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Original Article

Microbiology Section

Tobacco Agar as a Novel Medium for Chlamydospore Induction in *Candida* Species: A Cross-sectional Study

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ABSTRACT

Introduction: *Candida* species, especially *Candida albicans* and *Candida dubliniensis*, are major opportunistic pathogens in humans, distinguished by their ability to form chlamydospores. Traditional diagnostic methods rely on phenotypic traits, with chlamydospore production serving as a key differentiator. Tobacco agar has emerged as a potential low-cost, effective medium to induce chlamydospore formation, particularly useful in resource-limited settings.

Aim: To evaluate the efficacy of tobacco agar in inducing chlamydospore formation for the presumptive identification of *Candida* species.

Materials and Methods: This cross-sectional study was conducted in the Department of Microbiology at Shri MP Shah Government Medical College, Jamnagar, Gujarat, India over the period of six months from October 2019 to March 2020. A total of 100 non duplicate sputum samples from patients suspected of fungal respiratory infections were included based on Bartlett's criteria. Samples were cultured on Sabouraud Dextrose Agar, and isolates showing creamy white colonies were presumptively identified as *Candida* spp. These were further subcultured on in-house prepared tobacco agar using the Dalmau plate technique to induce chlamydospore formation and aid species-level identification. Microscopic evaluation began 48 hours post-inoculation, and isolates were classified based on germ tube and chlamydospore presence. Fisher's exact test assessed the association between incubation parameters and chlamydospore development, with a significance threshold of p-value < 0.05.

Results: *Candida* species were isolated from 30 (30.0%) of 100 sputum samples. Among these, 19 (63.3%) showed germ tube formation and chlamydospore production consistent with *C. albicans/C. dubliniensis*, while 11 (36.7%) were identified as non *albicans Candida*. Chlamydospore induction occurred in 19 (63.3%) isolates on both tobacco agar and Cornmeal Agar (CMA). Optimal induction was noted at 28°C after 48-72 hours, with no formation at 37°C or 24 hours.

Conclusion: Tobacco agar demonstrates reproducible morphological responses in Candida isolates under controlled conditions, which may support presumptive differentiation Its use may encourage broader adoption of low-cost, observation-based tools in diagnostic mycology.

Keywords: *Candida dubliniensis*, Chlamydospore formation, Phenotypic characterisation, Tobacco leaf

INTRODUCTION

Candida species are part of the normal human flora, residing predominantly in the mucosal surfaces of the skin, gastrointestinal tract, and genitourinary tract. While often harmless, they can act as opportunistic pathogens, causing infections that range from mild to severe, particularly in immunocompromised individuals. Candidiasis is an important clinical concern, as it can lead to a wide spectrum of infections, including cutaneous, mucocutaneous, and invasive candidiasis. The severity of these infections depends on factors such as the site of infection, the virulence of the infecting strain, and the host's immune status. *Candida albicans* is the most commonly implicated species in human infections, known for its ability to form biofilms, switch phenotypically between yeast and hyphal forms, and produce virulence factors that enhance tissue invasion and immune evasion [1,2].

The ability of *Candida* species to form chlamydospores, a type of thick-walled spore produced under nutrient-limited conditions, has been widely recognised as a critical phenotypic feature for species identification. Chlamydospores are typically formed on solid media when the organism is exposed to conditions such as low temperatures and limited nutrients. Among *Candida* species, *Candida albicans* and *Candida dubliniensis* are the primary clinically recognised species for reliable chlamydospore production under standard conditions, a characteristic that is crucial for their differentiation from non *albicans Candida* species [2,3].

Traditionally, the identification of *Candida* species has relied on a variety of phenotypic methods, such as germ tube formation, carbohydrate fermentation profiles, and chlamydospore production. However, with the advent of molecular techniques, commercial systems, and automated identification methods, the rapid and accurate detection of *Candida* species has improved. Despite the advantages of these newer techniques, phenotypic methods remain essential, especially in resource-limited settings where molecular diagnostic tools may not be readily available [4].

One of the simpler and more cost-effective phenotypic methods for *Candida* species identification is the use of tobacco agar, a specialised medium that induces the production of chlamydospores. Tobacco agar has garnered attention in mycological research due to its ability to differentiate *Candida albicans* and *Candida dubliniensis* from other *Candida* species based on their ability to form chlamydospores. Tobacco agar is prepared by boiling tobacco leaves in distilled water, followed by filtration, pH adjustment, and the addition of agar. The resulting medium, when inoculated with *Candida* isolates and incubated under light, promotes chlamydospore formation, particularly in species like *Candida albicans* and *Candida dubliniensis* [5].

