PCR-Internal Transcribed Spacer (ITS) genes sequencing and phylogenetic analysis of clinical and environmental Aspergillus species associated with HIV-TB co infected patients in a hospital in Abeokuta, southwestern Nigeria.

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Abstract

Background: Aspergillosis has been identified as one of the hospital acquired infections but the contribution of water and in-house air as possible sources of Aspergillus infection in immunocompromised individuals like HIV-TB patients have not been studied in any hospital setting in Nigeria.

Objective: To identify and investigate genetic relationship between clinical and environmental *Aspergillus sp.* associated with HIV-TB co infected patients.

Methods: DNA extraction, purification, amplification and sequencing of Internal Transcribed Spacer (ITS) genes were performed using standard protocols. Similarity search using BLAST on NCBI was used for species identification and MEGA 5.0 was used for phylogenetic analysis.

Results: Analyses of sequenced ITS genes of selected fourteen (14) *Aspergillus* isolates identified in the GenBank database revealed *Aspergillus niger* (28.57%), *A. tubingensis* (7.14%), *A. flavus* (7.14%) and *A. fumigatus* (57.14%). Aspergillus in sputum of HIV patients were *Aspergillus niger*, *A. fumigatus*, *A. tubingensis* and *A. flavus*. Also, *A. niger* and *A. fumigatus* were identified from water and open-air. Phylogenetic analysis of sequences yielded genetic relatedness between clinical and environmental isolates.

Conclusion: Water and air in health care settings in Nigeria are important sources of *Aspergillus sp.* for HIV-TB patients.

Keywords: Internal transcribed spacer genes, phylogenetic, genetic relationship, clinical and environmental fungi, HIV-TB.

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Introduction

Nigeria has the second largest number (3.1 million) of people living with HIV/AIDS (PLWHA) accounting for 10% of the global HIV burden with about 8% of the HIV-people being tuberculosis positive[1]. Tuberculosis is the most common opportunistic infections affecting HIV individuals. HIV and TB have synergistic interactions that speedily accelerate the weakening and decline of the host immune system, accentuating the progression of each other. People living with HIV/AIDS (PLWHA) are exquisitely vulnerable to TB and are 30-50 times more likely to progress to active TB. The likelihood of progressing to full blown AIDS also increases by 100 folds in HIV-TB co infected patients[2]. HIV-TB coinfection is on the rise with tuberculosis being the leading cause of death for people infected with HIV[3].

Aspergillosis in HIV patients is another opportunistic infection caused by inhalation of Aspergillus spores[4-7]. Aspergillus spores are numerous in the environment and the organism is the most common ubiquitous opportunistic pathogen affecting the lung[8]. Spectrum of aspergillosis are allergic bronchopulmonary aspergillosis,

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chronic necrotizing aspergillosis, aspergilloma and invasive aspergillosis[5] with the immune competence of the host largely determining the particular presentation.

Co infection of HIV, TB and aspergillosis[9-12] has been documented in the literature unlike reports on the duos of HIV-TB, Aspergillosis-TB and HIV-Aspergillosis. In Nigeria, high prevalence of 51.25% aspergillosis was associated with pulmonary symptoms in patients attending Infectious Diseases Hospital in Kano[13]. A cytologic assessment of pulmonary aspergillosis in the North-East Nigeria reported 46% in HIV, and 89% in TB patients[14]. Ogba et al[15] reported low incidence of aspergillosis in Calabar South-South Nigeria while Aliyu et al[16] reported yeasts and other molds in Benin, Nigeria.

Nosocomial outbreaks of invasive aspergillosis highlight the fact that Aspergillus spores are common in the hospital environment[17]. Genetic relationship between environmental and clinical strains has been a subject of several studies[18-23] with varied conclusion. Molecular characterization of fungi by polymerase chain reaction (PCR) amplification of internal transcribed spacer (ITS) region of the nuclear ribosomal repeat unit of the rRNA gene is a widely used tool. This ITS region has become the primary genetic marker for molecular identification and other species-level pursuits in many groups of fungi[24-25]. Its usefulness in sequence comparison are based on the PCR successes and high degree variation even between closely related species[26,27] and has been proven comparatively with other coding sequence on species resolution[28].

