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# Molecular characterization and antifungal sensitivity pattern of fungi species isolated from presumptive tuberculosis patients in university of port Harcourt teaching hospital

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**Abstract**
Tuberculosis (TB) and Mycoses has been around for centuries and remains a major health concern globally with its diverse environmental conditions and population dynamics, possess a unique epidemiological scenario for TB and its associated co-infections. This study was therefore aimed to molecularly character is Sputum specimens from 350 participants were collected and analyzed using molecular and conventional diagnostic methods for fungi. Antifungal susceptibility test was performed using the Kirby-Bauer Disc Diffusion method. The Molecular identification of fungal species isolated from presumptive TB patient used in this revealed diversity of fungal species such as *Curvularia caricae-papayae, Psudopithomyces chartum, Aspergillus udagawae, Penicillium sp. Trichoderma longibrachiatum, Candida albican, Cutaneotrichosporon curvatum, Trametes polyzona* and *Aureobasidium pullulans*. The results of antifungal profile of fungal isolated revealed that all the fungal isolates were susceptible to Ketoconazole except *Aspergillus udagawae,* the isolates also showed 100% susceptibility to Fluconazole except *Trichoderma longibrachiatum and Candida albican* that shown 100% resistant and intermediate, respectively to Fluconazole. *Curvularia caricae-papayae, Aspergillus udagawae, Penicillium sp. and Trametes polyzona showed 100% resistant to* itraconazole while *Psudopithomyces chartum, Aspergillus udagawae, Cutaneotrichosporon curvatum* and *Aureobasidium pullulans* revealed 100% resistant to Nystatrin**.** The study suggests the necessity for a dual-focused health strategy addressing TB, fungal infections co-infection. Furthermore, the variability in antifungal susceptibility profiles highlight the importance of individualized treatment plans based on local resistance patterns. This research contributes to bridging diagnostic and treatment gaps in TB, fungal infections co-infection, offering insights for refining public health interventions and optimizing patient care in Port Harcourt and similar settings.

**Keywords:** Antifungal susceptibility, molecular, fungal species, presumptive, tuberculosis

## Introduction
Tuberculosis (TB), a disease caused by *Mycobacterium tuberculosis*, remains a major health problem worldwide (Acharya *et al.*, 2022) [1]. Despite progress in medical science, TB continues to be a persistent problem, often complicated by other conditions that can affect its management and treatment outcomes (Agyeman *et al.*, 2017) [2]. In the bustling city of Port Harcourt, with its diverse environmental challenges and population dynamics, a unique epidemiological scenario for TB and its associated co-infections is observed. The symptoms of TB are notoriously nonspecific, often overlapping with those of various respiratory diseases, including fungal infections (Agyeman *et al.*, 2017; Aldardeer *et al.*, 2020) [2, 3]. The pathogenesis of TB involves the inhalation of aerosolized droplets containing *Mycobacterium tuberculosis*, leading to a primary lung infection (Acharya *et al.*, 2022) [1]. The ability of the bacterium to evade the immune system and persist within macrophages is a key aspect of its pathogenicity.

Concurrently, mycoses diseases caused by fungal agents—can occur in various parts of the body, including the lungs, the primary site of TB (Amiri *et al.*, 2017) [4]. Pathogenic fungi pose significant health challenges, but are often overlooked in the diagnosis of pulmonary infections (Arafa *et al.*, 2023) [5] The epidemiology of TB is closely linked to socio-economic factors and the prevalence of HIV/AIDS, which increases the risk and severity of the disease. Similarly, the incidence of invasive fungal infections has increased, particularly in individuals with compromised immune systems (Attia *et al.*, 2019) [6].

Recent studies have highlighted the complexity of TB treatment, noting the frequent occurrence of additional diseases that can interfere with standard therapeutic protocols. Fungal diseases, despite their significance, often receive insufficient attention during the diagnostic process of pulmonary infections, potentially leading to misdiagnosis and inappropriate treatment (Amiri *et al.,* 2017; Badali *et al.*, 2019) [4, 7].