Tobacco agar offers several advantages in clinical diagnostics: it is inexpensive, easy to prepare, and provides reliable differentiation of *Candida* species based on morphological characteristics [2,3]. However, its effectiveness can be influenced by environmental factors such as tobacco leaf source, incubation temperature, and light exposure, which may cause variability in results. Despite these limitations, tobacco agar remains a valuable tool for phenotypic identification, particularly in settings where advanced molecular methods are not accessible [6,7].

The accurate identification of *Candida* species is essential for effective clinical management, particularly in immunocompromised patients where species such as *Candida albicans* are common opportunistic pathogens. Although molecular diagnostic techniques offer high sensitivity and specificity, their use remains limited in many resource-constrained settings due to cost and technical requirements. Consequently, phenotypic methods continue to play a central role in routine fungal identification in clinical laboratories.

Tobacco agar is a traditional culture medium known to promote chlamydospore formation, a morphological feature associated with certain *Candida* species. Its low cost and ease of preparation make it a potentially useful tool for preliminary differentiation of species based on observable characteristics [2]. However, the application of tobacco agar in clinical diagnostics remains inadequately explored, particularly with respect to how environmental conditions influence its effectiveness.

This study aimed to evaluate the ability of tobacco agar to induce chlamydospore formation in clinical *Candida* isolates and to differentiate isolates based on chlamydospore production and colony morphology. In addition, the study investigated the impact of environmental factors, specifically incubation temperature and time, on the induction of chlamydospores and the overall clarity of morphological features on this medium. These findings provide insight into the practical utility of tobacco agar in diagnostic settings where molecular identification methods may not be accessible.

The primary objective was to assess the ability of tobacco agar to induce chlamydospore production and facilitate morphological differentiation of *Candida* isolates. The secondary objective was to evaluate the influence of incubation temperature and duration on chlamydospore formation and colony morphology on tobacco agar.

MATERIALS AND METHODS

This was a cross-sectional study conducted in the Department of Microbiology at Shri MP Shah Government Medical College, Jamnagar, Gujarat, India. The study period extended over six months, from October 2019 to March 2020. The study protocol was reviewed and approved by the Institutional Ethics Committee (IEC) of Shri MP Shah Government Medical College, Jamnagar. IEC Approval Number: (IEC/CERTI/18/01/2020). Informed consent was obtained from all patients prior to inclusion in the study. The confidentiality of patient data was maintained throughout.

Sample size and selection: A total of 100 non duplicate sputum samples were collected from patients attending various clinical departments, including outpatient and inpatient services. All samples received for routine mycological evaluation during the study period were included, making this a time-bound, exhaustive sample.

Inclusion criteria:

Sputum samples from patients of all age groups and sexes, samples received for suspected fungal respiratory infection, and samples meeting Bartlett's criteria [8] for acceptable sputum (i.e., >25 polymorphonuclear leukocytes per Low-Power Field (LPF) and <10 squamous epithelial cells/LPF) were included in the study.

Exclusion criteria:

Samples contaminated with saliva or showing poor quality on Gram stain, patients already on antifungal treatment and repeated samples from the same patient were excluded from the study.

Study Procedure

All laboratory procedures adhered to standardised protocols described in Koneman's Colour Atlas and Textbook of Diagnostic Microbiology [9] and Chandra J. Practical Mycology [10]. The overall workflow for sample processing is depicted in [Table/Fig-1].

One hundred sputum samples were inoculated onto Sabouraud Dextrose Agar (HiMedia Laboratories, Mumbai) and incubated aerobically at 37°C for 48 hours. Colonies exhibiting creamy-white, smooth morphology were presumptively identified as *Candida* species. Of the 100 samples, 30 (30.0%) yielded growth consistent with *Candida* spp. The 30 culture-positive isolates underwent species-level differentiation using germ tube testing and morphological assessment on tobacco agar and Dalmau Technique [11].

