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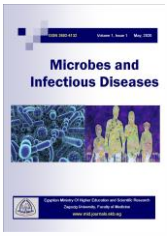

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Original article

Phylogeny of circulating strains of *Histoplasma capsulatum* isolated from Saye, Kaduna State, Nigeria

Rabiah Orchewa OSUMAH [1,2*], Muhammad Hassan Isa DOKO [1]

1- Ahmadu Bello University, Department of Microbiology, Zaria City, Kaduna State, Nigeria.
2- University of Benin, Department of Science Laboratory Technology, Benin City, Edo State, Nigeria.

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ABSTRACT

Background and rationale: *Histoplasma capsulatum* (*H. capsulatum*) is a saprophytic fungal which is dimorphic in nature and strive beautifully in soil rich in nitrogen. Inhaling of infectious spores by mammalian species can result to an infection which can be localized in the lungs infection or disseminated histoplasmosis. **Method:** Genomic DNA was extracted from the yeast cells with Pure gene DNA isolation kit and quantified by Nano drop technology. Conventional PCR procedure was used to amplify the ITS regions of *H. capsulatum* and primers (ITS1 and ITS2) used were based on the ITS sequence of *H. capsulatum*, deposited in the Gen Bank (accession number U18363), while Sequencing of the internal transcribed spacer (ITS) of the 16S rRNA genes of the *H. capsulatum* were examined for the nucleotide sequences of ITS1, the 5.8S rRNA gene, and ITS2 region. This was to determine the Sequence variations in both ITS regions which allowed the exploration of the diversity of *H. capsulatum* in the study area and to study the Phylogenies / epidemiology of the fungus. **Result:** Maximum likelihood and close relationship analysis was used to identify strains that fall under geographical clades that have been widely identified in literature. In our study, we were able to have isolates identifying with Eurasian clades (China 5.6 %; Spain 22.2 % and Malaysia 27.8 %) and Netherland Clade (5.6 %). **Conclusion:** All our isolates identified in this study are origin of African as literature had it that *Histoplasma capsulatum* variety duboisii is associated with African continent.

Introduction

Histoplasma capsulatum is a dimorphic fungus which occurs in the soil as saprophyte (at 25 °C) in form of mold and as a pathogen (at 37 °C) in yeast form in host cells [1]. It was first reported in 1905 by Samuel Darling when he was working on a patient from Martinique [1]. Has a high affinity for nitrogen/phosphate-rich soils associated with bird and bat guano [2]. Suitable ecological conditions for the growth of this fungus are moderate temperatures (18 - 28°C), constant humidity (>60%), and a low light environment [2]. At 25°C it reproduces asexually using microconidia and macroconidia [2] and when the soil is disturbed, fragmented hyphal cells can be inhaled by various vertebrate species, including humans, and upon reaching the alveoli undergo a rapid conversion to pathogenic yeast cells which persist in host lungs and may disseminate to other tissues causing histoplasmosis [3]. The pathogenic yeasts are ovoid and thick-walled and can be cultured in cysteine-enriched blood or brain-heart infusion media supplemented with glutamine

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* *Corresponding author:* Rabiah Orchewa Osumah
E-mail address: osuma.orchewa@uniben.edu.ng

(BHI). They also grow at 37°C in the tissues of an immunocompromised individual [3, 4]. The spectrum of disease varies from asymptomatic to symptomatic illness especially with pulmonary infection patients. Clinical presentation includes; cough, headache, muscle pain, sleeplessness, chill and fever in the infected patients [3, 5]. Immunocompetent persons can acquire the disease through tilling of soil, visiting caves infected with bats and birds, dilapidated buildings, reconstruction of old houses where birds nest (or build their roosting) [4,6]. Among the immunocompromised population with cellular immunity impairment (such as HIV), the disease is responsible for high rates of morbidity and mortality [6, 7]. The disease is mostly fatal without early diagnosis and proper treatment [8, 9]. In different parts of the world, *Histoplasma*-contaminated soils are often reported, especially in the presence of bat or bird guano. Infection of birds are sporicidal and could play role in its dispersal [10, 11]. The fungus can also be detected in wild mammals such as non-human primates (e.g., baboons) [9], mustelids (e.g., badgers and northern sea otter) [12] procyonids (e.g., raccoons) [13,14], as well as in domesticated animals such as equines [15], felines [16] and canines [13]. This broad host range and tight association with vertebrates suggests that mammals play an important role in the speciation and dispersal mechanisms of this species. *Histoplasma capsulatum* is a cosmopolitan fungus, and epidemiological knowledge has been improved by serology, culturing and molecular-based diagnostic methods [17].

