Heliyon 10 (2024) e38377

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Comparing ergosterol identification by HPLC with fungal serology in human sera

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ARTICLE INFO

Keywords:
Ergosterol
HPLC
Invasive fungal diseases
Diagnosis
Serology
Fungi

ABSTRACT

Background: Ergosterol, a predominant sterol in fungal cell membranes, holds promise as a specific marker for detecting fungal presence in human samples. This study investigated the performance of ergosterol detection compared to serological tests in identifying the presence of fungi in human sera.

Methods: Eighty-four non-duplicate human sera were analyzed by high performance liquid chromatography (HPLC) for ergosterol detection. Results were compared to serological tests for *Aspergillus* antigen, *Candida* antigen, *Cryptococcus* antigen, *Aspergillus* antibody and *Candida* antibody performed on the same patient sera.

Results: Out of the 84 serum samples, 51 (60.7 %) were positive for ergosterol. Among the 33 serology-positive sera, 26 (78.8 %) were also ergosterol-positive. In contrast, 26 out of 51 (51 %) serology-negative sera (including 20 negative controls) tested negative for ergosterol. Seven out of 33 (21.2 %) serology-positive sera were ergosterol-negative, while 25 out of 51 (49 %) serology-negative sera were ergosterol-positive. Compared to serological tests, HPLC detection of ergosterol had a sensitivity of 78.8 %, specificity of 51 %, positive predictive value of 51 %, negative predictive value of 78.8 % and overall accuracy of 61.9 %.

Conclusions: Ergosterol detection may serve as a useful supplementary tool for identifying fungi in human sera, acting as a broad-spectrum diagnostic marker. However, further research with larger sample sizes and clinical comparisons is needed to validate these findings.

1. Introduction

Ergosterol (5,7,22-ergostatrien-3-ol) is a sterol found in the cell membranes of fungi and some protozoa, playing a vital role in maintaining membrane integrity, permeability, and fluidity. Its absence in humans makes it an ideal target for fungal diagnostics and antifungal therapies. The global rise in immunocompromised populations has led to an increase in invasive fungal diseases (IFDs), emphasizing the need for rapid and accurate diagnostic methods. Traditional microscopy and culture techniques are now being

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https://doi.org/10.1016/j.heliyon.2024.e38377
Received 10 May 2024; Received in revised form 13 September 2024; Accepted 23 September 2024
Available online 24 September 2024
2405-8440/

complemented by advanced molecular tools, including PCR assays, next-generation sequencing, biosensors, and AI-based models, which will enhance the early detection of fungal pathogens [1]. Furthermore, the emergence of antifungal drug resistance and new pathogenic strains underscores the ongoing challenge of managing these infections. Crude mortality estimates for the most prevalent IFDs are 64 % for candidiasis, 85 % for aspergillosis, 42 % for *Pneumocystis* pneumonia and 76 % for cryptococcal meningitis [2]. Ergosterol detection has been successfully applied in diagnosing biocontamination, quantifying fungal biomass in soil, and identifying plant infections [3,4]. Fungal contamination of building materials can significantly contribute to indoor air pollution and lead to a range of health problems, including sick building syndrome and respiratory diseases [5]. Fungi thrive in damp indoor environments, releasing volatile organic compounds (VOCs), mycotoxins, and other bioaerosols that can irritate the respiratory system, trigger allergic reactions, and even cause chronic health conditions [6,7]. Ergosterol detection, a biomarker specific to fungal cell membranes, has emerged as a promising tool for diagnosing fungal infections in humans. While traditional diagnostic methods like microscopy, histology, and culture remain the gold standard, they often face limitations, including the need for invasive procedures, slow turnaround times, and suboptimal sensitivity and specificity [8]. Existing serological assays, such as *Candida* mannan, *Aspergillus* galactomannan, *Cryptococcus* glucuronoxylomannan, and β-D-glucan (BDG), are rapid but may not produce high specificity and/or sensitivity [9]. A comprehensive review of existing fungal diagnostic methodologies, including serological assays and newer non-culture-based tests indicate that some tests are excellent, however users need to be aware of the respective strengths and weaknesses. A combination of new approaches and conventional assays will give the most reliable and accurate diagnosis [1]. By addressing the limitations of conventional techniques, ergosterol detection offers the potential for more rapid, accurate, and less invasive diagnosis of fungal infections. This can lead to earlier initiation of appropriate antifungal therapy, ultimately reducing morbidity and mortality associated with these infections [10]. Given that ergosterol is exclusively found in fungal cell membranes and absent in human cells, its detection in human specimens may be an effective method for diagnosing IFDs. A previous study demonstrated the utility of ergosterol detection in diagnosing fungal nail infection (onychomycosis) [11]. However, the landscape of ergosterol-based diagnostic methods has evolved significantly, with advancements in technology and sensitivity [12]. Recent years have seen the emergence of chromatography-based methods, often combined with mass spectrometry (MS), as the most commonly employed and reliable techniques for detecting ergosterol [13]. For instance, researchers have employed Thin Layer Chromatography (TLC) and Ultra Performance Liquid Chromatography (UPLC) to detect the ergosterol structure in *G. boninense* mycelium [14]. Other researchers have utilized high performance liquid chromatography in combination with mass spectrometry (HPLC-MS) to detect ergosterol [11,15]. Additionally, another study described a method of ergosterol detection from fungal broth cultures using HPLC [16]. The detection of ergosterol in rat serum and plasma using liquid chromatography-based procedures has also been described [17,18].