This research addresses a critical gap in understanding TB and its mimics. Patients presenting with presumptive TB may actually have other respiratory conditions or co-existing diseases that affect treatment efficacy (Amiri *et al.,* 2017) [4]. Moreover, respiratory fungal infections, often overlooked in provisional diagnoses, warrant greater consideration due to their clinical resemblance to TB (Arafa *et al.,* 2023) [5] The urgent need to differentiate between pulmonary tuberculosis and fungal lung infections, which share overlapping clinical presentations, justifies this study (Bai *et al.,* 2021; Baluku *et al.,* 2019) [8, 9]. Without definitive diagnostic differentiation, inappropriate treatment decisions can negatively affect patient health. Thus, the study represents a pioneering effort within Port Harcourt, aiming to fill gaps in existing knowledge and contribute to refining diagnostic and treatment protocols for TB and its confounding co-infections. The confluence of TB and fungal infections presents a complex diagnostic and therapeutic challenge, requiring comprehensive diagnostic strategies to ensure accurate identification and effective management of these potentially fatal co-infections. This study focuses on the assessment of the mycological status of presumptive TB patients in Port Harcourt. The objectives include isolating and identifying fungal species in sputum specimens and investigate the sensitivity pattern of fungi species isolated from of presumptive tuberculosis patients in University of Port Harcourt Teaching Hospital.

## Materials and Methods

### Study Area

This study was carried out at the University of Port Harcourt Teaching Hospital (UPTH), a tertiary healthcare institution. UPTH is situated in Obio/Akpor local government in the neighborhood of the bustling city of Port Harcourt, the capital of Rivers State, Nigeria. As a tertiary hospital, UPTH provides a high level of medical care, offering a wide range of specialized health services. Its location in the neighbourhood of Port Harcourt, a major urban center, allows it to serve a diverse population, making it an ideal setting for this study.

### Study Design

This cross-sectional study was conducted to molecularly characterize fungi isolates in presumptive TB patients. From November 2022 to October 2023, a total of 350 patients suspected of having TB were enrolled in the study. Sputum samples were systematically collected from each participant to perform comprehensive examinations for fungi. In addition to conventional diagnostic methods, molecular analyses were employed to characterize the specific strains of fungi present in the sputa.

### Inclusion and Exclusion Criteria

Patients with provisional diagnosis of tuberculosis, particularly of those with a persistent cough for more than three weeks and are able to produce sputum at the time of sample collection were recruited in the study. Only patients who consented to participate in the study with evidence of signed written consent were included. Apparently, healthy individuals with no clinical symptoms of pulmonary infection, and those who are under antifungal or tuberculosis treatment were excluded from the study.

### Sampling Methods

The sampling method employed in this study was simple random sampling, which is a foundational technique to ensure unbiased selection. Each presumptive TB patient was assigned a binary numbering system, consisting of the digits "1" and "0". Participants were then asked to pick a number at random. Those who picked a "1" were included in the study sample, while those who picked a "0" were not selected.

### Laboratory Assessment of Fungi Culture

For the laboratory assessment of fungi culture, Sabouraud Dextrose Agar with Chloramphicol (50mg/l) plates were meticulously prepared and labeled accordingly following a standardized protocol (Bao *et al.,* 2020) [10]. Using a sterile inoculation loop, sputum specimen was carefully collected and then streaked evenly across the surface of the SDA plates, in duplicate. The inoculated SDA plates were incubated at room temperature (25 -27 °C). The incubation period ranged from 2 to 5 days, during which the plates were regularly monitored for the emergence of fungal colonies, indicative of fungal presence and growth (Prescott *et al.,* 2011) [19].

### Identification of the Fungal Species

#### Microscopy (Lactophenol Staining)

To further characterize fungal colonies identified during culture, a microscopy technique employing Lactophenol Cotton Blue staining was meticulously performed (Berkow *et al.,* 2020). A drop of Lactophenol Cotton Blue stain was first placed in the center of a clean glass slide to prepare the specimen. A small fragment, approximately 2-3mm in size, was then carefully removed from the edge of the fungus colony using an inoculating needle. This fragment was placed in the drop of stain and gently teased apart to disperse the cells. A coverslip was carefully placed over the smear without exerting pressure or tapping, to avoid disrupting the sample. The prepared slide was then examined under a microscope using a x 40 objective lens, allowing for the detailed observation of the fungal structures and morphology.

