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ABSTRACT

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MOLECULAR AND IMMUNOLOGICAL DETECTION OF GLIOTOXIN GENES IN *ASPERGILLUS FUMIGATUS* ISOLATED FROM PATIENTS WITH INVASIVE PULMONARY ASPERGILLOSIS

Introduction: *Aspergillus fumigatus*, a filamentous fungus, is a leading cause of invasive pulmonary aspergillosis (IPA), a severe condition predominantly affecting immunocompromised patients. Gliotoxin, a secondary metabolite crucial to *A. fumigatus* virulence, is produced through a biosynthetic pathway regulated by several genes, including *GliP*, *GliA*, and *GliJ*. It plays a significant role in its pathogenicity, affecting host immune responses and contributing to disease progression. This study aimed to molecularly and immunologically detect gliotoxin-related genes in *A. fumigatus* isolates from IPA patients.

Methods: Forty patients diagnosed with IPA were recruited, and sputum and plasma samples were collected to identify fungal isolates and assess plasma interleukin-17 (IL-17) levels. Molecular detection of *A. fumigatus* involved extracting genomic DNA from isolates, followed by polymerase chain reaction (PCR) using primers for the gliotoxin biosynthetic genes *GliP*, *GliZ*, and *GliA*. Additionally, plasma IL-17 levels were measured in IPA patients to assess their immunological response and gliotoxin production was confirmed using thin-layer chromatography.

Results: Of the 40 isolates, 20 were confirmed to be *A. fumigatus*. PCR analysis revealed that 85% of these isolates harbored the *GliP* gene, while 75% carried the *GliZ* gene. Also, 40% of the isolates demonstrated gliotoxin production. The IL-17 levels in patients with IPA (1660.10±103.65 pg/mL) were significantly higher compared to the control group (807.92±101.51 pg/mL). The elevated IL-17 levels observed in IPA patients underscores the role of this cytokine in the immune response against *A. fumigatus*. The detection of gliotoxin-

producing isolates varied, with genetic and environmental factors influencing gliotoxin biosynthesis. Isolates lacking either *GliP* or *GliZ* failed to produce gliotoxin, while some isolates with both genes exhibited reduced toxin production, likely due to regulatory influences.

Conclusion: The study found that a combination of molecular and immunological methods offers a promising diagnostic tool for detecting IPA and understanding *A. fumigatus* virulence. Future research could enhance diagnostic tools' sensitivity and specificity, apply them in clinical settings, and develop non-invasive methods like biomarkers for improved patient outcomes.

Keywords: *Aspergillus fumigatus*, immunity, invasive pulmonary aspergillosis, diagnostics.

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INTRODUCTION / ВСТУП

Nearly 90% of cases of invasive aspergillosis are caused by the extremely pathogenic filamentous fungus *Aspergillus fumigatus* [1]. The three clinical types of pulmonary aspergillosis are invasive, allergic, and saprophytic. Among these, invasive aspergillosis (IA) is the most dangerous, as it penetrates tissues and can lead to severe infections, particularly in immunocompromised individuals or patients undergoing immunosuppressive therapy, such as those receiving organ transplants [2]. Research by Machado *et al.* (2024) [3], Seyedmousavi et al. (2018) [4], and Ben-Ami *et al.* (2009) [5] has linked the virulence of *A. fumigatus* to secondary metabolites produced during lung infections. Gliotoxin, a key secondary metabolite of A. fumigatus, plays a significant role in this process [6]. Epipolythiodioxopiperazines (ETPs) are immunosuppressive compounds that enhance phagocytosis while inhibiting the activation of nuclear factor-kappa B (NF-κB) and suppressing phagocytosis in macrophages and lung epithelial cells [7]. Gliotoxin plays a crucial role in the progression of IA, as noted by Lanier *et al.* (2020) [8]. The gliotoxin biosynthetic cluster, described by Nielsen *et al.* (2022) [9], controls its production in *A. fumigatus* through 13 genes. One of these genes, *GliP*, encodes a non-ribosomal peptide synthase that catalyzes the first step in gliotoxin biosynthesis [10]. *GliA* is crucial for establishing gliotoxin tolerance and protecting *A, fumigatus* from exogenous metabolites by exporting the toxin. This mechanism helps the fungus avoid the harmful effects of its gliotoxin production. Additionally, GliA secretes gliotoxin, which increases the pathogenicity of A. fumigatus [11]. Furthermore, the GliJ gene produces a metal-dependent dipeptidase, one of four enzymes in the gliotoxin production pathway that transform glutathione conjugates into transannular disulfide bridges [12].

