms, who consented to the study, were above the age of 10 years and who were on current anti-retroviral therapy while the exclusion criteria were out-patients who did not meet all the conditions in the inclusion criteria.

Phlegm collection

One hundred out-patients of the FMC, Jalingo, Taraba State, were each given a dry, clean, leak-proof 20ml sample bottle labelled with identification numbers to produce sputum which was immediately collected. The sex and age of each patient was appended on the bottle label and the sputum macroscopically examined.

Culture, isolation and identification of fungal colonies

Subsequently, an inoculum of sputum was streak platted on sabouraud dextrose agar (SDA) using an inoculating loop. The SDA culture plate was incubated at 25^0C and examined after three days of growth till an extended period of 7 days. Isolated microbial colonies were picked off and aseptically transferred to sterile SDA media to obtain pure colonies that were eventually examined macroscopically and microscopically. Pure colonies were characterized based on their colonial morphology: form, margin, shape, colour, topology, opacity, surface texture and diffusible pigments while the wet, and lactophenol cotton blue (LCB) mounts were used to identify hyphal morphology including the spores.[19,20] Data obtained was expressed as percentages.

DNA extraction and amplification

The basic local alignment search tool (BLAST), was used to examine the DNA and protein sequences two distinct genera of these isolates, infer functional and evolutionary relationships between sequences and help to identify members of gene families and species. Genomic DNA was extracted from each pure fungal culture using the Quick-DNA™ Miniprep Plus kit (Zymo Research, Catalogue No. D4068). The OneTag® Quick-load® 2x Mater Mix (NEB, Catalogue No. M0486) was used to amplify the COI target with ITS primers. The target of ITS-1 and ITS-4 was ITS rDNA while the sequence (5` to 3`) of ITS-1 and ITS-4 was TCCGTAGGTG AACCTGCGG and TCCTCCGCTTATTGATATGC respectively.[14,15]

DNA sequencing and purification

The PCR products were run on a gel and enzymatically cleaned up using the EXOSAP method. The extracted fragments were sequenced in the forward and reverse direction using (Nimagen, BrilliantDye™ Terminator Cycle, Sequencing Kit V3.1, BRD3-100/1000) and purifed (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit, Catalogue No. D4050). The purified fragments were analysed on the AB1 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific) for each reaction and every fungal sample. Thereafter, BioEdit Sequence Alignment Editor Version 7.2.5 was used to analyse the ab1 files generated by the AB1 3500XL Genetic Analyzer. The results obtained by a BLAST search from the national center for bioinformatics information (NCBI) were used to confirm the identities of these strains.[14,15]

Statistical analyses

The frequency, percentage, rate, or prevalence of occurrence was expressed as a percentage.

RESULTS

Table I shows the colony (morphological) and microscopic characteristics of *Aspergillus* and *Penicillium* sp. recovered from all sample cultures. There were observational variances in their cultural and cellular appearances on SDA plates and the microscope respectively.

Table- I: Morphological characteristics of fungi

S/N	Colonial characteristics	Reverse surface reaction	Microscopic appearance	Probable organism
1.	Wooly at first white to yellow, then turning to dark brown black	White to yellow	Double cover, entire vesicle form "radiate" head	*Aspergillus* sp.
2.	Velvety, yellow to green	Golden to red brown	Single and double cover, entire vesicle point out in all directions	*Aspergillus* sp.
3.	Blue-green pigmentation with suede-like surface consisting of a dense felt of conidiophores	Pale yellow	A single series of phialides, rounded and rarely conidia	*Aspergillus* sp.
4.	Greenish-blue with whitish edge	Yellow to brown	Septate hypha, columnar conidial head	*Aspergillus* sp.
5.	Tan to brown	Brown	Globose conidia with varying sizes that are slightly roughed	*Aspergillus* sp.
6.	White colour	Pale yellow	Singly phialide, branched metulae Singly phialide, branched metulae	*Penicillium* sp.