### Molecular Identification of Fungi

For molecular identification, DNA was extracted from the fungal isolates using a standard protocol. The extracted DNA was then subjected to PCR amplification targeting the internal transcribed spacer (ITS) regions, which are commonly used for fungal identification. PCR products were purified using the QIA quick PCR purification kit (Bao *et al*., 2012) [10] PCR products were sequenced. The sequences obtained were compared with those in the NCBI Gene Bank database to determine the molecular identity of the fungal isolates (Landeweert *et al*., 2003; Javadi *et al*., 2012) [17, 15].

### Antifungal Susceptibility Testing:

The antifungal susceptibility of the isolated fungi was

assessed using the disc diffusion assay. Antifungal discs containing nystatin (10mg), itraconazole (10µg/ml), fluconazole (10mg/ml), and ketoconazole (10mg/ml) were prepared for this purpose (Bongomin *et al.*, 2017) [11]. By dissolving 200mg of each drug in 10ml of sterile distill water to get 20mg of the drugs which was further diluted to 10mg by 2-fold serial dilution. Fungal colonies were suspended in 5 ml of sterile 0.85% normal saline, and the suspension was vortexed. The turbidity was then adjusted to match 0.5 McFarland standards, corresponding to a concentration of 1. 5 x $10^6$ cells/ml. A sterile cotton swab moistened with the inoculum suspension was used to inoculate Sabouraud dextrose agar plates, which were supplemented with 10 ml of chloramphenicol. After allowing the plates to dry for 5-15 minutes, the antifungal disks were placed on the agar. The plates were then incubated at 37°C for 24 - 48 hours. Slowly growing isolates were re-examined after 48 hours. The zones of inhibition were measured in millimeters. Interpretation of the results was conducted in accordance with the National Committee for Clinical Laboratory Standards (Carabali-Isajar *et al.*, 2023) [12], and the Clinical and Laboratory Standards Institute (Chahine *et al.*, 2022) [13], zone interpretative criteria, which define susceptibility as a diameter of ≥ 19 mm, resistance as ≤ 14 mm, and dose-dependency as a diameter between 15 and 18 mm for fluconazole (Chahine *et al.*, 2022) [13].

## Results and Discussion

### Results

Results of molecular identification of fungal and bacterial isolated with their ascension numbers is presented in Table 1. Eight out of the ten fungal isolates shown 100% similarity to the fungi stored in the NCBI genebank, others two fungal isolates shown 99% and 98% similarity while the two bacterial isolates showed 100% similarity. Phylogenetic tree showing the evolutionary distances between the Fungal isolates and percentage relatedness with their close relatives in the gene bank is presented in Figure 1. Plate 1 is an agarose gel electrophoresis amplified ITS gene of the ten fungi isolated and identified in this study. The ITS gene is specific to all fungi species. All of the isolates were found to possess the ITS gene according to the electrophoresis data, proving that they are all fungal isolates.

**Table 1:** NCBI Genebank Accession Number of the Fungal

| Iso. Code | Ascension Number | Molecular Identity | % Similarity |
|---|---|---|---|
| V7 | OW986428 | *Curvularia caricae-papayae* | 100 |
| V10 | OP676036 | *Psudopithomyces chartum* | 100 |
| V3 | OR143378 | *Aspergillus udagawae* | 99 |
| V6 | OR095997 | *Penicillium sp.* | 100 |
| V8 | OP157556 | *Trichoderma longibrachiatum* | 100 |
| V1 | OP601573 | *Candida albican* | 98 |
| V9 | OK267690 | *Candida albican* | 100 |
| V2 | OW984461 | *Cutaneotrichosporon curvatum* | 100 |
| V4 | OR100374 | *Trametes polyzona* | 100 |
| V5 | KX023301 | *Aureobasidium pullulans* | 100 |