Invasive pulmonary aspergillosis, primarily caused by *A. fumigatus* remains a major cause of morbidity and mortality, especially among immunocompromised individuals, such as those undergoing organ transplantation or receiving chemotherapy [13]. Early diagnosis and effective treatment are crucial for improving patient outcomes, but current diagnostic methods often lack sensitivity and specificity [14, 15]. Identifying gliotoxin-producing *A. fumigatus* strains is essential for understanding the virulence mechanisms driving IPA. Gliotoxin, a potent immunosuppressive compound, disrupts immune system function and promotes fungal survival, making it a critical factor in the progression of IPA [16]. While gliotoxin, a key metabolite, is linked to IPA progression, gaps remain in understanding its genetic regulation and environmental influences. The molecular mechanisms behind gliotoxin-related genes (*GliP*, *GliA*, *GliJ*) in clinical isolates are poorly characterized. Moreover, few studies integrate molecular and immunological methods to improve IPA diagnosis. Bridging this gap is crucial for advancing knowledge of *A. fumigatus* virulence and enhancing diagnostic tools for IPA.

The present research combines molecular and immunological methods to study gliotoxin-related genes in *A. fumigatus* isolates from patients with IPA. It uniquely examined molecular markers (*GliP*, *GliA*, *GliJ*) and immune responses, particularly IL-17, to understand disease progression. By analyzing sputum and plasma samples, the study provides new insights into the relationship between gliotoxin biosynthesis and immune activation, advancing the understanding of IPA pathogenesis. Furthermore, the research investigated gliotoxin production variability among clinical isolates of *A. fumigatus*, revealing factors contributing to virulence. This understanding could aid in developing targeted diagnostic tools and therapeutic interventions for IPA. In

the present study, it was hypothesized that gliotoxin-related genes in *A. fumigatus* contributed to IPA pathogenesis, and combining molecular and immunological techniques enhances IPA diagnosis by detecting genetic markers and IL-17 responses.

This study aimed to identify and characterize the presence of gliotoxin-related genes in *A. fumigatus* isolates from patients with IPA through molecular and immunological techniques.

MATERIALS AND METHODS

Source of fungal strain

Aspergillus fumigatus strains (ATCC 4345 [wild type] and strain ATCC12348) were obtained from the American Type Culture Collection (ATCC), USA.

The samples were collected in the period between **1/10/ 2022 and 20/1/ 2023.**

Study population

This study comprised 40 patients with an IPA diagnosis who were admitted to Madinat Al Tib's Department of Respiratory and Critical Care Medicine between September 2022 and December 2023.

Inclusion and exclusion criteria

Participants in this study included individuals aged 18 years and older with a confirmed diagnosis of IPA, based on clinical and laboratory evidence. Only those with confirmed *A. fumigatus* infection in their clinical samples were included, as adults are generally more susceptible to IPA compared to children. Exclusion criteria encompassed individuals without confirmed *A. fumigatus* infection, those with respiratory infections unrelated to IPA, patients with severe comorbid conditions not associated with aspergillosis, those who had received antifungal treatment before sample collection, and pregnant individuals. Informed consent was obtained from all participants before inclusion in the study.

Ethical approval

Ethical approval for the study was obtained from the Ethics Committee, Department of Biology, College of Science, University of Kirkuk, Iraq.

Sample collection

Sputum and plasma samples were collected from each patient between October 1, 2022, and January 20, 2023, to identify the causative agent. Plasma samples were obtained before treatment initiation to detect IL-17 levels. Plasma IL-17 concentrations were measured using the Human IL-17 enzyme-linked immunosorbent assay (ELISA) kit (Abcam) following the manufacturer's instructions.

Isolation of *Aspergillus fumigatus* from specimens

Sputum samples were cultured on potato dextrose agar (PDA; Himedia, India) in the Department of Biotechnology laboratory at the University of Baghdad. *Aspergillus fumigatus* strain ATCC 4345 (wild type) was used as the positive control, while strain ATCC12348 served as the negative control. The cultures were incubated at 37°C for 7–10 days and subsequently sub-cultured onto Sabouraud dextrose agar (SDA; Oxoid, UK).