Epidemiology

The endemicity of histoplasmosis varies from low (Europe and Oceania), to moderate (Africa and South Asia), to high (Americas) prevalence areas. The Midwestern and Southeastern regions of the United States, specifically the Ohio, St Lawrence and Mississippi River regions are considered highly endemic [18]. In Latin America, the high prevalence areas range from Uruguay to Mexico, mostly in countries with a moderate climate and constant humidity [19]. In Africa, two well-delimited areas are considered endemic-southern Africa, which includes South Africa, Tanzania and Zimbabwe, and western/central Africa which include Nigeria [20]. In southern Asia, histoplasmosis is found in China, India and Thailand based on very few clinical reports; however, skin test surveys suggest that the fungus also occurs in Malaysia, Indonesia, Myanmar and the Philippines [21,3]. Epidemiological surveys suggest that under-surveyed areas of the disease can have moderate to high levels of *Histoplasma* natural infections and the impact of the disease outside of the current known endemic regions should be investigated. Based on phenotypic characteristics (host, morphology and pathogenicity), the genus *Histoplasma* was split into three varieties: *H. capsulatum var. capsulatum*, *H. capsulatum var. duboisii* and *H. capsulatum var. farciminosum*. The capsulatum variety is the most broadly dispersed, and the lungs are the main compromised organs, and in isolated cases the phagocytic mononuclear system is involved in disseminated forms. The *var. duboisii* occurs in tropical areas in Africa, causing cutaneous, subcutaneous and bone lesions and the *var. farciminosum* isolates are known to occur in Europe, Northern Africa, India and Southern Asia, commonly infecting horses and mules [20].

Advances in molecular genotyping, techniques such as Restriction Fragment Length Polymorphisms (RFLP), DNA hybridization, Random Amplified Polymorphic DNA (RAPD) and ITS1/2 sequencing all revealed high genetic diversity in *Histoplasma* and some indicating geographical association [22]. Kasuga and colleagues [23] suggested at least 8 clades with 7 phylogenetic species: North America clade 1 (NAm1), North America clade 2 (NAm 2), Latin America clade A and B, (which is LAmA and LAm B), Australia, Netherlands, Eurasia, and Africa. The aim of this research is to diagnose patients with pulmonary diseases who are thought to be suffering from tuberculosis and treated with tuberculosis drugs but fail to respond to treatment and also to know the phylogenetic relationship between *H. capsulatum* isolates circulating in the study area, as knowing the variety (ies) of *H. capsulatum* in circulation may also provide data on epidemiology of Histoplasmosis in the study area and Nigeria at large.

Methods

Study area

This study was conducted in Zaria, Nigeria. The ancient city of Zaria formally known as Zazzau is a major city in Kaduna State, in Northern Nigeria as well as being a Local Government Area. It is one of the major seven Hausa City States and was also a capital of the Hausa kingdom of Zazzau and the capital of Zazzau emirate [24,25]. Zaria is located

between latitudes 11004'00"N and 11008' 800"N and Longitude 7036 ' 00"E and 7044 ' 00"E [26]. The total land covered is about 2638.20Km2 with an altitude of about 615 meters above sea level [27]. It is bounded in the North by Kudan, in the East by Soba, in the West by Giwa and in the South by Igabi. The study area consists of one distinct settlement, Saye in Zaria metropolis. The main occupation of Zaria people is farming, trading and some are civil servants.