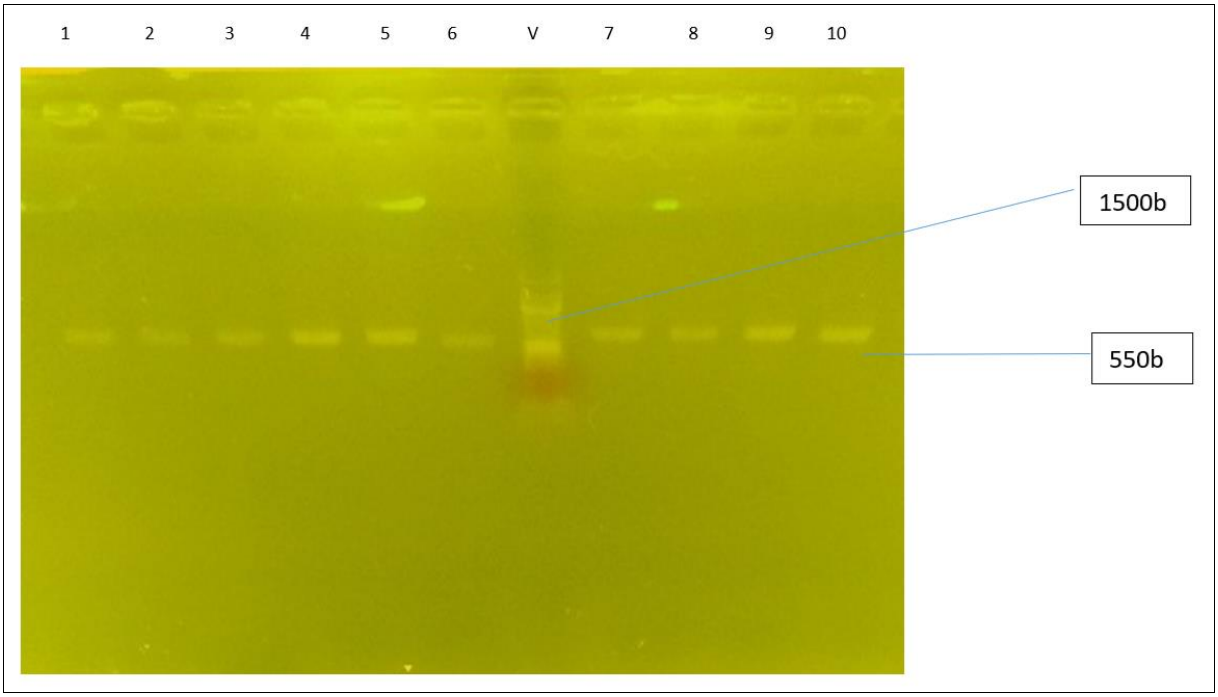


**Plate 1:** Agarose gel electrophoresis of ITS gene of Fungi isolates. Lanes 1-10 represent the ITS gene bands (550bp). Lane V represents the 100bp DNA Ladder of 1500bp.