Since hospitalized HIV-TB co infected patients are at the risk of exposure to nosocomial infections such as aspergillosis, there is a need to identify the different environmental *Aspergillus* in hospital settings and to investigate the contribution of water and open-air to Aspergillus dissemination by determining their genetic relationship to those from sputum of HIV-TB patients using PCR-ITS gene sequencing and phylogenetic techniques.

Methods

Study design

This cross-sectional study was conducted between September 2013 and March 2014 in the Tuberculosis (TB) ward of a semi-private hospital in Abeokuta, Ogun State, Nigeria. An Ethical clearance was obtained from the Ethical Committee of the hospital. The hospital has facilities for tuberculosis and HIV/AIDS diagnosis, treatment and monitoring. Patients that were already diagnosed of HIV-TB coinfection were considered eligible for the study. Informed consent (oral) was obtained from the subjects and only consented candidates were enrolled in the study. Demographic characteristics were obtained using a structured form from a total population of thirty (30) HIV-TB patients.

Sampling and sample collection

Details of fungal sampling and isolation from environmental (water and open-air) and clinical (sputum) samples and their morphological classification were as described Shittu et al[29]. Briefly, water samples were collected from the taps and storage water in the hospital into sterile bottles using standard techniques. Also, different bottled and sachet water sources for drinking water that are sold within and around the hospital premises were purchased for fungal analysis. Also, open-air sampling of the corridor, and indoor (corners and centre) of the wards, nursing section, and bathroom was conducted by exposing duplicate Sabouraud Dextrose Agar (SDA, BIOLAB, Hungary) plates for 2h. Also, sputum samples from HIV-TB patients were collected into sterile universal bottles.

Isolation of fungi from environmental and clinical samples

Membrane filter technique was employed in the isolation of fungi from water; by filtering 100 ml in duplicate, using 47 mm membrane filters (Millipore, Bangalore) with a pore size of 0.45µm. The filter papers were placed on both SDA and potato dextrose agar (PDA, LAB M, UK) plates. Also, SDA plates exposed to air were withdrawn after 2h. For fungi isolation from clinical samples, a loopful of sputum was cultured on PDA. All SDA and PDA plates were incubated for 3-5 days at 25^{o}C for growth of fungi.

DNA extraction, purification, amplification and sequencing

DNA extraction, amplification and sequencing were conducted at the International Institute for Tropical Agriculture (IITA), Ibadan, Nigeria. DNA extraction and purification was done with QIAamp DNA Mini Kit (Qiagen, Valencia, CA) with catalogue number 51304 following manufacturer's instruction. The Extracted Nucleic

Acid was then treated with RNase and 3µl of the DNA was loaded on 1% agarose and ran at 96-100 volts using 1xTBE for 1h.

DNA amplification primers, ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') were purchased from Integrated DNA Technologies, Belgium. PCR amplification was performed using GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA) protocol. PCR Amplification was carried out in a PCR System Thermocycler (Applied Biosystems) according to standard protocol of White et al[26]. This involved using 5µM of each primer, 2µl genomic DNA and nuclease free water adjusted to a final volume of 50µl. The PCR protocol consisted of initial denaturation of 94°C/5mins, 30 cycles of 72°C/45secs, 56°C/30secs and final extension/elongation of 72°C/5mins. The PCR was purified with absolute ethanol and 70% ethanol respectively. PCR products were separated by electrophoresis on agarose 2.5 -3.0% gels. Gels were photographed with a gel documentation system (Gel Doc 1000, BioRad) and the sizes of the PCR products were calculated by comparison with internal mol. wt standards. Finally, 2.5µl of the purified PCR products were sequenced using the Applied Biosystems ABI PRISMTM 3100 DNA sequence Analyzers with the BigDye® Terminator v3.1 Cycle Sequencing kit and protocols.