Considering the limited utility and performance of conventional methods and serological assays, ergosterol detection in human specimens emerges as a promising approach for diagnosing IFDs, attributed to its absence in human cell membranes. The primary issue in this research was to determine whether ergosterol detection by HPLC serves as a reliable diagnostic tool for identifying fungal infections in human sera compared to existing serological methods. The hypothesis was that ergosterol detection by HPLC would demonstrate comparable or superior sensitivity and specificity for identifying fungal presence in human sera compared to conventional serological assays. Since ergosterol is a specific component of fungal cell membranes and is absent in human cells, it should provide a more direct and reliable indication of fungal infections. Therefore, the main objective of this study was to assess the performance of ergosterol detection by HPLC in comparison to existing serological methods for the presence of fungi in human sera.

2. Materials and methods

2.1. Collection of human sera

We collected eighty-four human sera left-overs that were sent as part of clinical investigations to the microbiology laboratories of Hospital Canselor Tuanku Muhriz (HCTM) in Kuala Lumpur and Hospital Sultanah Aminah (HSA) in Johor Bahru. These sera were obtained from patients suspected to have invasive fungal infections, as determined by their treating physicians, along with twenty sera samples from patients with no indication of fungal infection. The sera had already undergone mycology serological tests in according to the manufacturers' instructions; including *Aspergillus* galactomannan antigen (ELISA by Platelia BioRad), *Candida* mannan antigen (ELISA by Platelia BioRad), *Cryptococcus* glucuronoxylomannan antigen (latex agglutination by MiraVista Diagnostics), *Aspergillus* antibody (immunodiffusion by IMMY Diagnostics) and *Candida* antibody (immunodiffusion by IMMY Diagnostics). All sera samples were transported via cold chains to the laboratory of the Department of Biosciences, Universiti Teknologi Malaysia, where HPLC procedures were performed.

2.2. Ergosterol extraction procedure for human serum samples

We used an extraction procedure modified from Chen et al. [19]. We prepared the serum samples by combining 100 μL of serum with 30 μL of chloroform and 270 μL of 0.3 mol/L sodium chloride solution in a 1/5 mL-capped centrifugal tube. After mixing for 5 min, we added and mixed 200 μL of 5 % Triton X-114 (v/v) aqueous solution for another 5 min. We then incubated the mixture at 40 °C for 20 min. We achieved phase separation by centrifuging at 10,000 rpm for 15 min, resulting in the surfactant-rich phase settling at the bottom after removing the water phase. Then we added 200 μL of mobile phase to the surfactant-rich phase, followed by centrifugation at 16,000 rpm for 10 min. This led to the precipitation of most surfactants and co-extractants like hydrophobic proteins at the bottom of the tube. Finally, we injected 20 μL of the supernatant fluid into the HPLC system for analysis. We then evaluated the ergosterol extraction procedure for its efficacy in a pre-test using non-infected serum samples spiked with either fungal or bacterial cells ($5.0 \cdot 10^2$ to $2.5 \cdot 10^3$ cells per mL).