Sputum samples (n = 100)

↓

Primary Culture on SDA (37°C, 48 h)

↓

Candida-like Colonies Isolated Creamy white, smooth colonies (n = 30;30.0%)

↓

Subculture for Species Differentiation
Inoculation on Corn Meal Agar (CMA) and Tobacco Agar
Incubation: 28 °C, ambient light, 48–72 h

↓

Microscopic Examination
- Germ tube formation
- Chlamydospore production (40×)
- Semi-quantitative chlamydospore count (average of 10 fields)

↓

Classification of Isolates
- *C. albicans/C. dubliniensis*-like (positive for germ tube and chlamydospores)
non *albicans Candida* spp. (negative for both tests)

[Table/Fig-1]: Sample processing workflow.

Tobacco Agar Preparation

Tobacco agar was prepared in-house as follows [1,3,6]:

- **Extraction:** Fifty grams of dried tobacco leaves (90% purity, procured from a local vendor) were boiled in one litre of distilled water for 30 minutes.
- **Filtration and pH adjustment:** The extract was cooled, filtered through muslin cloth, and pH adjusted to 6.0.
- **Medium formulation:** The volume was readjusted to one litre with distilled water, then 20 grams of agar-agar. The mixture was sterilised by autoclaving at 121°C for 15 minutes.
- **Plating:** The sterile medium was cooled to 45-55°C and dispensed into sterile petri dishes under aseptic conditions. For quality control, *Candida albicans* ATCC 90028 was inoculated and showed robust growth and chlamydospore formation without inhibitory effects.

Inoculation and Incubation

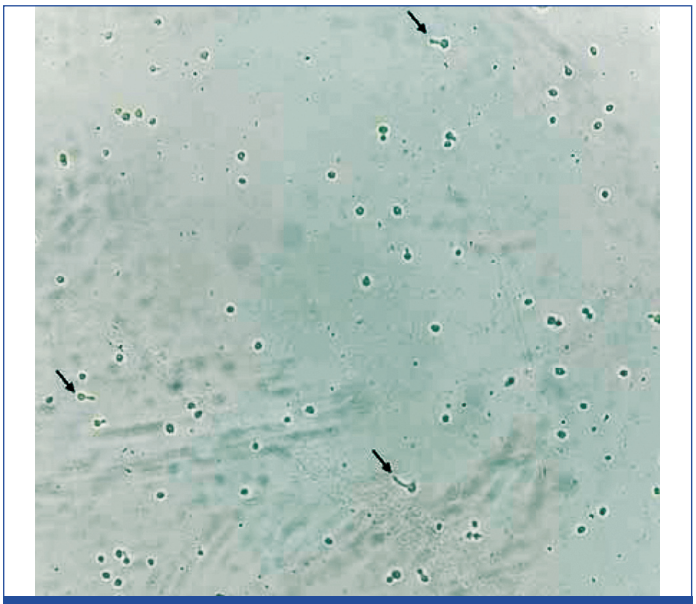
Candida isolates were inoculated onto tobacco agar following procedures described in the existing literature [1-3,6]:

- Parallel shallow streaks were made using a sterile inoculation wire.
- Sterile glass cover slips were gently placed over the streaks to facilitate chlamydospore induction.
- All plates were prepared in duplicate to ensure reproducibility.

Plates were incubated at 28°C under ambient light (exposed to natural laboratory lighting) without sealing. Based on preliminary observations, no chlamydospores were detected at 24 hours; thus, routine microscopic monitoring commenced from 48 hours onward, with chlamydospore formation reliably observed within 48-72 hours. Incubation at 37°C was excluded from the optimised protocol, as no chlamydospore induction was observed at this temperature. For comparison, isolates were subcultured on conventional CMA prepared in accordance with standard guidelines [9,10]. Chlamydospore induction efficiency and associated morphological characteristics were compared across the different media.

Microscopic examination: Microscopic evaluation was performed daily from day 2 onward to assess germ tube formation and chlamydospore development beneath and around the cover slips using lactophenol, cotton blue, and saline wet mounts.

- **Germ tubes** were identified as tube-like projections emerging from yeast cells without any constriction at the point of origin [Table/Fig-2].



[Table/Fig-2]: Germ tubes observed as tube-like outgrowths without basal constriction in unstained wet mount preparations (40x).

- Chlamydospores were identified as large, thick-walled, spherical structures located terminally or intercalarily on pseudohyphae, consistent with established descriptions [2,3,5].
    - For semiquantitative evaluation, chlamydospore production was assessed under the 40× objective lens. A minimum of 10 randomly selected fields per isolate were examined, and the average semiquantitative grade (limited [+], moderate [++], abundant [+++]) was recorded, following methods adapted from Zavalza-Stiker A et al., [12].
    - Isolates were subsequently classified as:

      ***Candida albicans*/*Candida dubliniensis*-like:** positive for both germ tube formation and chlamydospore production.

      Non *albicans Candida* species: negative for both germ tube formation and chlamydospore production.