Molecular identification of sequenced ITS-18S rRNA genes

Fasta formatted sequences obtained from ABI automated sequencer were edited and aligned to generate a consensus sequence using BioEdit Sequence Alignment Editor (version 7.1.9). Consensus sequences were then aligned with sequences deposited in the National Centre for Biotechnological Information (NCBI) genebank by using the Basic Local Alignment search Tool (BLAST) to establish identities of the fungal isolates.

Phylogenetic analysis

To identify homologies, multiple sequence alignment was done using ClustalW alignment algorithm[30]. Aligned sequences were arranged with MEGA 5.0 for phylogenetic analyses[31]. The evolutionary history was inferred using the Neighbor-Joining method[32]. The evolutionary distances were computed using the Maximum Composite Likelihood method[33] and were in the units of the number of base substitutions per site. Two thousand bootstrap replicates were performed. All positions containing gaps and missing data were eliminated.

Results

HIV-TB patients' characteristics and fungi isolation

Table 1 shows the sex distribution of HIV-TB patients and positive fungi isolation observed in this study. From a total of thirty patients investigated, there were more female (66.7%) with 95% of culture positive sputum while male (33.3%) had 50% of positive sputum samples.

Table 1. Sex distribution of HIV-TB patients and fungi isolation

Sex	Frequency (%)	Culture Positive n (%)	Culture Negative n (%)
Male	10 (33.33)	5(50)	5(50)
Female	20 (66.67)	19(95)	1(5)
Total	30 (100)	24(80)	6(20)

A total of 24 (80%) out of thirty HIV-TB patients were culture positive. Fig. 1 shows the age distribution of HIV-TB co infected patients and fungi isolation with age group 21-30 giving 25% of positive culture.

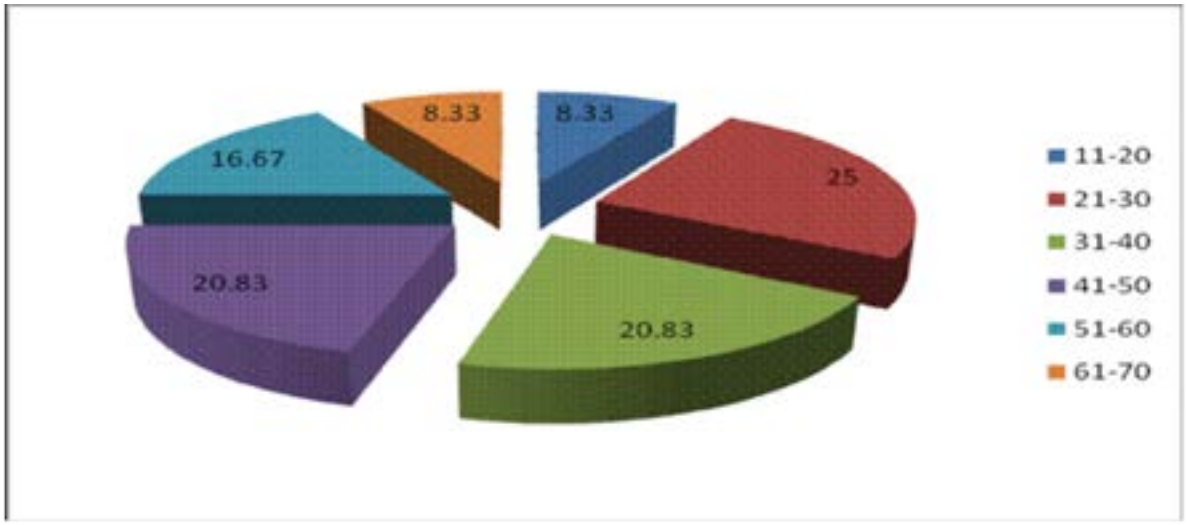

Fig 1. Age distribution of HIV-TB co infected patients with percentage fungal infection

PCR amplification and DNA sequences results

Polymerase Chain Reaction (PCR) amplification of ITS region of rRNA genes with ITS1 and ITS 4 primers yielded distinct DNA bands for all representative isolates investigated (Plate 1).