2.3. Preparation of standard solutions of ergosterol

Ergosterol with a purity of >95.0 % (HPLC standard) was procured from Sigma-Aldrich [CAS Number 57-87-4, Empirical Formula $C_{28}H_{44}O$, Molecular Weight 396.65]. Standard solutions were prepared by dissolving appropriate quantities of ergosterol in chloroform at concentrations ranging from 5 to 100 parts per million (ppm). These standard solutions were stored in darkness at 4 °C to minimize degradation or any potential chemical alterations that could affect the stability of ergosterol.

2.4. Chromatographic conditions

Similar to a previous study [19], we conducted the HPLC analysis using Waters 2695 Alliance Separation Modules. These included a Waters 2487 dual Diode Array Detector, a column oven and a quaternary pump. The chromatographic separation utilized an Ascentis C18 HPLC Column (25 cm × 4.6 mm, 5 μm) with the column temperature maintained at 30 °C. A 98:2 (v/v) isocratic elution of methanol (A) and water (B) was carried out over 23 min with the flow rate set at 1.0 mL/min. The injection volume was 20 μL and 283 nm was set as the detector wavelength (the maximum absorption wavelength of ergosterol). Empower software was used in the data collection. The Ascentis C18 column was selected for its high efficiency and ability to effectively separate non-polar compounds like ergosterol. Previous studies have demonstrated the reliability of C18 columns in ergosterol detection, providing sharp and well-resolved peaks [16,19]. The column temperature of 30 °C was chosen to maintain the stability of ergosterol and ensure consistent retention times. The isocratic elution with methanol and water was optimized to achieve a balance between resolution and run time, as supported by earlier work where similar solvent systems were used successfully for ergosterol analysis [13,14]. To ensure the robustness of the method, we conducted preliminary experiments comparing different mobile phase compositions and flow rates. These experiments indicated that a 98:2 methanol-water ratio provided the best peak shape and separation efficiency for ergosterol, minimizing baseline noise and maximizing sensitivity. Additionally, the chosen flow rate of 1.0 mL/min was found to be optimal for maintaining column integrity while providing adequate resolution within a reasonable run time.

2.5. Optimization of chromatographic conditions and sample preparation

Chromatographic optimization of HPLC conditions was carried out with respect to mobile phase conditions (methanol-water), stationary phase (Ascentis C18 HPLC Column (25 cm × 4.6 mm, 5 μm) with the column temperature set at 30 °C, and peak shape. The test results showed that the peak shapes of ergosterol were improved by the solvent system of methanol and water.

2.6. Selectivity

We evaluated the method selectivity by studying independent sources of blank serum samples added with ergosterol standards [19]. This was done to assess any interference from the serum samples that could impact the analytes or ergosterol standards. The results showed no detectable interfering peaks, indicating good selectivity of the method.

2.7. Linearity, limit of detection, and lower limit of quantification

We prepared calibration standards at seven different concentrations of ergosterol (5, 10, 20, 40, 50, 80 and 100 ppm). The calibration curve linearity was confirmed by plotting the peak-area ratios of ergosterol against the ergosterol concentrations and performing a 1/x-weighted least-squares linear regression analysis. The limit of detection was established as the lowest concentration that generated a signal-to-noise ratio of 3, indicating the minimum level at which the analyte could be reliably detected. Additionally, the lower limit of quantification was defined as the lowest concentration on the calibration curve where the precision was within 20 % and the accuracy was within the acceptable range of 100 ± 20 % [19]. Calibration curve of ergosterol was linear over the ergosterol standards (Table 1).

Table 1
HPLC results for ergosterol standards.

Ergosterol (ppm)	HPLC retention time (minutes)	HPLC Peak Area	HPLC Peak height
5	5.123	93787	3836
10	5.213	237786	6782
20	5.091	378169	13169
40	5.121	601954	23829
50	5.108	875348	34215
80	5.107	1207565	45482
100	5.108	1504803	56517

3. Results

3.1. Evaluation of ergosterol extraction procedure

The HPLC analysis of the spiked serum samples showed that ergosterol was detected in all ten fungal species tested (*Aspergillus fumigatus, Aspergillus niger, Candida albicans, Candida tropicalis, Cryptococcus gattii, Cryptococcus neoformans, Fusarium solani, Mucor* sp., *Penicillium* sp. and *Rhizopus* sp.). However, ergosterol was not found in any of the four serum samples containing bacterial species (*Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus*) (Table 2). These findings confirmed the reliability and effectiveness of the extraction process.