Table II represents the prevalence and distribution of fungi. The overall prevalence of fungi infection was 79.0. The sex- and age-related distribution profile of fungi shows that 80.0% males and 78.3% females were infected. In both sexes, fungi were isolated in 92.9% of patients that were between 30 and 39 years and 64.7% in those between 15 and 29 years.

Table- II: Prevalence and distribution of fungi

Age group (years)	Number examined			Number infected		
	Male	Female	Total	Male	Female	Total
10-19	2(5.00)	5(8.30)	7(7.00)	1(3.50)	2(2.80)	3(42.9)
20-29	4(10.0)	6(10.0)	10(10.0)	3(7.50)	5(8.30)	8(80.0)
30-39	12(30.0)	16(26.7)	28(28.0)	10(23.3)	16(28.0)	26(92.9)
40-49	9(22.5)	13(21.6)	22(22.0)	7(17.1)	9(15.2)	16(72.7)
50-59	5(12.5)	9(15.0)	14(14.0)	4(11.2)	7(10.9)	11(78.6)
60-69	8(20.0)	11(18.3)	19(19.0)	7(16.6)	8(13.8)	15(78.9)
Total	40(40.0)	60(60.0)	100(100)	32(80.0)	47(78.3)	79(79.0)

Table III states the molecular BLAST of pure fungal isolates; here the molecular identification of the two queried *Aspergillus* species that were culturally alike using the BLAST technology showed that the similarities between them and biological sequences in the NCBI database, was 99.7%. Ultimately, their identity was confirmed as *Aspergillus flavus* and *Saccharomyces cerevisiae.*

Table- III: Molecular BLAST of pure fungal isolates

S/N	Culturally characterized pure isolate	GenBank Accession Number	Percentage ID (%)	Predicted fungi
1.	*Aspergillus flavus*	XR- 002086443.1	99.7	*A. flavus*
2.	*Aspergillus niger*	NR- 132207.1	99.7	*S. cerevisiae*

Table IV contains the prevalence and distribution of fungal genera. In both sexes, the recovery rates of *A. flavus* and *S. cerevisiae* was 48.1% and 24.1% respectively. In all cases of fungal infection, 47 (78.3%) females were infected, where males were 32 (80.0%) more. *Penicillium* species were also 22 (27.8%) and *A. flavus* were 38 (48.1%).

Table- IV: Prevalence and distribution of fungal genera

Sex	Number examined	Number infected	Number positive			Total
			A. flavus	S. cerevisiae	Penicillium sp.	
Male	40	32	17(21.3)	6(7.50)	9(11.3)	32(80.0)
Female	60	47	21(26.8)	13(16.6)	13(16.6)	47(78.3)
Total	100	79	38(48.1)	19(24.1)	22(27.8)	79(79.0)

Table V shows that the prevalence of non-concomitant *A. flavus* and *S. cerevisiae* in 57% male and female patients in six (6) age categories. There was no established pattern of infection across ages and sexes. However, the overall number of infected females was 61.4%) and infected males was 38.6%.

Table- V: Prevalence and distribution of Aspergillus flavus and Saccharomyces cerevisiae