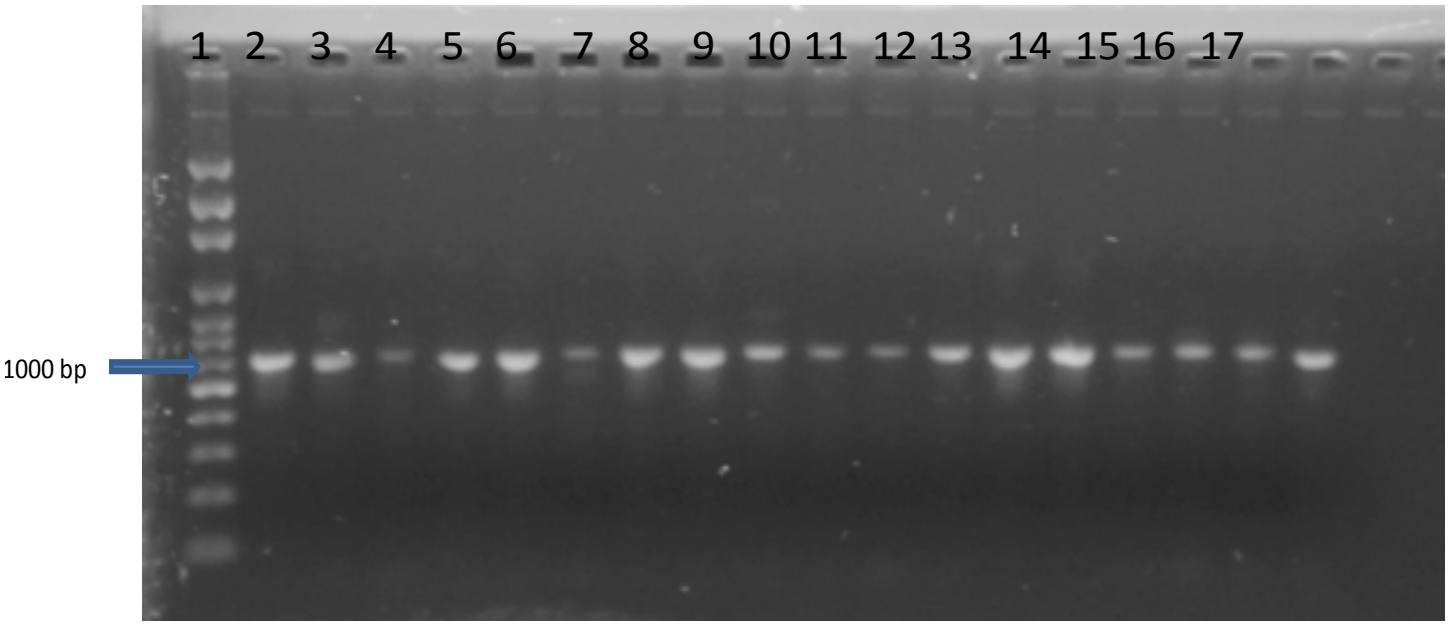

Plate1. 1% agarose gel electrophoresis of PCR amplicons of the Internal Transcribed Spacer (ITS) region genes of the fungal isolates.

Key: Lane 1- 1kb DNA marker; Lane 2, 3, 14 - Isolate from open air culture; Lane 4, 5, 6, 16, 17 – Isolate from sputum; Lane 7, 8, 13 – Isolates from storage water; Lane 9, 10 – Isolates from tap water; Lane11 – Isolates from bottled water; Lane 12, 15 – Isolates from sachet water.

DNA sequences of *Aspergillus sp.* identified by similarity searches in the GenBank gave *A. fumigatus* (57.6%), *A. niger* (28.6%), *A. tubingensis* (7.1%), and *A. flavus* (7.1%) from all sources investigated (Table 2). *Aspergillus niger* and *A. fumigatus* were identified from the three environments examined, i.e. water, air, and sputum while *A. tubingensis* and *A. flavus* were identified from sputum only.