3.2. Ergosterol detection in human serum samples

A total of 84 unique human serum samples were gathered from two locations, HCTM (n = 60) and HSA (n = 24), and scrutinized for the presence of ergosterol. Among these samples, 51 (60.7 %) tested positive for ergosterol. Within the 33 serum samples that showed positive serology, 26 (78.8 %) also exhibited ergosterol presence. Conversely, 26 out of 51 (51 %) serology-negative serum samples, which included 20 negative control sera, were devoid of ergosterol. Seven out of the 33 (21.2 %) serology-positive serum samples did not contain ergosterol, while 25 out of the 51 (49 %) serology-negative serum samples did show ergosterol presence (Table 3). Representative chromatograms of ergosterol detection in human serum samples can be observed in Fig. 1.

3.3. Statistical analysis

The performance of ergosterol detection via HPLC was compared to conventional serological tests. Due to the small number of samples in each test category, all test results were grouped together as a single category of serological tests. Consequently, HPLC was only compared to this aggregated group, referred to as 'serological tests'. Therefore, no corrections for multiple comparisons were performed or needed. A confusion matrix was utilized to evaluate the performance of HPLC against serological tests. In this context, a true positive (TP) test indicates that ergosterol was detected by HPLC when the serological test was also positive. A false positive (FP) test means that ergosterol was detected by HPLC while the serological test was negative. A true negative (TN) test indicates that ergosterol was not detected by HPLC when the serological test was negative, whereas a false negative (FN) test means that ergosterol was not detected by HPLC when the serological test was positive. The sensitivity, specificity, accuracy, positive predictive value (PPV), negative predictive value (NPV), and likelihood ratios, along with their 95 % confidence intervals (CIs), were calculated using MedCalc software [20]. The key findings for ergosterol detection by HPLC were as follows: sensitivity 78.79 % (95 % CI = 61.09–91.02 %), specificity 50.98 % (95 % CI = 36.60–65.25 %), positive predictive value 50.98 % (95 % CI = 42.75–59.16 %), negative predictive value was 78.79 % (95 % CI = 64.60–88.32 %), and overall accuracy 61.90 % (95 % CI = 50.66–72.29 %) (Table 4). These results suggest that while ergosterol detection via HPLC had a reasonably high negative predictive value, indicating its usefulness in ruling out fungal infections, its overall performance was limited by lower specificity and positive predictive value compared to conventional serological tests.

4. Discussion

Ergosterol detection has emerged as a promising method for diagnosing invasive fungal infections. The presence of ergosterol was found in both serology-positive and serology-negative serum samples. However, the percentage of ergosterol-positive samples was

Table 2
Concentration of ergosterol in serum with fungal and bacteria isolates.

Sample No.	Sample in Serum	HPLC Result	Conc. (ppm)	HPLC RT[a] (minutes)	HPLC Peak Area	HPLC Peak Height (AU)
1- S1	Standard Ergosterol	Positive	38.7170	5.110	708763	30001
2- S2	Standard Ergosterol	Positive	39.8268	5.099	728110	36890
3- SAf	*Aspergillus fumigatus*	Positive	32.8982	5.132	607324	34659
4-SAn	*Aspergillus niger*	Positive	6.6433	5.105	186551	9037
5-SCa	*Candida albicans*	Positive	26.4787	5.118	495412	28187
6-SCt	*Candida tropicalis*	Positive	28.5678	5.157	531831	31205
7-SCrg	*Cryptococcus gattii*	Positive	35.3673	5.159	650368	39012
8-SCrn	*Cryptococcus neoformans*	Positive	25.0387	5.333	409085	17508
9-SFs	*Fusarium solani*	Positive	40.2090	5.127	734772	39108
10-SMu	*Mucor* sp.	Positive	33.8558	5.142	624018	34646
11-SPe	*Penicillium* sp.	Positive	70.1781	5.119	1284894	67500
12-SRh	*Rhizopus* sp.	Positive	29.2230	5.133	427189	27045
13-SBs	*Bacillus subtilis*	Negative	0	–	–	–
14-SEc	*Escherichia coli*	Negative	0	–	–	–
15-SPse	*Pseudomonas aeruginosa*	Negative	0	–	–	–
16-SSte	*Staphylococcus aureus*	Negative	0	–	–	–

[a] RT, retention time.