Saye is a locality in Zaria where the National Tuberculosis and Leprosy Training Center and Referral Hospital (NTLTCRH) is located. It is a small community with farming and rearing of live-stock mainly as the occupation of the people. The NTLTCRH is the only referral Mycobacterial tuberculosis (TB) and Leprosy Centre in Nigeria where people from different geographical locations visit for TB treatments. The hospital is well staffed with Doctors, Nurses and trained Laboratory personnel. The Center has a laboratory, well equipped with reagents, media, gene Xpert machines, supplied by the World Health Organization in collaboration with the Federal Republic of Nigeria.

Source: Ministry of Land and Survey Kaduna State (2000).

Study design

This was cross sectional and Hospital based. Sputum sample were collected from patients and structured questionnaires were administered.

Ethical clearance

Ethical clearance (NBTL/TR6/ZA/182/vel3v) for the study was obtained from the Ethical Committees of National Tuberculosis and Leprosy Training Center and Referral Hospital Saye, Nigeria. Similarly, informed consent was obtained from each of the participants prior to recruitment into the study.

Study population

This comprised patients with signs and symptoms of pulmonary infection attending National Tuberculosis and Leprosy Training Centre and Referral Hospital, Saye.

Inclusion criteria and exclusion criteria

Inclusion criteria

Patients attending National Tuberculosis and Leprosy Training and Referral Hospital, Saye presenting with pulmonary diseases who gave consent.

Exclusion criteria

Non-pulmonary disease patients presenting at National Tuberculosis and Leprosy Training and Referral Hospital Saye and those patients who did not give consent.

Collection of samples

Sputum samples were aseptically collected in universal containers. Pulmonary disease patients who were attending the Referral Hospital were educated by a qualified Medical Laboratory Personnel on how to collect early morning sputum. A total of 322 sputa samples were collected with universal containers.

Isolation and identification of *H. capsulatum*

Preparation of sputum

Sputum samples were diluted with sterile distilled water and centrifuged to obtain a homogeneous solution prior to culturing [28, 27].

Culturing of sputum samples

The sputum samples were streaked unto Brain heart infusion agar (BD Difco, Sparks, USA) enriched with 10 % sheep blood and supplemented with 5 % glutamine, 12 mg/ml penicillin and 40 mg/ml streptomycin and incubated at 37° C for *H. capsulatum* yeast (pathogenic) form [29].

Confirmation of isolates with Polymerase chain reaction (PCR)

Deoxyribonucleic acid (DNA) extraction

Pure culture of yeast cells of *H. capsulatum* was grown at 35 °C in yeast peptone broth (in a water shaker at 120 rpm for 3 days) to obtain a log phase growth [30]. The yeast cells were harvested and washed three times in sterile distilled water by centrifugation at 2,000 rpm. Genomic DNA was extracted from the yeast cells with Pure gene DNA isolation kit (Gentra Systems, Inc. Minneapolis, Minn.) following the manufacturer's instructions. DNA was quantified by Nano drop technology. (Nano drop Technologies, Oxford shire, UK).

Conventional polymerase chain reaction (PCR)

This was performed to amplify the entire ITS region. Amplification was done as described by White et al. [31]. The PCR cocktail mix consist of 2.5 µl of 10x PCR buffer, 1µl of 25 mM of MgCl2, 1µl each of forward and reverse primers, 1µl of DMSO, 2 µl of 2.5 mM DNTPs, 0.1µl of 5 µ/µl Taq DNA polymerase and 3 µl of 10 ng/µl DNA. The total reaction volume was made up to 25 µl using 13.4 µl Nuclease free water. Negative controls, without the presence of DNA, were subjected to amplification simultaneously. The amplification was performed in a master cycler thermocycler (Maun. Therm.)

involving initial denaturation of 94 °C for 5minutes followed by 36 cycles of denaturation at 94 °C for 30 seconds annealing at 54 o C for 30 seconds and extension at 72 °C for 45 seconds, and one cycle of final extension at 72 °C for 7minutes and holding temperature of 10 °C.