Table 2. DNA Sequences and organisms identified by Blast on NCBI

Isolate codes	Length of query sequences (bp)	NCBI Best Match (Identified organisms)	Sources of isolate
A1	1037	*A. niger* SUMS0061	Air
A2	1004	*A. fumigatus* HF11	Air
A3	568	*A. niger* SUMS0061	Sputum
A4	1037	*A. fumigatus* 871435	Sputum
A5	1077	*A. flavus*	Sputum
A6	689	*A. niger* SUMS0061	Storage Water
A7	1038	*A. fumigatus* SUMS0106	Storage Water
A8	727	*A. fumigatus* SUMS0106	Tap Water
A9	720	*A. fumigatus* SUMS0106	Bottled Water
A10	1032	*A. fumigatus* SUMS0106	Sachet Water
A11	1046	*A. fumigatus* SUMS0106	Storage Water
A12	571	*A. niger* 20-10	Air
A13	559	*A. fumigatus*W1-25	Sachet Water
A14	391	*A. tubingensis* G1-18	Sputum

Phylogenetic relationship between isolates

Figure 2 shows an unrooted phylogenetic tree in cladogram format with branch lengths using a neighbor joining method. The optimal tree with the sum of branch length = 3.78942342 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary relationship between the DNA of the isolates sequenced depicted distinct clades. Analysis of sequences also revealed inter and intra species diversity among *A. fumigatus* and *A. niger* from same and different sources. DNA sequences of *A. fumigatus* from sputum and air showed same branch length and were genetically related with one another.