Table 3
Comparison of serological tests and ergosterol detection by HPLC in serum samples.

Serological tests (n)	HPLC ergosterol positive (%)	HPLC ergosterol negative (%)
Aspergillus antibody positive (10)	7	3
Aspergillus antigen positive (8)	7	1
Aspergillus and *Candida* antibody positive (6)	6	0
Candida antibody positive (6)	4	2
Cryptococcus antigen positive (2)	2	0
Candida antigen positive (1)	0	1
Total no of positive serological tests (33)	26 (78.8)	7 (21.2)
Total no of negative serological tests (51)	25 (49.0)	26[a] (51.0)
Total no of sera tested (84)	51 (60.7)	33 (39.3)

[a] 20 out of 26 were negative control sera.

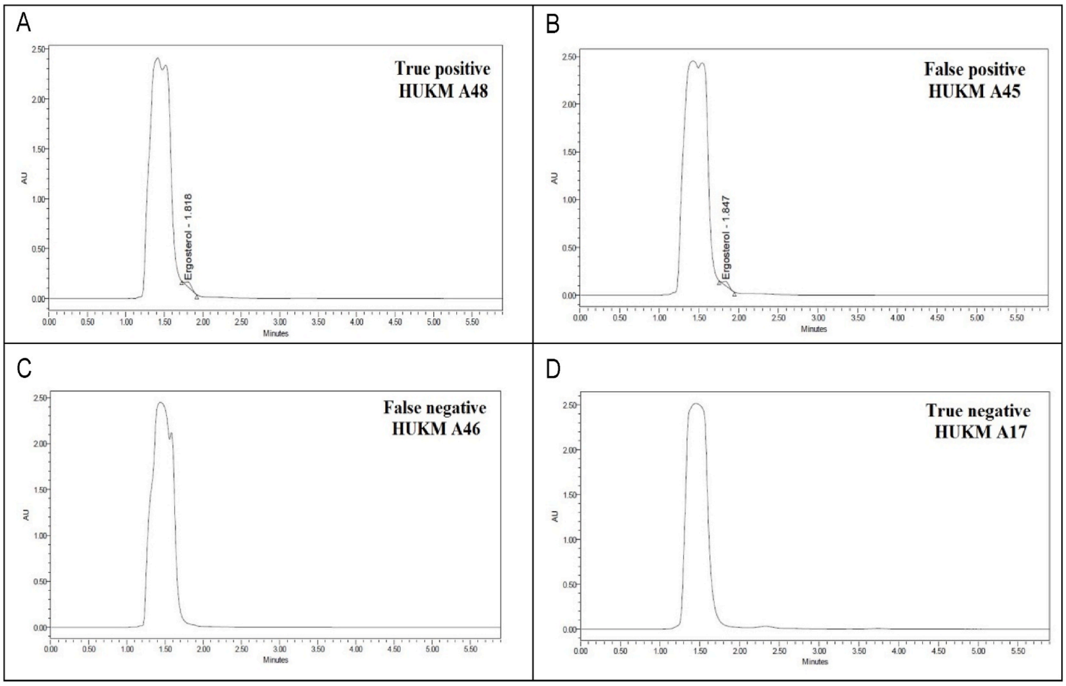

Fig. 1. HPLC chromatograms in human serum samples. A. Positive ergosterol detection in a serum with a positive serological test (True Positive). B. Positive ergosterol detection in a serum with a negative serological test (False Positive). C. Negative ergosterol detection in a serum with a positive serological test (False Negative). D. Negative ergosterol detection in a serum with a negative serological test (True Negative).

Table 4
A confusion matrix on the performance of ergosterol detection by HPLC as compared to serological tests.

