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RESEARCH ARTICLE

RT-PCR detection of ASP1 gene in Candida albicans isolated from Meningitis cases

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Abstract

Objective : The main objective of the sampling is to isolate fungi associated with cases of meningitis ,study of some Virulence factors of fungi and sensitivity to antifungal agents.

Methods: 100 CSF sample were suspected of meningitis culture ,biochemical test and API-System for diagnosis of fungi in sample ,Real-time PCR based on SYBR Green I fluorescent dye for detection of sap gene in Candida albicans.

Results: Depending on agricultures characteristics ,Biochemical tests and Api System the results showed that 32 samples, 32% were given the positive result for yeast growth, Candida albicans found in these samples by 87.5% and Cryptococcus neoformans 12.5% and 25(89.28%) of C.albicans isolates had Sap gene. C. albicans showed the lowest MICs (1 µg/ml) for amphotericin B. and higher MICs for fluconazole (4 - 32 µg/ml) and Nystatin (8-32 µg/ml) While C.neoformans showed low MICs for all antifungal agents used in this study ranged from (0.06-0.5 µg/ml) for amphotericin B and (0.25 - 1 µg/ml) for fluconazole and (1-4 µg/ml) for nystatin

Conclusion: Fungal Meningitis which caused by Candida albicans which have SAP gene that consider as virulence factors and Resistant to antifungal agents and Cryptococcus neoformans is a debilitating and potentially deadly disease that affects patients with both intact and impaired immune systems. early diagnosis and treatment is essential for optimal outcomes. Patients with Fungal Meningitis have multisystem disturbances and require a well-organized and executed plan of care.

INTRODUCTION

Fungal meningitis is rare and usually the result of spread of a fungus through blood to the spinal cord. Although anyone can get fungal meningitis, people with weakened immune systems, The most common cause of fungal meningitis for people with weakened immune systems is *Candida* and *Cryptococcus*. This disease is one of the most common causes of adult meningitis (1).

Meningitis is an acute inflammation of the protective membranes covering the brain and spinal cord, known collectively as the meninges (2). In spite of the fact that bacteria are the most widely implicated pathogens, reports of fungal infections, especially due to *Candida* sp., have increased in recent years. Their reported frequency ranges between 6% and 17%. Many factors have been implicated in the pathogenesis of Candida meningitis, such as broad spectrum antibiotics used in the treatment of a bacterial meningitis, steroids and indwelling bladder and intravenous catheters.(3; 4)

Candida meningitis is also a rare clinical situation; although it is recently becoming more frequent, especially in the cases with immunosuppressive conditions, such as drug addiction, malignancies, organ transplantation, and HIV infection (5). Central nervous system (CNS) involvement could be occurring as a complication of neonatal candidiasis, which is a major challenge in establishing diagnosis and adequate treatment and can lead to neurodevelopment morbidity and death in a case of delay in diagnosis (6).

So *Cryptococcus* sp consider a major case of Cryptococcal meningitis which is believed to result from dissemination of the fungus from either an observed or unappreciated pulmonary infection. Often there is also silent dissemination throughout the brain when meningitis is present. *C. neoformans v. grubii*, and *v. neoformans* usually only cause clinically evident infections in persons with some form of defect in their immune systems People with defects in their cell-mediated immunity, Cryptococcosis is often fatal, even if treated.(7)

All pathogenic microorganisms have developed mechanisms that allow successful colonization or infection of the host (8.). As a result, most pathogens, including *Candida* species, have developed an effective battery of putative virulence factors and specific strategies to assist in their ability to colonize host tissues, cause disease, and overcome host defenses .One factor that contributes to the process of virulence is hydrolytic enzyme production, which is known to play a central role in the pathogenicity of bacteria , protozoa (9), and pathogenic yeasts (10) The three most significant extracellular hydrolytic enzymes produced by *C. albicans* are the secreted aspartyl proteinases (*Sap*), phospholipase B enzymes, and lipases. Of these, the Sap proteins, the existence of 10 *SAP* genes in *C. albicans* and their controlled expression and regulation raises a number of questions concerning the roles and functions of these proteinases during the infective process. The complexity of *Sap* involvement in *C. albicans* virulence has been highlighted by the fact that Sap production is associated with a number of other putative virulence attributes of *C. albicans* including hyphal formation, adhesion, and phenotypic switching encoded by a family of 10 *SAP* genes (11).

Candida albicans proteinases may also evade host defenses by directly degrading molecules such as salivary lactoferrin, lactoperoxidase, cathepsin D (an intracellular lysosomal enzyme of leukocytes), and complement (12). In addition, Sap2 can degrade α_2-macroglobulin, a natural proteinase inhibitor in human plasma (13), and cystatin A, a cysteine proteinase inhibitor found in human epidermal tissues and fluids (14). Furthermore, the proinflammatory cytokine interleukin-1β can be activated from its precursor by Sap1, suggesting a role for proteinases in the activation and maintenance of the inflammatory response at epithelial surfaces in sites of infection(15). Similarly, Sap1 may also act on the blood clotting system by activating coagulation cofactor X (, clotting factor XII, or prothrombin, which may in turn result in the generation of thrombin and hence blood clotting (16).