Age group (years)	Number examined (n =100)			Number infected (n=57)			Distribution of A. flavus (n = 38) and S. cerevisiae (n = 19)						Total
	Male	Female	Total	Male	Female	Total	Male			Female			
							A. flavus	S. cerevisiae	Total	A. flavus	S. cerevisiae	Total	
10-19	2(5.00)	5(8.30)	7(7.00)	0(0.00)	1(1.40)	1(14.3)	0(0.00)	0(0.00)	0(0.00)	1(1.00)	0(0.0)	1(57.0)	1(14.3)
20-29	4(10.0)	6(10.0)	10(10.0)	1(2.50)	4(6.70)	5(50.0)	1(5.00)	0(0.00)	1(11.4)	2(2.50)	2(5.5)	4(45.6)	5(50.0)
30-39	12(30.0)	16(26.7)	28(28.0)	5(11.7)	9(15.8)	14(50.0)	3(8.40)	2(5.60)	5(20.4)	5(7.70)	4(6.2)	9(36.6)	14(50.0)
40-49	9(22.5)	13(21.6)	22(22.0)	6(14.7)	7(11.8)	13(59.1)	4(8.70)	2(4.30)	6(26.3)	5(9.30)	2(3.7)	7(30.7)	13(59.1)
50-59	5(12.5)	9(15.0)	14(14.0)	3(8.40)	6(9.30)	9(64.3)	3(9.00)	0(0.00)	3(19.0)	4(6.00)	2(3.0)	6(38.0)	9(64.3)
60-69	8(20.0)	11(18.3)	19(19.0)	7(16.6)	8(13.8)	15(78.9)	4(8.60)	3(6.40)	7(26.6)	6(11.3)	2(3.8)	8(30.4)	15(78.9)
Total	40(40.0)	60(60.0)	100(100)	22(55.0)	35(58.3)	57(57.0)	15(38.9)	7(18.1)	22(38.6)	23(37.5)	12(19.5)	35(61.4)	57(57.0)

Figures in parentheses represent percentages

DISCUSSION

Aspergillosis has been associated with significant morbidity and mortality among immune-compromised patients.[21,23] This present study provides current microbiological evidence that pulmonary *Aspergillus* infection is more common with HIV infected persons undergoing treatment than other fungal infections. However, this study highlighting the high recovery of *S. cerevisae* should elicit concerns in the management of both diseases as this is the first record of its occurrence in Nigeria in those immunosuppressed due to HIV or AIDS and who are currently and regularly receiving antiretroviral treatment.

The results herein interestingly show that *A. flavus* and *S. cerevisae* infections are not age- or sex dependent. The implication of this is that transmission can equally occur in any person irrespective of his or her age.[16] More worrisome is the fact that the prevalence of *A. flavus* obtained in this current study was higher than those of Ogba *et al.*[23] and Nasir *et al.*[24] who respectively recorded occurrences of 47.1% and 12.7% in Southern and Northern Nigeria, as well as those of Kaur *et al.*[16] and Prakash *et al.*[25] who reported a prevalence of 16.9% and 16.5% respectively in India. However, these discrepancies might be as a result of the differences in the sample size, defining inclusion criteria and methodologies employed.

Several genetic techniques have been used to classify different *Aspergillus* species, namely the ITS (internal transcribed spacer) region, the aflatoxin gene cluster, and random amplification of polymorphic DNA (RAPD).[14,23] In this current study, the genomic DNA containing 16srDNA specific primers that were helpful in amplifying medically important fungi were used. All the fungal isolates tested appeared very heterogenous. This allowed the exclusion of a common source of infection. The

non-relatedness of *A. niger* sequential isolates could suggest a pattern of re-infection rather than relapse unlike those of *A. flavus*.

Following the recommendation of the World Health Organization.[20] that diagnostic services for confirmation of their causative role in AIDS and mortality be strengthened, it is therefore necessary to identify and carefully monitor patients with high risk factors for developing AIDS and to initiate diagnostic procedures as soon as possible in these patients. The results of the study showed that the BLAST method is diagnostically significant in the characterization of closely related *Aspergillus* and *Saccharomyces* species found in the respiratory tract of humans infected with HIV and whose presence may account for the morbidities observed even while they are currently receiving anti-retrovirals. Considering that as at the end of December 2022, the Joint United Nations Programme on HIV/AIDS (UNAIDS) documented the fact that 29.8 million people were accessing ART up from 7.7 million in 2010.[26]

CONCLUSIONS

Accurate and timely diagnoses of these fungal infections are likely to positively affect HIV treatment outcomes as well as the epidemiological pattern of HIV/ AIDS. In this regard, all HIV-positive individuals, regardless of whether they have cough symptoms or not, should be appropriately tested early for respiratory tract fungal pathogens. Success can be increased by collaborative groups of expertise in diagnostics.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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