Electrophoresis of the PCR products

The PCR products were electrophoresed through 1.5 % agarose (Sigma Chemical Co., St. Louis, Mo.) dissolved in Tris-borate-EDTA buffer (0.1 M Tris, 0.09 M boric acid, 0.001 m EDTA [pH 8.4]). Electrophoresis was conducted at 80 V for 60 min, with 5 µl of each PCR amplicon plus 1µl of tracking dye added to each well; the bands were visualized with a UV trans illuminator after ethidium bromide staining [31].

Sequencing of the internal transcribed spacer (ITS) of the 16S rRNA genes of the *H. capsulatum*

Isolates of *H. capsulatum* were examined for the nucleotide sequences of ITS1, the 5.8S rRNA gene, and ITS2 region. This was to determine the sequence variations in both ITS regions which allowed the exploration of the diversity of *H. capsulatum* in the study area and to study the phylogenies / epidemiology of the fungus. Conventional PCR procedure was used to amplify the ITS regions of *H. capsulatum*. These primers (ITS1 and ITS2) used were based on the ITS sequence of *H. capsulatum*, deposited in the Gen Bank (accession number U18363).

The PCR products were purified using the GFX PCR DNA and gel band purification kit (GE Healthcare Life Sciences). The ITS1–5.8S–ITS2 region was sequenced using primers ITS1 and ITS2. The sequencing reactions were then analyzed by Bio. edit. The determined sequences were compared to those already deposited in the GenBank database using the BLAST program (www.ncbi.nlm.nih. gov/Blast. cgi) [32]. The program MEGA7 (version 7.0) [33] was used for phylogenetic analysis.

Phylogenetic analysis of *H. capsulatum* isolates circulating in the study population

The phylogenetic relationships between the ITS1–5.8S–ITS2 sequences from the clinical *H. capsulatum* isolates used in this study and corresponding sequences from other *H. capsulatum* strains circulating in the world, was obtained, using Bio edit 7.2.5 software (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and Cluster W version and the alignment was manually adjusted [34]. The neighbor joining tree was constructed with Mega version 7.0.21 using Kimura's two parameter method [35], including gaps. The confidence value of branches was determined by a bootstrap's analysis [36]. with 500 replicates. The corresponding fungal isolates were assigned strain names after comparison with representative ex-type sequences available in National Centre of Biotechnology Information USA (NCBI) Gen Bank database. All the sequences from this study were deposited in Gen Bank.

Results

Amplification of the ITS region of *Histoplasma capsulatum* DNA

Plate 1 shows the Photomicrograph of amplified ITS region of the *H. capsulatum* isolates obtained in the study by polymerase chain reaction. From left to right, M is the 50bp molecular ladder and 1 to 18 are the samples with amplicons corresponding to 650bp (ITS region) at Lanes 1,2,5,6,8,9,10,11,12,13,15,16,17,18.

Phylogeny of *H. capsulatum* isolates Genetic similarities of *H. capsulatum* isolates obtained from this study and those from the Gen Bank are shown in **table (1)** with their query identity, query cover, e-value and accession numbers

Figure 4 is the representative phylogenetic tree based on the sequence analysis of the ITS1 – 5.8S – ITS2. The consensus tree topology was obtained by maximum likelihood bootstrap method using5036 tax id, for *H. capsulatum* strains circulating in study area and in some parts of the world. Bootstrap value of 50% (based on 500 replication) are shown. More of the strains in our study clustered highly with strains from Malaysia while some clustered with strains from different parts of the world.

Table 1. Details of the *H, capsulatum* strains whose ITS1–5.8S–ITS2 sequences were used in the study and their places of origin in world.