For quality control, *Candida albicans* ATCC 90028 and the non-*albicans* strain *Candida tropicalis* MTCC 184 were used.

Cost analysis: A basic cost-analysis was conducted to compare the preparation costs of tobacco agar (prepared from dried tobacco leaves procured locally) with those of CMA. The costs were calculated based on the material expenses required to prepare one liter of medium.

While molecular identification remains the gold standard for differentiating *Candida albicans* from *Candida dubliniensis*, the absence of such facilities at our institution necessitated reliance on conventional phenotypic methods.

STATISTICAL ANALYSIS

Statistical analysis was conducted using Fisher's exact test to assess associations between incubation parameters and chlamydospore formation. Additional descriptive statistics, and frequency distributions, were also calculated. All analyses were performed using Statistical Package for the Social Sciences (SPSS) version 24.0.

RESULTS

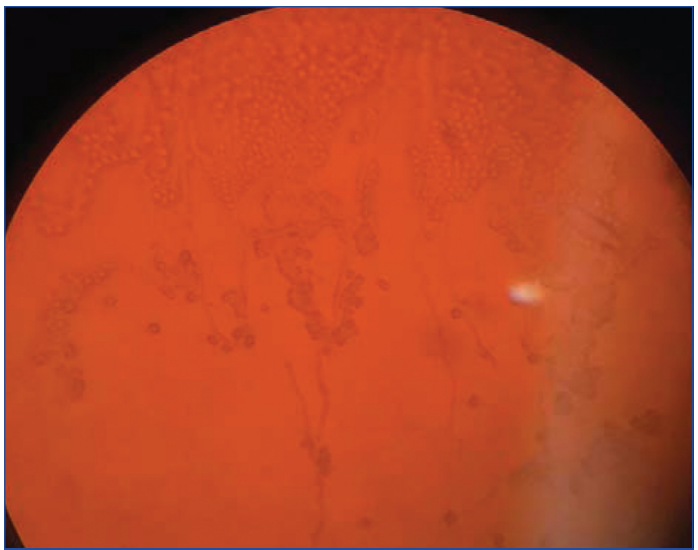
Out of 100 sputum samples, *Candida* spp. were isolated in 30 (30.0%). All 30 (100.0%) isolates produced creamy-white, smooth colonies on SDA after 48 h at 37°C. SDA did not permit species-level differentiation. On tobacco agar, all 30 (100.0%) isolates showed raised, creamy-white colonies along the inoculated streaks [Table/Fig-3].

[Table/Fig-3]: Colony morphology of *Candida* spp. on tobacco agar showing raised, creamy white growth in streak patterns.

For presumptive identification, all isolates were subjected to germ tube testing and microscopic examination for chlamydospore formation [Table/Fig-4,5].

Category	Isolates, n (%)
Germ tube (+)/chlamydospore (+) - *C. albicans/C. dubliniensis*-like	19 (63.3)
Germ tube (–)/chlamydospore (–) - non *albicans Candida* spp.	11 (36.7)

[Table/Fig-4]: Distribution by germ tube and chlamydospore status.



[Table/Fig-5]: Chlamydospore formation in *Candida* spp. on tobacco agar (40×, LPCB mount).

Comparison with Cornmeal Agar (CMA): Parallel inoculation on conventional CMA (CMA; HiMedia) was performed under identical conditions. Chlamydospore induction was observed in 19 of 30 isolates (63.3%), which was comparable to the yield obtained on tobacco agar. Microscopic examination at 40× magnification was independently assessed by two observers, with grading performed on the basis of 10 representative fields per isolate using a published qualitative scale [12] (limited [+], moderate [++], abundant [+++]). Although absolute counts were not recorded, the relative grading patterns were consistent across both media, ensuring reliability of interpretation [Table/Fig-6].

Qualitative grades assessed	Concordance across tobacco agar and CMA	Notes
Limited (+), Moderate (++), Abundant (+++)	Concordant for each positive isolate	No discordant pairs observed among 19 chlamydospore-positive isolates

[Table/Fig-6]: Semiquantitative chlamydospore grades at 40× (qualitative scale).