Due to the lack of study in the province of Qadissiyah dealt with same projec The aim of our work is to isolate the fungi associated with meningitis and the study of some virulence factors using the Real-time PCR technique

Material and methods

1-Collection of Samples

One hundred samples were collected from Spinal cord of the inpatients and reviewers clinics suffering from meningitis after infection diagnosed clinically by the specialist doctors in Diwaniyah Teaching Hospital and Maternity& child hospital in Diwaniyah province for the period from 2013 to 2015
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CSF samples were collected by a specialist doctor depending on Aspiration process by introducing special needle between the fourth and fifth paragraphs of the spine until it reaches the cerebrospinal fluid withdrawn. This process has been under sterile conditions since been withdrawn 2-5 mL of liquid spinal cord and then distributed to the three pipes clean and sterile in each tube about 2 ml and sent to the laboratory, as the first tube sent to the hematology department to conduct a numbers of cells and studied. The second tube was sent to the Department of microbiology to cultured Sabourauds dextrose agar, and the third tube sent to the Chemistry Department for measuring the concentration of glucose and protein.

2. Identification of *Candida albicans*

Candida albicans were identified by colony in pure cultures grown in Sabouraud Agar and microscopic morphologies and using a germ tube test in BHI that was supplemented with 10% horse serum and the **Api candida system** (BioMerieux, Durham, NC, USA).

3 .Identification of *Cryptococcus neoformans*

Unease tests were performed on 24-hour on Sabouraud dextrose agar plus 0.005% chloramphenicol (SAB+C) cultures using Remel rapid urea broth (Remel, Lenexa) according to the manufacturer's instructions. Briefly, 3 ml of broth was heavily inoculated with yeast culture and incubated at 37°C. Tubes were inspected at 4 and 24 h (Remel) for pink color development, indicating positive urea hydrolysis. the **Api *candida* system** (BioMerieux, Durham, NC, USA) also was used as diagnostic tool.

4-Detection of capsule in *Cryptococcus neoformans*

Mix the specimen with a small drop of India on a clean glass slide. Place a cover slip over the smear and press gently. The preparation should be brownish, not black. Using reduced examine the smear microscopically (100X) for the presence of encapsulated cells as indicated by clear zones surrounding the cells(17)

5- Genomic DNA extraction

Candida albicans genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) DNA extraction from pure cultures. Stock cultures of yeasts were sub cultured on Sabouraud dextrose agar (Difco, Detroit, Mich.) and incubated at 37°C. Colonies of these strains were suspended in saline to obtain the turbidity of a 0.5 McFarland standard at a 530-nm wavelength. Two micro liters of cell suspension was added to 18 µl of microlysis solution (Microzone Limited, East Sussex, United Kingdom) in a 0.2-ml Eppendorf tube and overlaid with 20 µl of sterilized mineral oil. the lyses solution-DNA mixture was stored at −20°C for further use in Real-Time PCR.

6-Real-Time PCR Amplification of the SAP Genes

The *SAP*1 primer (*SAP*1F- TCAATCAATTTACTCTTCCATTTCTAACA) and(*SAP*1R-CCAGTAGCATTAACAGGAGTTTTAATGACA) were used for the real-time PCR assay. The Rotor-Gene SYBR Green PCR kit (Qiagen) was used for the real-time PCR experiments. A final volume of 25 µl was used for each reaction and contained 12.5 µl of SYBR Green Master Mix, 1 µl of forward primer (1 µm), 1 µl of reverse primer (1 µm), 2 µl of cDNA (20 ng), and 8.5 µl of RNase-free water. The amplification conditions were 95˚C for 5 minutes followed by 95˚C for 5 seconds with an annealing/extension combination step at 60˚C for 10 seconds for 40 cycles.

7-Antifungal susceptibility

the antifungal susceptibilities of *Candida albicans* and *Cryptococcus neoformans* were determined by the broth micro dilution method according to the EUCAST guidelines. In brief, a Candida density of 0.5 McFarland, corresponding to 1×10^6 to 5×10^6 CFU/ml, was used to prepare the final concentration of 1×10^5 to 5×10^5 CFU/ml in RPMI 1640. Aliquots of 100 µl of serial 2-fold dilutions of each antifungal were dispensed into micro titer plate wells, followed by the inoculation of 100 µl of fungal suspension. The plate was read by spectrophotometer (at 530 nm) after 24 h of incubation at 37°C. The MIC was defined as the lowest antifungal concentration inhibiting ≥50% of growth, except for Amphotericin B, whose MIC was defined as growth inhibition of ≥90%. Experiments were performed in triplicates (18).