Isolates (Codes)	Quarry Identity (Q.I)	Quarry Covered (Q.C %)	E – value	Identity Number (IDT %)	Accession No. (Country)
297	17931	32	$8e^{-62}$	91.43	KY646015.1 Mexico
210	19329	32	$5e^{-55}$	91.43	KM912594.1 Netherland
188	1305	32	$1e^{-45}$	94.41	KX646015.1 Spain
303	25303	43	$1e^{-60}$	90.91	KM2225280.1 India
55	14363	34	$6e^{-60}$	90.86	MF105781.1 Indonesia
318	32299	18	$8e^{-69}$	96.27	KX646015.1 Spain
22	42935	43	$1e^{-60}$	90.91	KY684059.1 Malaysia
231	11505	32	$8e^{-62}$	91.43	KY684059.1 Malaysia
317	441859	43	$1e^{-62}$	91.43	KY684059.1 Malaysia
61	387365	32	$8e^{-62}$	91.43	KY684059.1 Malaysia
193	104073	32	$8e^{-62}$	91.43	FM105781.1 Indonesia
313	4185	32	8^{-62}	90.34	KY684059.1 Malaysia
300	20083	17	$8e^{-70}$	96.27	KY684059.1 Malaysia
268	62897	32	$8e^{-60}$	91.43	FJ011535.1 China
225	30637	32	$7e^{-61}$	96.27	KX646015.1 Spain
263	429806	32	$8e^{-62}$	91.43	KX646015.1 Spain

Table 2. Primer used in the PCR reaction.

PCR primer	Nucleotide Sequences	Amplicon Size(bp)	Reference
ITS1	TCCTCCGCTTATTGATATGC	330	[31]
ITS2	GGAAGTAAAAGTCGTAACAAGG	315	

Conventional Polymerase Chain Reaction (PCR)

Figure 1. Map of Nigeria showing Kaduna State.

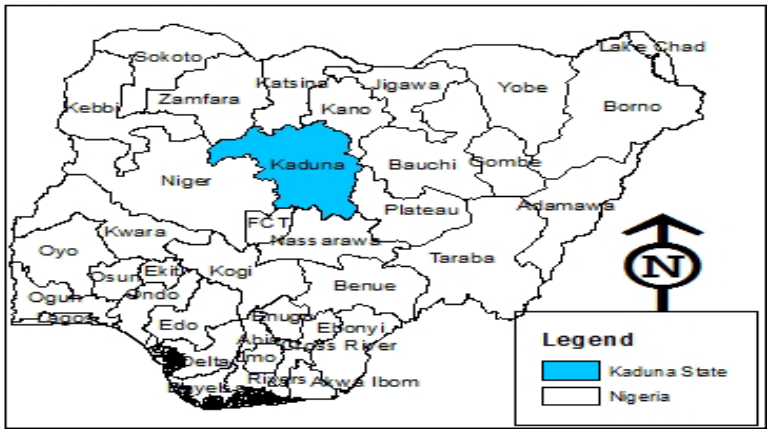


Figure 2. Map of Kaduna State showing Zaria.

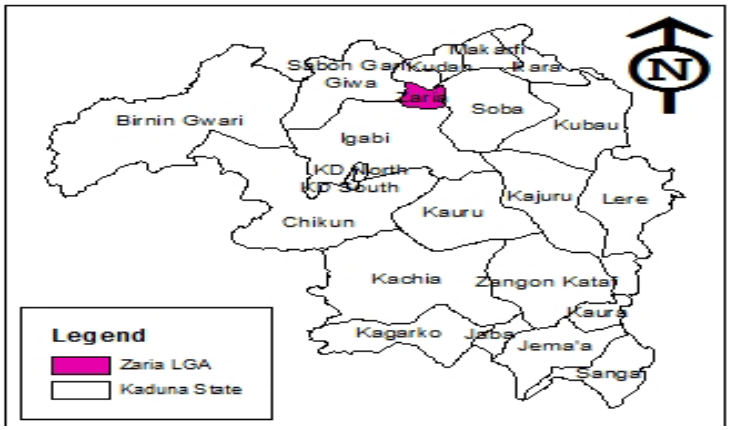


Figure 3. Map of Zaria showing Saye where the study was conducted.