Molecular identification of *Aspergillus fumigatus* and detection of gliotoxin genes

Using the genomic DNA reagents kit (Geneaid, Taiwan), genomic DNA was isolated from *A. fumigatus*. After extraction, the quality and concentration of the DNA were assessed using a Nanodrop spectrophotometer. Electrophoresis on a 0.8% agarose gel, ethidium bromide staining, and UV detection were used to verify integrity [17, 18]. Five oligonucleotides were lyophilized by dissolving them in sterile deionized double-distilled water (DDW) until they reached a final concentration of 10 pmol/µl. One set of primers was used to identify A. fumigatus, and four primers were utilized to detect gliotoxins. Table 1 shows the nucleotide sequences of the primers used. A thermal cycler (Eppendorf, Germany) was used for the polymerase chain reaction (PCR) reaction with PCR mix (Promega) in a final reaction volume of 25 µl. The following PCR program was employed: initial strand separation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 40 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for 1 second. A final extension step was carried out at 72°C for 10 minutes. This program was optimized for improved performance. Electrophoresis was carried out on 1.2% agarose gels for 90 minutes at 5 V/cm using 1X Tris-borate buffer in order to separate around 7 µl of amplified PCR products. Following ethidium bromide staining of the gels, a UV transilluminator was used to view the PCR products, and a gel documentation system was used to take pictures. A DNA ladder marker (100–2000 bp) was used to compare the amplified products' sizes (Bioneer) [19, 20].

Extraction and purification of gliotoxin

Aspergillus fumigatus produced gliotoxin after being incubated for 7 days at 37°C in yeast extract sucrose (YES) broth, consisting of 20 g yeast extract and 40 g sucrose dissolved in 1000 mL distilled water, with the pH adjusted to 5.80. Using Whatman No. 1 filter paper, the *Aspergillus* hyphae were removed from the YES culture medium. The filtrate was extracted three times by adding an equal volume (30 mL) of chloroform to the filtrate and mixing continuously for 30 minutes. The recovered chloroform fraction was then evaporated at 50°C, resuspended in 250 µl methanol, and stored at -70°C until analysis. Thin layer chromatography (TLC) plates were activated in a 100°C oven for 1 hour before use. Dry extracts were redissolved in 200 µl of methanol. Using a solvent mixture of toluene, ethyl acetate, and formic acid (5:4:1) as the mobile phase, a silica gel 60 plate was spotted with 20 µl of each extract and developed in a TLC tank until the solvent front reached 10 cm. Following air drying, gliotoxin was observed at 365 nm under a UV lamp.

Statistical analysis

The Statistical Analysis System (SAS) was used to analyze the data, and the results are shown as means ± standard errors of the mean. Analysis of variance (ANOVA) was used to assess group differences. The threshold for statistical significance was $p < 0.05$.

Table 1 – The names and sequences of specific primers

Primers	Sequence (5'-3')
AfmiF1	GCCCGCCGTTTCGACCCCCCCAAAAA
AfmiR1	CCGTTGTTGAAAGTTTTAACTGATTAC
GliAf	AAAATTTGCGATCAACGAACTCTG
GliAr	AAAACCCTTGACGGACTGGAAGTA
GliJf	CTCTGATCGACGGCCATAATAAAAAA
GliJr	TCGAGCTGTTGGAGTGTCTGCCCCCCC
GliPf	AAACCCCTGTGAATGCAGACAAAAAA
GliPr	CCCCTTGAGATGAAAGGTGACCCCCCC
GliZf	TCCAGAAAAGGGAGTCGTTGTTTTTTTCCC
GliZr	ACGACGATGAGGAATCGAACCCCCCCAAA

RESULTS AND DISCUSSION

Immunological detection

Forty (40) patients were included in the study, 20 of whom were diagnosed with IPA and had culture-positive results for *A. fumigatus*. Table 1 compared levels of IL-17 between the IPA and the control groups (healthy individuals). The results showed that levels of IL-17 were significantly elevated in the IPA group compared to the control group.

Identification of *A. fumigatus* isolates

Genomic DNA was efficiently extracted using a reagent genomic DNA kit. Standard methods were employed to assess purity and concentration, with DNA yields ranging from 65 to 210 ng/µl and purity values between 1.5 and 1.9. The PCR technique was applied to confirm the identity of *A. fumigatus* isolates. The Afumi primer proved sensitive and specific for detecting *A. fumigatus* through PCR. Agarose gel electrophoresis of the PCR products revealed a 136 bp amplicon in all *A. fumigatus* samples. The genomic DNA of all isolates was successfully detected and confirmed to be complementary to the Afumi gene sequence, as indicated by the presence of a single 136-bp band (Figure 1).