	Serology positive	Serology negative	Total
HPLC ergosterol positive	26 (TP)	25 (FP)	51
HPLC ergosterol negative	7 (FN)	26 (TN)	33
Total	33	51	84

TP, true positive; TN, true negative; FP, false positive; FN, false negative.

higher among serology-positive samples, suggesting that ergosterol detection could potentially serve as a complementary diagnostic tool in serological tests for fungal infections. Among the seven serology-positive samples that tested negative for ergosterol, it may indicate that ergosterol detection is not universally effective and should be utilized alongside other diagnostic methods. The higher percentage of ergosterol-positive samples in serology-negative samples may also point to the possibility of false-negative serological results. Notably, none of the 20 negative control sera tested positive for ergosterol. Furthermore, six sera tested positive for both *Aspergillus* and *Candida* antibodies, and all of which were also positive for ergosterol. However, it remains unclear which specific test the ergosterol positivity correlated with. To validate these initial findings, further studies with larger sample sizes are imperative.

In this study, the overall performance of ergosterol detection by HPLC in diagnosing IFDs was deemed moderate. A comparison with Almeida-Paes et al. revealed a relatively superior performance of *Aspergillus* galactomannan (Bio-Rad Platelia Sandwich ELISA) with a sensitivity of 87 %, specificity of 78 %, and accuracy of 82.5 % based on a 0.52 cut-off index [21]. Dupuis et al. reported similar values for serum BDG in predicting invasive candidiasis, with sensitivity, specificity, and accuracy of 0.85, 0.46, and 0.5 at a cut-off value of 80 pg/mL, and 0.3, 0.81, and 0.75 at a cut-off value of 250 pg/mL, respectively [22]. Mikulska et al. [23] conducted 14 studies involving patients with invasive candidiasis to assess the performance of the sandwich enzyme-linked immunosorbent assay (Platelia™, Bio-Rad Laboratories, Marnes-la-Coquette, France). The findings highlight encouraging results for the combined use of mannan antigen (Man-Ag) and antimannan antibody (anti-Man-Ab) testing, demonstrating superior diagnostic accuracy compared to the individual assays. However, it is important to acknowledge the limitations of these tests. While the combination improves sensitivity and specificity, it cannot differentiate between fungal species, necessitating additional diagnostic methods for comprehensive pathogen identification. Specifically, the sensitivity and specificity of the Man-Ag assay were 58 % and 93 %, respectively, while the anti-Man-Ab assay exhibited a sensitivity of 59 % and specificity of 83 %. When used in combination, the sensitivity increased to 83 % and specificity to 86 %.

Among the serological tests that are currently in use, only serum BDG serves as a pan-fungal marker. BDG is a polysaccharide component of the fungal cell wall that may be detected in infections caused by *Aspergillus, Candida, Fusarium, Trichosporon, Saccharomyces, Acremonium* and *Pneumocystis jiroveci*. However, *Cryptococcus* species, the yeast phase of *Blastomyces dermatitidis* and some Mucorales species do not produce BDG, and consequently, will not be detected by the serum BDG assay [24]. Another drawback of BDG testing is the presence of glucan in large amounts in almost all environments, necessitating stringent laboratory precautions to minimize contamination. Additionally, a high rate of false positivity has been observed in patients, which was attributed to concurrent bacteraemia, the use of haemodialysis or treatment with human immunoglobulin [25]. Ergosterol is the dominant sterol in the cell membrane of most fungal pathogens, with the notable exception of *Pneumocystis jiroveci* [26]. Unlike BDG, ergosterol is not found in plant-based material or detectable in bacteria, and therefore may be a better pan-fungal marker of infection in patients, with potential prognostic value.

A key finding in this study is that ergosterol was not detected by HPLC in all expected negative samples (i.e., samples not indicated for fungal testing; n = 20), which are considered the true negative samples. It is crucial to highlight this observation, as negative results for serological diagnosis do not necessarily indicate a lack of fungal infection, as in most cases, only one serology test was performed. While the sensitivity and negative predictive value of ergosterol detection were high, the specificity and positive predictive value were relatively low, suggesting that false positives may be a concern with this method. The serological tests used as comparison in this study were limited to the detection of antigens of *Aspergillus*, *Candida*, *Cryptococcus* and antibodies against *Aspergillus* and *Candida*. Although these have been the major fungal pathogens causing IFDs worldwide, common causes of fungemia in Malaysia include *Talaromyces marneffei* and *Histoplasma capsulatum* [27]. Additionally, Basidiomycete fungi, including the pathogen *Schizophyllum commune*, have been isolated from blood cultures [28]. Therefore, the 25 false positive results may be due to infection or colonisation by other fungal species, and a lack of sensitivity of the serological test used as comparison.