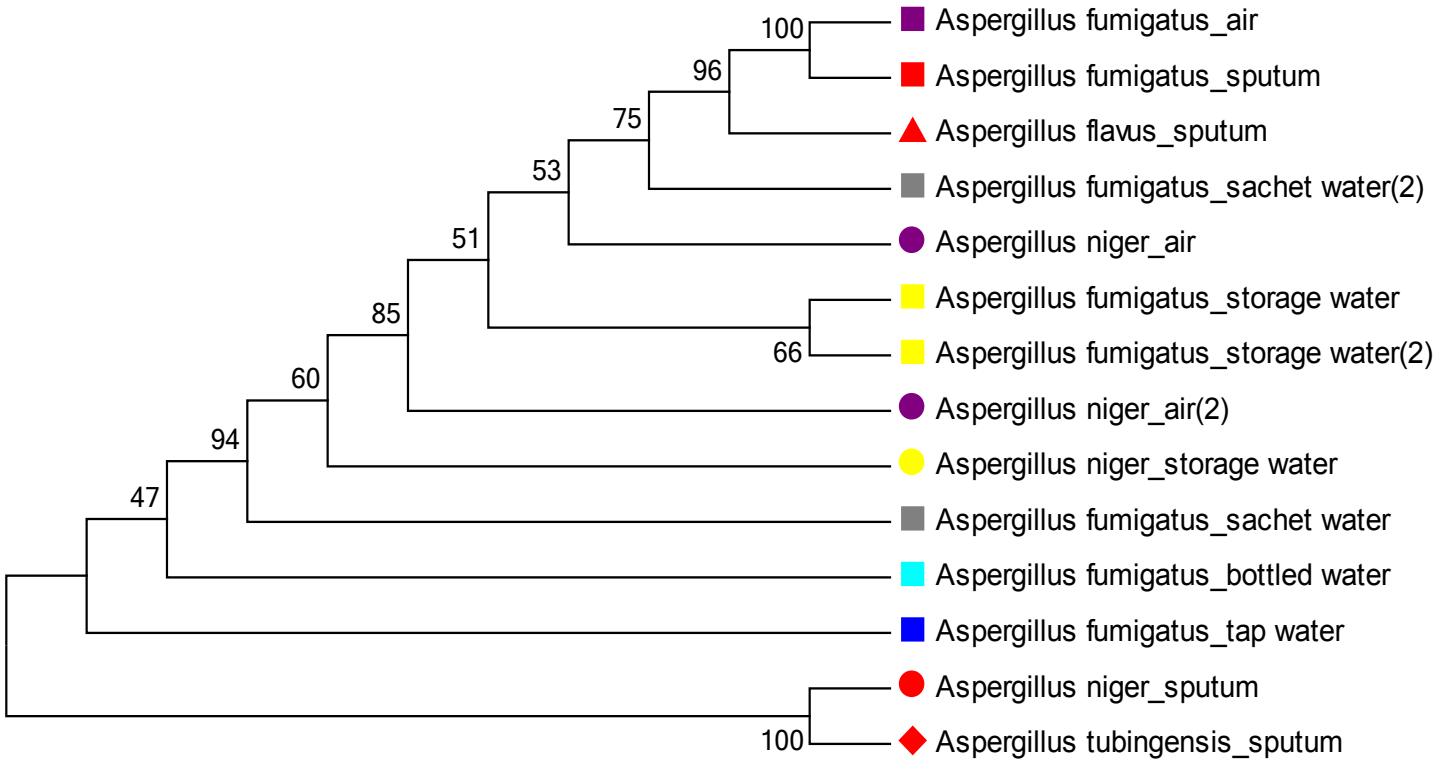

Fig. 2. Neighbor joining phylogenetic bootstrap consensus tree of PCR-ITS DNA sequences of environmental and clinical Aspergillus species. The percentage of replicate trees in which the associated taxa related together in the bootstrap test (2000 replicates) is shown next to the branches. Same species are shown in the same shape and sources of isolates in same color.

Discussion

DNA sequences of the ITS regions of fourteen (14) Aspergillus isolates in the GenBank database yielded *A. fumigatus, A. niger, A. flavus* and *A. tubingensis* from both clinical and environmental fungal isolates. The same strain of *A. fumigatus* was isolated from storage water, tap water, sachet water, and bottled water. Notable is the predominance of *A. fumigatus* in sputum samples. In immunocompromised hosts, *A. fumigatus* represents a major cause of morbidity and mortality[34] and it is the commonest etiologic agent of various clinical forms of bronchopulmonary aspergillosis including allergic, acute invasive and chronic pulmonary aspergillosis[8-9,19-22].

In this study, *A. tubingensis* initially identified as *A. niger* by microscopic and macroscopic morphology was discovered from sputum samples of patients. This reclassification of *A. niger* as *Aspergillus tubingensis* by sequencing agrees with Perrone et al[35]. This organism was implicated to have caused infectious keratitis[36] and osteomyelitis of the maxillary bone of an immunocompromised patient after tooth extraction[37].

Phylogenetic analysis in this present study showed that environmental and clinical isolates proceeded from a common ancestor which is similar to that of Lee et al[23]. Sequences yielded distinct clades revealing inter and intra species similarity and diversity among *Aspergillus sp.* DNA sequences of *A. fumigatus* from sputum, air and water were genetically related while *Aspergillus tubingensis* from sputum was also genetically related with *A. niger* from air.

This present study has not only revealed the co-existence of *Aspergillus sp* in 80% of HIV-TB co infected patients, but emphasizes that water and air in health care settings are important sources of *Aspergillus* portraying serious health risk for HIV-TB co- infected patients.

There is therefore a need to further investigate clinical cases of aspergillosis in HIV-TB patients in Nigeria, a TB endemic region.

To our knowledge, this is the first study on molecular identification of clinical and environmental fungi in Nigeria, a tropical and low incomecountry. This is also the first report on genetic relationship of *Aspergillus sp* from environmental and clinical sources.

Conclusion

This investigation has shown that both open-air and water in health care settings in Nigeria are important contributors to the transmission of *Aspergillus sp.* to immunocompromised host.

Conflict of interest

The authors declare that there was no conflict of interest during this study.

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