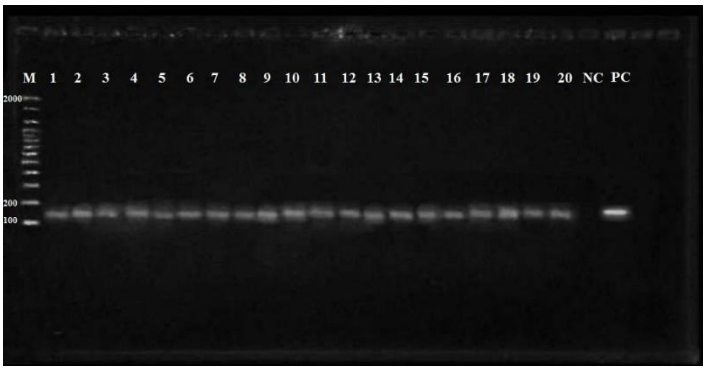

Figure 1 – PCR product for Afumi primer for DNA samples of *Aspergillus fumigatus* on 1.2% agarose gel bromide. M: 100 bp DNA ladder. NC: Negative Control; PC: Positive Control

Production of gliotoxin in *Aspergillus fumigatus* isolates

The screening test aimed to demonstrate that after 7 days of incubation in YES medium at 37°C, the studied isolates produced gliotoxin. Only eight isolates were found to produce gliotoxin (Figure 2). Since both environmental and genetic factors can influence the mycotoxin production capabilities of the isolates, it is essential to optimize conditions for metabolite production and to enhance growth conditions to promote mycotoxin production [21]. These outcomes support those of [22], who found that A. fumigatus strains differed significantly in their ability to produce gliotoxins [22]. Only 18% of the fifteen clinical strains surveyed

were positive for gliotoxin production, which may be attributed to geographic or patient-specific factors. Nonetheless, clinical isolates generally exhibit a higher tendency for gliotoxin production than environmental isolates, consistent with previous studies [23].

Only five *A. fumigatus* isolates were identified as capable of producing gliotoxin under solid-state fermentation [20]. In contrast, Dos-Santos *et al*. (2003) reported that only a low level of gliotoxin production was observed in clinical *A. fumigatus* strains from the Azores [24]. *A. fumigatus* was isolated in over 90% of clinical cases. The screening test aimed to verify that the test isolates could produce gliotoxin after 7 days of incubation in YES medium at 37°C. Chromatographic analysis using TLC identified eight isolates suitable for gliotoxin production as displayed in Figure 2. Since both environmental and genetic factors can influence the efficiency of parasitic functions involved in mycotoxin production, it is crucial to optimize conditions for the formation of secondary metabolites and enhance food systems to reduce mycotoxin contamination [25]. However, the results indicated that clinical isolates contain more gliotoxin-producing strains of *A. fumigatus* than environmental isolates, which aligns with previous studies [21]. Only five *A. fumigatus* isolates were found capable of producing gliotoxin through solid-state cultivation. In contrast, over 90% of clinical *A. fumigatus* isolates from IA cases in tertiary-care cancer treatment settings were found to be gliotoxin producers, as reported by Lewis *et al*. (2005) [25]. Additionally, the total gliotoxin produced by *A. fumigatus* varied between strains, and not every strain from IA cases in tertiary-care cancer settings was a gliotoxin producer, as reported by Lewis *et al*. (2005) [25]. Moreover, gliotoxin production by *A. fumigatus* was strain-dependent, with only certain strains acting as gliotoxin producers [25].