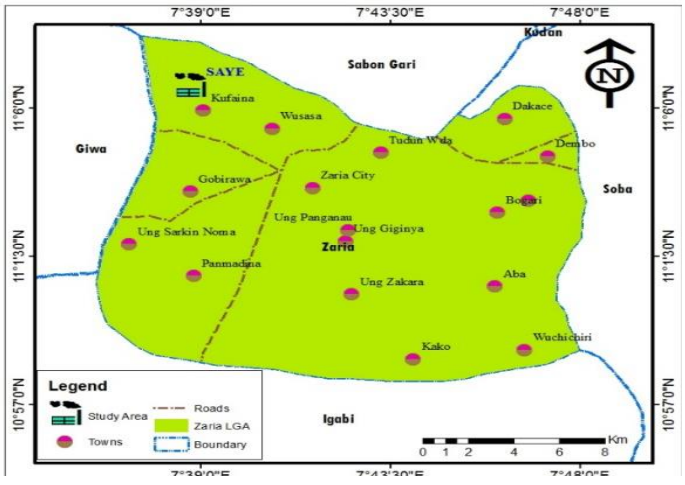


Figure 4. Phylogenetic analysis by Maximum Likelihood Method.

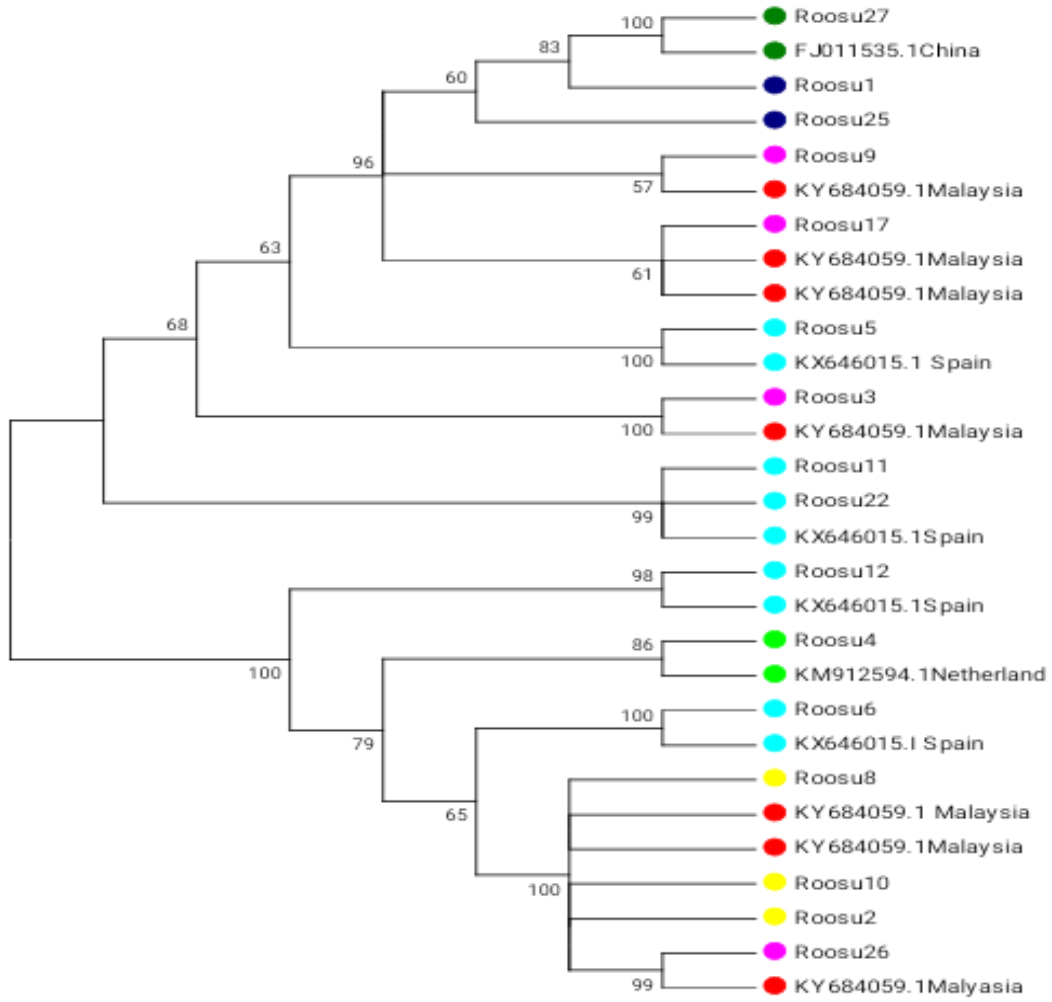
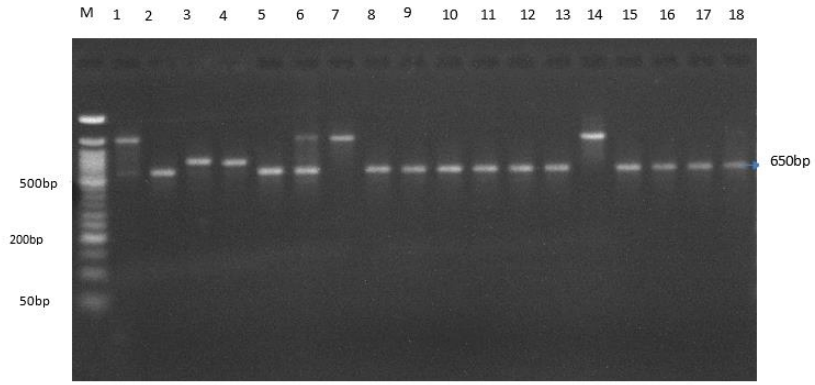