When serum samples are used for diagnostic testing, a variety of factors – including the host, the microbe and the laboratory setting – can impact the test's sensitivity and specificity. Fungi are part of the human microbiota and can act as either colonizers or pathogens. The positive detection of antigens or the presence of antibodies must be interpreted in conjunction with the patient's clinical presentation. Consequently, a minimum of two consecutive positive samples is recommended as the initial screening strategy for IFDs in patients [29]. Evidence suggests that, aside from the *Cryptococcus* antigen lateral flow assay, no other serological test can conclusively confirm or exclude an IFD. However, persistent positive results may indicate a refractory fungal infection. Therefore, a combination of diagnostic tests is recommended for the detection of IFDs [30].

The false negative results were observed more frequently when ergosterol detection was compared to the positivity of antibodies against *Aspergillus* and *Candida* (5 samples). Antibody levels are known to remain elevated in patients, corresponding to a favorable recovery prognosis [31], even when fungal antigen may not be detectable. The ergosterol negative results in two samples, which were positive for *Aspergillus* and *Candida* antigens, could be attributed to a positive response to azole treatment in patients, as this treatment can reduce ergosterol content in the cell membranes of azole susceptible *Candida* species [32] and *A. fumigatus* [33]. Conversely, elevated ergosterol content is linked to azole resistance [34]. Furthermore, the quantity of ergosterol in *Candida* species correlated with in vivo outcome in a murine model [32]. Therefore, detecting ergosterol in sequential patient samples, prior to azole therapy and post-therapy, may provide additional information on the antifungal susceptibility of the infecting fungal pathogen.

Detecting ergosterol in serum samples via HPLC-MS is highly sensitive but also time-consuming and labor-intensive, making it impractical for routine diagnostic use in hospitals. Our study, however, demonstrated that ergosterol serves as a specific marker for fungal presence in clinical sera, as it was detected exclusively in fungal samples and not bacterial ones (see Table 2). Therefore, further research is needed to develop more practical methods for detecting ergosterol in clinical samples. This could involve modifications to HPLC techniques or the exploration of alternative methods, such as lateral flow assay or a specific sensor for ergosterol. While no such assay is available commercially, a biosensor-based method to detect ergosterol in clinical samples has been patented [35]. One significant limitation of this study was the small sample size, which hindered the ability to conduct in-depth analysis and draw definitive conclusions. However, the explorative nature of this study could be enhanced through larger-scale studies in the future.

5. Conclusions

Ergosterol detection has the potential to serve as a supplementary tool for identifying the presence of fungi in human sera. While it may not distinguish between different fungal species, ergosterol can function as a broad-spectrum diagnostic marker, particularly when used in conjunction with serological assays. Nonetheless, further research with larger sample sizes, utilizing sequential serum samples and comparing results with patients' clinical presentations, is necessary to confirm and validate these findings.

Funding disclosure

This work was supported by the Center of Desert Studies at University of Anbar, Iraq and Universiti Teknologi Malaysia (UTM) and University Research Grant (GUP) No. Q. J130000.2545.14H56.

Ethics declarations

This study was conducted after receiving approval from the Medical Research and Ethics Committee, Ministry Of Health, Malaysia (NMRR-16-1299-31493).

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Ahmad SH.A. Lafi: Writing – original draft, Methodology, Investigation. **Mohd Nizam Tzar:** Writing – review & editing, Supervision, Formal analysis, Data curation, Conceptualization. **Jacinta Santhanam:** Writing – review & editing, Supervision, Formal analysis, Data curation, Conceptualization. **Fahrul Huyop:** Writing – review & editing, Supervision, Formal analysis, Data Curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The authors would like to acknowledge support from both microbiology laboratories of Hospital Canselor Tuanku Muhriz UKM in Kuala Lumpur and Hospital Sultanah Aminah in Johor Bahru, Malaysia.

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