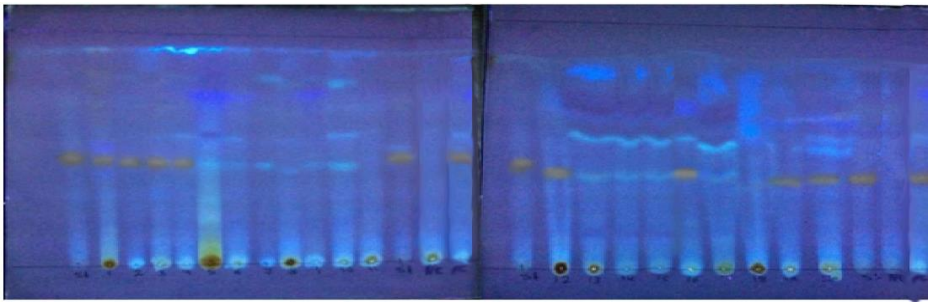

Figure 2 – Detection of the gliotoxin production ability of 20 *Aspergillus fumigatus* isolates on YES medium. Using TLC analysis, the mobile phase was toluene: ethylacetate: formic acid (5:4:1), gliotoxin appeared as a brown colour, Lanes 1 to 20: *A. fumigatus* isolates; St: Standard gliotoxin

In the genome of A. fumigatus, Fan et al. (2020) [26] discovered a 12-gene cluster that is similar to the genes involved in the manufacture of gliotoxins. In A. fumigatus, mutations in this gene cluster are linked to decreased virulence factors. Before being coated with silver nitrate reagent, gliotoxin on TLC plates looked brown when exposed to visible light and yellow when exposed to UV light at 365 nm. Following spraying, it looked dark brown when exposed to UV light at 365 nm and brown when exposed to visible light.

Optimal conditions for gliotoxin production included a water-to-substrate ratio of 5:1 (w/v), an inoculum size of 6×10^6 spores, and incubation for 10 days at 37°C. Polymerase chain reaction results using the *GliP* primer showed positive results in 20 isolates (1, 2, 3, 4, 5, 7, 9, 10, 11, 12, 13, 14, 16, 17, 18, and 19), indicated by the presence of a 168-bp single band. However, isolates 6, 8, 9, and 15 exhibited negative results, with no 168-bp band observed (Figure 3). Also, PCR results using the *GliZ* primer showed positive results in isolates 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 19, and 20, indicated by the presence of a 162-bp single band. However, isolates 11, 13, 15, 17, and 18 were negative, with no band observed at this molecular weight (Figure 4).

IL-17 is a soluble type II transmembrane protein that binds to the β-glucan in the *Aspergillus* cell wall. Research has shown that following *A. fumigatus* infection, IL-17 production correlates with patients' immune response, leading to the migration and activation of immune cells against the fungal infection. IL-17 may play a key role in innate immunity to opportunistic infections. In the present study, IPA detection was more sensitive and specific when based on IL-17 levels in plasma, as fungal infections led to elevated cytokine levels [27, 28]. Biomarkers, including IL-17, were significantly higher in infected patients compared to the control group. The findings in the present study align with previous research in mice, which also showed increased IL-17 levels during fungal infections [29, 30]. Two transcriptional regulators, LaeA and *GliZ*, control gliotoxin production [19]. These regulators play crucial roles in gliotoxin synthesis, working together with non-

ribosomal peptides to synthesize the enzyme responsible for catalyzing the first step in the process. Gliotoxin is classified as a non-ribosomal peptide toxin [23]. Given that 85% of the isolates tested possessed the *GliP* gene, 75% had the *GliZ* gene, and 75% had both, it is reasonable to conclude that the presence of these key genes underpins gliotoxin production. Conversely, the absence of either of these genes results in a loss of gliotoxin production capability. Sugui [30] demonstrated that deletion of the *GliP* gene in *A. fumigatus* led to a significant reduction in gliotoxin synthesis. Additionally, the loss of gliotoxin production associated with *GliP* disruption confirms the role of *GliP* in gliotoxin biosynthesis [20]. It was established that gliotoxin production was reduced both in vivo and in vitro upon deletion of GliZ, a potential transcription factor found in the gliotoxin gene cluster. The lack of an essential

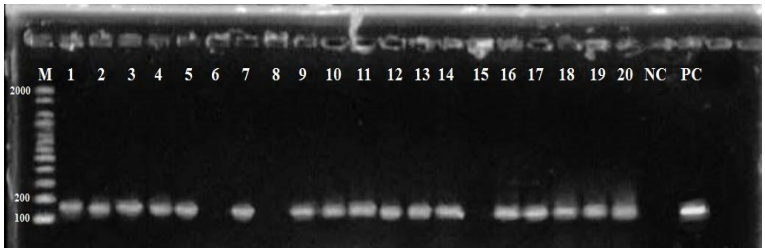

Figure 3 – PCR products for the *GliP* primer for DNA samples of *A. fumigatus* spores on 1.2% agarose gel bromide. M: 100 bp DNA ladder