Plate I. Photomicrograph of *H. capsulatum* amplified ITS region.



Discussion

Amplicons of approximately 650bp were produced with a conventional PCR performed on the DNA of 18 culture positive samples. This confirmed the organism under investigation as *H. capsulatum*. **White et al.** [37] also used this same primer (ITS1 and ITS2) to confirm *H. capsulatum*.

Our phylogenetic analysis of *Histoplasma capsulatum*, from Kaduna State, Northern Nigeria revealed similarity between our strains and those from seven different countries supported by bootstrap (Spain, Malaysia, India, China,

Netherland, Indonesia and Mexico). Our strain cluster with Eurasian clade which is made up of India and China, as shown in **figure (4).** One of the isolates; Roosu 4 clustered with a strain from Netherland with bootstrap value of 89% indicating strong relationship. With this it may belong to the Netherland clade. Majority of the strains identified in this study nested within strains from Malaysia hence could be natives of Malaysia. Only one of the strains clustered with the strain from Mexico, one with and one with China showing that their origin was from these countries. However, four of our strains clustered with those from Spain, two clustered with Indonesian strains and with a single strain clustering with the ones from India. It is possible that the reason why we have so many of our strains clustering with those from Malaysia as a result of educational pursuit, international trade among countries, tourism and visitation, hence opening a window of interaction among individuals from these two different parts of the world. Migration could also be a reason. In Nigeria there is no adequate medical personnel to conduct medical assessment of immigrants especially those from endemic countries, therefore no attention is been paid to persons that are migrating into the country on daily bases, this agrees with **Teixeira et al.** [38].

Conclusion

The strains were similar to those from Malaysia (4 isolates), China (1 isolate), Netherland (1 isolate) and Spain (4 isolates) showing that they originated from these countries.

Recommendation

Patients presenting symptoms of pulmonary infection similar to tuberculosis should be screened for Histoplasmosis with which it shares symptoms

Study with wider coverage should be undertaken to assess the National distribution of Histoplasmosis since no much data is available as such.

As a re-emerging disease in Nigeria, Histoplasmosis should be included in awareness campaign and implementation of surveillance program with a view of controlling and preventing its spread.

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Authors contributions

All authors contributed equally in making this research successful.

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Conflicting interest

There was no any competing interest.

Authors contributions

All authors contributed equally in making this research successful

Ethical approval

Ethical clearance (NBTL/TR6/ZA/182/vel3v) for the study was obtained from the Ethical Committees of National Tuberculosis and Leprosy Training and Referral Hospital Saye, Nigeria. Similarly, informed consent was obtained from each of the participants prior to recruitment into the study.

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