Both tobacco agar and CMA supported chlamydospore formation only under specific conditions. Induction was seen consistently at 28°C after 48-72 hour of incubation, whereas no chlamydospores developed at 24 hour or at 37°C on either medium. This indicates that 28°C with an incubation window of 48-72 hour provides the most favourable environment for sporulation. Importantly, the use of locally sourced dried tobacco leaves did not interfere with colony growth or chlamydospore production [Table/Fig-7]. These associations were statistically significant by Fisher's exact test [Table/Fig-8].

Factor	Condition	Tobacco agar: Isolates with chlamydospores n (%)	CMA: Isolates with chlamydospores n (%)	Observation
Temperature	28°C	19 (63.3)	19 (63.3)	Optimal induction on both media
	37°C	0	0	No induction
Incubation time	24 h	0	0	Insufficient
	48-72 h	19 (63.3)	19 (63.3)	Optimal window on both media
Medium-specific factor	Tobacco source	Growth: 30 (100.0); Sporulation: 19 (63.3)	–	No inhibition; good morphology (tobacco agar only)

[Table/Fig-7]: Comparative effect of incubation parameters on chlamydospore induction in tobacco agar and Cornmeal Agar (CMA).

Factor	Condition	Chlamydospores formed, n (%)	Not formed, n (%)	Total, n	p-value
Temperature	28°C	19 (63.3)	11 (36.7)	30	<0.001
	37°C	0	30 (100.0)	30	
Incubation time	48-72 h	19 (63.3)	11 (36.7)	30	<0.001
	24 h	0	30 (100.0)	30	

[Table/Fig-8]: Fisher's exact test for incubation parameters The same 30 isolates were tested under each condition; values reflect within-group outcomes.

Cost Comparison of Media (per 1 L batch): A basic cost analysis was carried out to compare CMA; HiMedia with in-house prepared tobacco agar. This analysis demonstrates that tobacco agar is not only cheaper than CMA but also performs equally well in inducing chlamydospores [Table/Fig-9].

Medium	Inputs	Estimated cost (₹)	Notes
Cornmeal Agar (CMA; HiMedia)	17 g dehydrated CMA per litre	238	Price includes CMA
Tobacco agar (in-house)	50 g dried tobacco leaves + 20 g agar-agar	193	Tobacco ≈ ₹10; agar ≈ ₹183

[Table/Fig-9]: Ingredient-level cost comparison for 1 L medium.
Prices reflect distributor and local vendor rates during the study period and may vary by location.

DISCUSSION

The findings of the present study are congruent with prior literature highlighting the diagnostic utility of tobacco agar for presumptive identification of *Candida albicans* and *Candida dubliniensis*. Kumar CPG and Menon T, demonstrated that 96% of *C. albicans* isolates and all *C. dubliniensis* strains produced chlamydospores within 24 hours on tobacco agar, affirming the medium's high sensitivity [3]. Notably, this contrasts with the extended incubation period (48-72 hours) observed in the present investigation for optimal chlamydospore development, highlighting potential variability in incubation dynamics across laboratories.

Khan ZU et al., emphasised the critical role of incubation temperature, reporting that *C. dubliniensis* formed rough, yellowish-brown colonies with peripheral hyphal fringes and abundant chlamydospores at 28°C, whereas *C. albicans* consistently exhibited smooth, white-to-cream colonies devoid of chlamydospore formation even after prolonged incubation [6]. These findings corroborate the present study's observation that 28°C facilitated chlamydospore induction, while 37°C failed to support sporulation, reinforcing the established temperature dependency of chlamydospore production.

Morales-López SE et al., and Steyn AE et al., extended this understanding by demonstrating that *C. albicans* and *C. albicans* var. africana reliably produced smooth, white-to-cream colonies without chlamydoconidia at both 25°C and 37°C, irrespective of tobacco brand [2,13]. In contrast, *C. dubliniensis* exhibited brand-dependent variations in colony pigmentation and morphology, with darker pigmentation and more robust chlamydoconidia formation on Marlboro Classic, Gold, and Fusion agars. While the present study utilised commercial cigarette tobacco without stratifying by brand, no adverse effects on growth or morphology were observed, suggesting that basic species differentiation may be achievable independent of specific brand formulations under standard conditions.

Silveira-Gomes F et al., provided additional nuance by identifying isolates initially classified as *C. albicans* that demonstrated growth inhibition in hypertonic Sabouraud broth- a phenotype characteristic of *C. dubliniensis* [1]. However, 10.1% (8/79) of these inhibited isolates retained typical *C. albicans* morphology on tobacco agar, indicating occasional overlap in phenotypic traits and the inherent limitations of relying solely on morphology for species differentiation.