Figure 4 – PCR products for the *GliZ* primer for DNA samples of *A. fumigatus* spores on 1.2% agarose gel bromide; M: 100 bp DNA ladder

biosynthetic gene within the gliotoxin gene cluster was identified as the cause of this impairment. As stated by Kupfahl and colleagues (2007) [29], gliotoxin plays a key role in the toxicity of *A. fumigatus*, as demonstrated by their finding that *GliP* mutants exhibited reduced virulence in two different mouse strains. Furthermore, GliP mutants show a reduced capacity to trigger apoptosis in mammalian cells and inhibit the oxidative burst in human neutrophils in in vitro tests, according to Sugui et al. (2007) [31].

The inability to produce gliotoxin is associated with the absence of the *GliP* and/or *GliZ* genes. However, some isolates with both *GliP* and *GliZ* genes still fail to produce gliotoxin, as shown by TLC screening. This lack of production can be due to various genetic and environmental factors. Other genes, such as LaeA, which regulate multiple secondary metabolites including gliotoxin, may also influence gliotoxin production. These factors are important in understanding the pathophysiology of *A. fumigatus* and the development of invasive aspergillosis linked to gliotoxin [23, 30]. Despite lower *GliP* gene expression and reduced gliotoxin accumulation, Dhingra *et al.* (2012) [29] reported higher *GliZ* gene expression in the veA strain [29]. Overexpression of *GliZ* in the presence of veA led to significant increases in both *GliP* production and gliotoxin levels. Thus, veA in *A. fumigatus* may influence the expression of structural genes in the gliotoxin cluster through mechanisms beyond its effect on *GliZ* gene expression. Wang *et al.* (2014) proposed that *A. fumigatus* may utilize mechanisms to protect itself from the harmful effects of its gliotoxin [11]. They suggested that *GliA* might offer additional protection by periodically neutralizing the toxin, thereby mitigating its potentially detrimental effects on the fungus. Disruption of *GliA* significantly increased the parasite's vulnerability to extracellular gliotoxin. Additionally, the levels of gliotoxin in both extracellular and intracellular compartments were reduced, indicating that *GliA* disruption markedly diminished gliotoxin synthesis.

GliJ, a product of the *GliJ* gene, is one of the four key components in the gliotoxin production pathway. It encodes a metal-dependent dipeptidase that converts glutathione to transannular disulfide bonds [22].

Table 2 – The levels of IL-17 in patient and control groups

Group	IL-17 levels (ml/pg)
Invasive pulmonary aspergillosis (IPA)	1660.10±103.65
Control	807.92±101.51
T test	225.01**
P value	P ≤ 0.01, NS: Non-significant

Table 2 summarizes all PCR results related to gliotoxin genes. The TLC results indicate that only 40% of the isolates were able to produce gliotoxin. This could be explained by a number of environmental factors that affect the production of gliotoxins, including the extraction method, temperature, incubation period, water-to-substrate ratio, inoculum size, and culture media [20]. These circumstances may lead to the lack of expression of the genes that produce gliotoxins, as was previously described.

CONCLUSION

Thin-layer chromatography screening revealed that only 40% of *A. fumigatus* isolates produced gliotoxin. While *GliA* and *GliJ* were present in all isolates, the absence of *GliP* and/or *GliZ* in some suggests these isolates cannot produce gliotoxin. Combining immunological and molecular markers presents a promising new diagnostic approach for IPA. The combination of molecular (PCR-based) and immunological (cytokine-based) methods for gliotoxin detection holds promise for early diagnosis of IPA. Future research could work on refining these diagnostic tools, increasing sensitivity and specificity, and applying them in clinical settings. Developing non-invasive diagnostic methods, such as biomarkers detectable in bodily fluids, may also improve patient outcomes.

ARTIFICIAL INTELLIGENCE DISCLOSURE/ ВИКОРИСТАННЯ ШТУЧНОГО ІНТЕЛЕКТУ

The authors declare that this manuscript was prepared without the assistance of artificial intelligence (AI) tools, except for paraphrasing some sentences. All the contents were generated through thorough research, critical analysis, and original writing by the authors.

AUTHOR CONTRIBUTIONS / ВКЛАД АВТОРІВ

Study conception and design: SM literature review: AH data acquisition: BM data analysis and interpretation: SM manuscript preparation: AH manuscript proofreading and revision BM.

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The authors declare no conflict of interest.

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