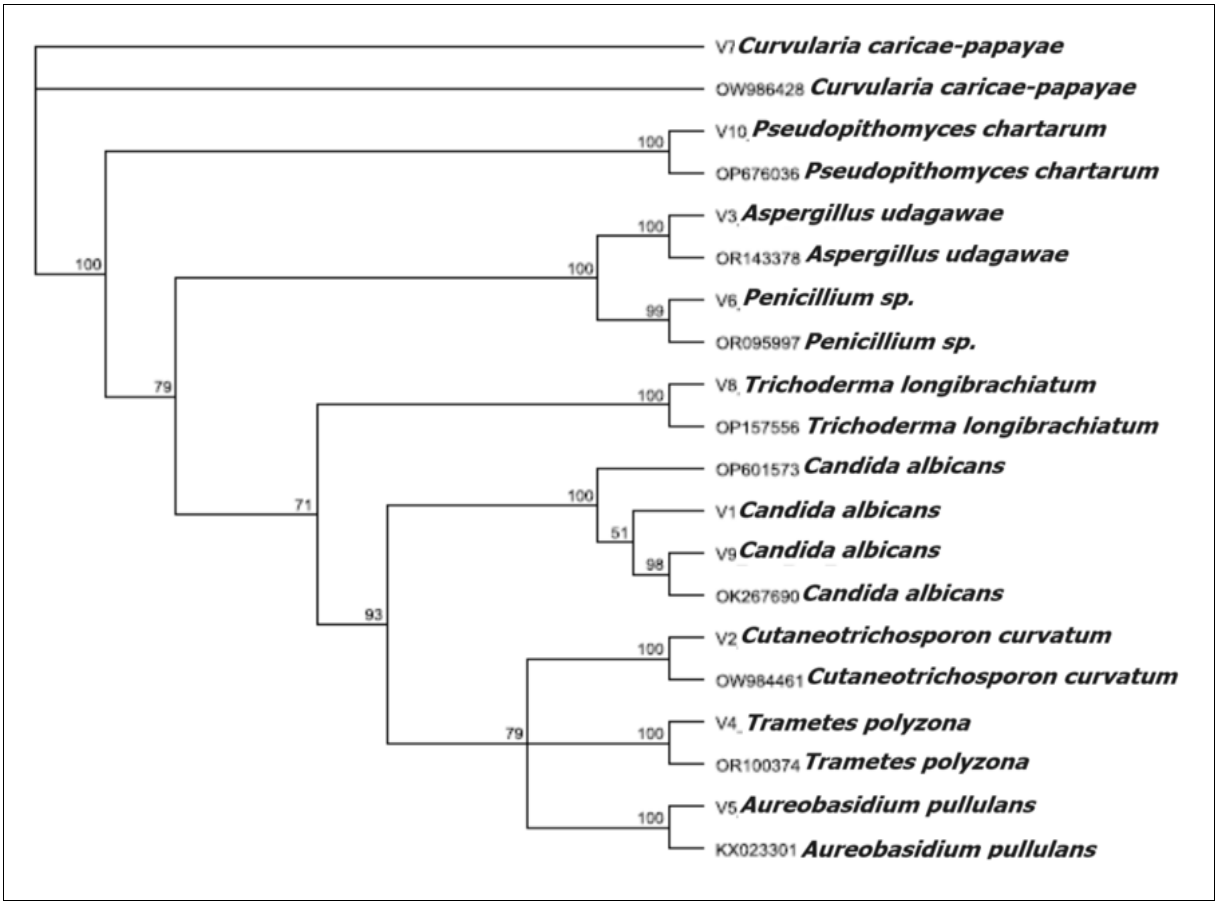


**Fig 1:** Phylogenetic Tree Showing the Evolutionary Distance between the Fungal Isolates

Results of the antifungal sensitivity profile as presented in (Table 2), revealed that all the fungal isolates were susceptible to Ketoconazole except *Aspergillus udagawae*, the isolates also showed 100% susceptibility to Fluconazole except *Trichoderma longibrachiatum and Candida albican* that shown 100% resistant and intermediate to Fluconazole. *Curvularia caricae-papayae, Aspergillus udagawae, Penicillium sp. and Trametes polyzona showed 100% resistant to* Clostrinazole while *Psudopithomyces chartum, Aspergillus udagawae, Cutaneotrichosporon curvatum* and *Aureobasidium pullulans* revealed 100% resistant to Nystatrin

**Table 2:** Antifungal Sensitivity Profiles of isolates from patients

| Iso. Code | Microscopic and Microscopy Identity | Molecular Identity | NY | IT | FLU | KET |
|---|---|---|---|---|---|---|
| V7 | *Candida* sp | *C. caricae-papayae* | I | R | S | S |
| V10 | *Mucor* sp | *P. chartum* | R | S | S | S |
| V3 | *Aspergillus* sp. | *A. udagawae* | R | R | S | R |
| V6 | *Penicillium* sp. | *Penicillium sp.* | S | R | S | S |
| V8 | *Penicillium* sp. | *T longibrachiatum* | S | S | R | S |
| V1 | *Candida* sp. | *C. albican* | S | S | I | S |
| V2 | *Rhyzopus* sp. | *C. curvatum* | R | S | S | S |
| V4 | *Mucor* sp. | *T. polyzona* | I | R | S | S |
| V5 | *Candida* sp. | *A. pullulans* | R | S | S | S |

**KEY: R**- Resistance, S- Sensitivity, I-Intermediate, NY-Nystatrin, IT-Itraconazole, KET-Ketoconazole, Flu-Fluconazole, Zone interpretative criteria for Susceptible (diameter, ≥ 19 mm), Intermediate (diameter ≥15 mm). Resistant (diameter, ≤ 14 mm) or dose dependent (diameter between 15 and 18mm) for fluconazole (CLSI, 2012) [14].