Morin A and Joly J focused on broader yeast identification, reporting uniform white to off-white colony morphology for *Candida* spp. on tobacco agar, with pigmentation primarily characteristic of *Cryptococcus* [14]. This observation aligns with the non pigmented colony morphology noted in the current investigation's isolates.

Collectively, these comparative data affirm the reliability of tobacco agar as a low-cost, accessible medium for the presumptive identification of *C.albicans/C.dubliniensis*, while also emphasising the critical influence of incubation parameters and potential brand-specific effects. The incorporation of adjunct phenotypic tests or molecular methods remains advisable for definitive species identification, particularly in settings where precise differentiation between closely related species is clinically significant [1-3,6,13].

Tobacco agar represents a practical, cost-effective medium for the presumptive identification of *Candida albicans* and *Candida dubliniensis*, based on their ability to form chlamydospores. Its simplicity of preparation using readily available materials and its effectiveness in promoting species-specific morphological features make it particularly well-suited for use in laboratories with limited resources. By enabling early phenotypic differentiation, tobacco agar can contribute to more timely and targeted antifungal therapy, potentially improving patient management and reducing unnecessary or inappropriate antifungal use.

In future applications, further work is warranted to standardise the preparation of tobacco agar, including evaluation of different tobacco sources and formulation protocols, to ensure consistency and reproducibility across diagnostic settings. For commercial implementation, such standardisation could extend to the development of a ready-to-use dehydrated base or standardised extract, which would reduce inter-laboratory variability and enhance reproducibility, while also facilitating wider clinical acceptance. Beyond standardisation, future research should focus on integrating phenotypic approaches with molecular confirmation strategies such as PCR-based assays or sequencing methods to validate species identity and overcome the limitations of morphology-based

identification. Such an integrated workflow would help balance accessibility with diagnostic precision, particularly in clinical settings where accurate species differentiation has therapeutic implications. Additionally, incorporating tobacco agar into broader diagnostic frameworks alongside rapid phenotypic and molecular methods may enhance the accuracy and efficiency of *Candida* species identification, especially in low and middle-income healthcare environments.

Similar low-cost media have been successfully applied in identifying dermatophytes [15], isolating pathogenic yeasts [16], and screening antimicrobial activity of plant-based substances [17], reinforcing the role of accessible laboratory tools in strengthening microbiological diagnostics in resource-limited settings.

The chief strength of this study lies in its practical evaluation of tobacco agar as a low-cost medium for the presumptive identification of *Candida* species, directly addressing diagnostic needs in laboratories with limited resources. The medium's reliance on easily obtainable raw materials shows its accessibility and adaptability, while the side-by-side comparison with CMA enhances the robustness of the findings. Importantly, the assessment of environmental factors such as temperature and incubation time provides concrete guidance on optimising chlamydospore induction, which may improve reproducibility in everyday diagnostic practice. The incorporation of a cost analysis further adds translational value, demonstrating that the approach is not only technically feasible but also economically sustainable.

Limitation(s)

The modest sample size reduces the generalisability of the findings, and the sole use of phenotypic criteria carries the risk of misclassification between closely related species such as *C. albicans* and *C. dubliniensis*. The study employed a single unbranded tobacco source, which, while economical, may limit reproducibility in other settings. Although prior reports using branded cigarettes suggest greater consistency, such an approach would inevitably raise costs and weaken the advantage of affordability. Cost estimates themselves may also vary according to local market availability. Chlamydospore production was assessed in a semiquantitative manner, and more precise quantification (e.g., number per field, size measurements) should be pursued in future studies. Finally, the absence of molecular confirmation restricts definitive species identification. Future work should therefore aim to expand the sample pool, incorporate more rigorous quantitative analysis of chlamydospores, explore standardised yet cost-conscious formulations, and integrate molecular validation to strengthen the reliability and wider applicability of tobacco agar as a diagnostic tool.

CONCLUSION(S)

The study contributes to the growing interest in utilising readily available materials to enhance laboratory diagnostics. By demonstrating consistent morphological responses, the findings support further exploration of simple media-based approaches in clinical mycology. Continued research into such low-cost innovations may help bridge gaps in diagnostic capacity across diverse healthcare settings.

Authors' contribution: All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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