## Discussion

Fungal species isolated and identified from presumptive tuberculosis patients in this study includes *Curvularia caricae-papayae, Psudopithomyces chartum, Aspergillus udagawae, Penicillium sp. Trichoderma longibrachiatum, Candida albican, Cutaneotrichosporon curvatum, Trametes polyzona* and *Aureobasidium pullulans.* Most of the fungal isolates identified from this study are saprophytic in water, air and soil and skin. These organisms have been implicated in various diseases; *Curvularia caricae-papayae* Can cause severe respiratory infections, exacerbating TB symptoms. *Pseudopithomyces chartarum* Associated with allergic reactions, respiratory issues, and neurological problems. *Aspergillus udagawae* Can cause invasive aspergillosis, particularly in immunocompromised patients. *Penicillium* sp.: Penicillium sp has been implicated in penicilliosis disease in humans and can also cause respiratory infections, allergies, and mycotoxin production. *Trichoderma longibrachiatum* Can cause respiratory infections, skin

lesions, and allergic reactions. *Candida albicans* Can cause opportunistic infections, particularly in immunocompromised patients. *Cutaneotrichosporon curvatum* Associated with skin and nail infections. *Trametes polyzona* Can cause respiratory infections and allergic reactions. *Aureobasidium pullulans* Associated with respiratory infections, allergies, and biofilm formation (Kibbler *et al.* 2019; Obire and Alali, 2015) [16, 18].

Sensitivity profile is crucial for selecting appropriate antifungal treatments and for understanding the resistance patterns that may be present in the local fungal population. The study showed a range of sensitivity and resistance across the fungal species to four common antifungal agents: Nystatin (NY), Itraconazole (IT), Fluconazole (FLU), and Ketoconazole (KET). *Aspergillus udagawae* showed intermediate (I) sensitivity to Nystatin, resistance (R) to Itraconazole, and sensitivity (S) to Fluconazole and Ketoconazole. This pattern suggests that while some treatments may be ineffective, others could be viable options for *Aspergillus* infections. On the other hand, *Candida sp.*, a common cause of opportunistic infections, exhibited resistance to Nystatin, which is noteworthy given Nystatin's frequent use in treating Candida infections (Rodrigues and Nosanchuk, 2020) [20]. However, its sensitivity to the other three antifungals indicates alternative treatment options are available.

*Pseudopithomyces chartarum*. and *Trichoderma longibrachiatum*., both associated with mucormycosis (Rudramurthy *et al.*, 2023) [21], showed varied responses to the antifungals, with *Psudopithomyces chartum.* Being the most drug resistant. This highlights the challenge of treating infections caused by these species and the importance of susceptibility testing. The sensitivity profile for *Penicillium sp*., and *Trametes polyzona* also varies, with each showing resistance to at least one antifungal agent. This variability underscores the need for individualized treatment plans based on specific antifungal susceptibility patterns. In comparison to previous studies (Verma 2021 WHO 2021; Wiederhold 2017) [22-24], the resistance patterns observed in this study may reflect local prescribing practices, environmental factors, or inherent species-specific characteristics.

## Conclusion

This study offers a comprehensive assessment of the mycological status of presumptive TB patients in Port Harcourt, Nigeria. The findings underscore the complexity of pulmonary infections in this population, emphasizing the need for precise diagnostic strategies and integrated treatment approaches. This study also revealed valuable insights into fungal pathogens associated with presumptive tuberculosis patients, informing diagnosis, treatment, and prevention strategies. The diversity of fungal species identified underscores the importance of a multifaceted approach to diagnosis and treatment.

Moreover, the fungal co-infections raise awareness of the challenges posed by overlapping clinical presentations and the need for tailored therapeutic interventions. The prevalence rates of fungal infections within the study cohort underscores the importance of a dual-focused health strategy addressing both bacterial and fungal diseases.

The sensitivity profiling of fungal isolates against commonly used antifungal agents reveals varying susceptibility patterns, highlighting the necessity for individualized treatment strategies guided by accurate susceptibility testing. This underscores the importance of ongoing surveillance to monitor antifungal resistance trends and inform empirical treatment protocols.

## Consent

Informed written consent was obtained from each participant before their enrollment in the study. This process ensured that all subjects were fully aware of the study's nature, potential risks, and benefits, and voluntarily agreed to participate, thereby upholding the ethical principle of autonomy.

## Ethical Consideration

Prior to the commencement of the study, ethical approval was secured from the Ethics Committee of the University of Port Harcourt Teaching Hospital (UPTH), with the reference number UPTH/ADM/90/S. II/VOL.XI/1659.

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