Results and Discussion

The main objective of the sampling is to isolate fungi associated with cases of meningitis and then study of some Virulence factors of fungi and for that 100 CSF samples were collected from Spinal cord of patients severed from meningitis .

Depending on Agricultures characteristics ,Biochemical tests and Api System the results showed that 32 samples, 32% were given the positive result for yeast growth, *Candida albicans* found in these samples by 87.5% and *Cryptococcus neoformans* 12.5% Table (1)

Table (1)Number and percentage of samples that have fungal growth

Total number of samples	No .of samples contain fungal growth	(%)	Fungi	(%)
100	32	32	*C.albicans*	87.5
			C. neoformans	12.5
	32	32		100

The results of our study agreement with his findings (19), who pointed out that the *C. albicans* is responsible for the occurrence of 70% of the meningitis infection , SO the results of this study are consistent with his findings (20), who pointed out that the yeast C. *neoformans* is responsible for the events of 15.2% of the meningitis infection.

Colonies of *Cryptococcus neoformans* appeared on the Sabourauds dextrose agar, in the form of white colonies contain elevated scar in the center of the colony(image 1) and when using Indian ink dye to detect the capsule surrounding the yeast above the capsule is greenish featured image (2) due to the overlap of Indian ink components with the components of the capsule, (21). *C. neoformans* virulence is mediated predominantly by a polysaccharide capsule that surrounds its cell wall and has multiple effects on the host immune system. This structure provides a physical barrier that interferes with normal phagocytosis and clearance by the immune system. Capsule components inhibit the production of proinflammatory cytokines, deplete complement components (by efficiently binding them), and reduce leukocyte migration to sites of inflammation (22). The capsule also constitutes the major diagnostic feature of cryptococcosis, because its components can be detected in the bloodstream and it can be visualized with light microscopy by using India ink staining, the capsule excludes the ink particles and forms characteristic halos whose diameters are often several times that of the cell. The elaborate structure of the capsule may also be appreciated by electron microscopy
(23.).

Image (1) *Cryptococcus neoformans* on Sabourauds dextrose agar

Image (2)capsule in *Cryptococcus neoformans* by using Indian ink

Candida albicans is a major fungal pathogen of humans (24). It exists as a commensal in many individuals, generating no obvious pathology, but can cause a range of infections in patients whose immune defenses have been compromised. *C. albicans* is an opportunistic pathogen whose invasion correlates with changes in environmental factors such as alterations to host immunity, competition from other saprophytes and physical perturbation of its niche, for example through surgery. *C. albicans* of the most important causes of fungal infection meningitis in immunosupprised patients as children newborns and patients taking drugs immunosuppressive and those with other diseases such as diabetes, cancer and other (25).

The neoformans (n) variety is found predominantly in contaminated soil and in bird droppings, and is responsible for the majority of Cryptococcus meningitis cases in immunosuppressed patients (26; 27) .*C. neoformans* are acquired through inhalation and reach to lung In the case of weakened immune system of the patient, it spread to other places, such as central nervous system, especially the brain, causing meningitis (28). If intact, the immune system forms a mucous capsule around the fungus to isolate it, thus offering some protection to the host. When the yeast spreads to the brain in immunocompetent hosts, the encapsulated fungus spurs a local granulomatous response, and the fungus becomes "walled off," which may appear on computed tomography (CT) as a ring-enhancing lesion.(22).

Fungal infection of brain more dangerous than bacterial infection due to lack of response to treatments used first and not to the attention of specialist doctors for this type of injury, especially the similarity of symptoms of infection between them and the injury caused by bacteria, as are injuries fungal responsible for the death of 40% of patients with meningitis (29).

2- Study of the physical and chemical changes of the spinal cord fluid

History and physical exam are useful to help decide the likelihood of a CNS infection and to determine if further diagnostic testing is indicated. History and physical examination alone cannot confirm the diagnosis, and therefore if a considerable amount of uncertainty remains. therefore we study the physical and chemical description of patients with fungal meningitis in this study .

A- Physical description

CSF variation that has been collected from patients was clear in 5% from patients to muddied by 48% and 40% slant to yellowing and 7% bloody as it was due turbidity cause yellowing in the cerebrospinal fluid to the high

number of white cells or the presence of bacteria or fungi in many numbers This is the first injury diagnosis meningitis. As for some of the samples the color appearance of clear, this means that the number of white cells or down a little bit and that's what happens at the beginning of the disease or injury first evidence for the absence of this disease may be. And the color of the blood samples is attributed to the high number of as a result of rupture of blood vessels in the area with a needle prick clouds (30; 31).

B- Chemical description

1-Glucose level

glucose with respect to rate the level of sugar in the liquid spinal cord was between 15-38 mg / dl in 32 (32%) of the patients table(2). To measure and determine the percentage of glucose in CSF explains resulting from injury membranes meningitis, which leads to imbalance in the glucose transmission to CSF .Dysfunction also refers to the excess sugar consumption nervous system as well as by white blood and microorganisms that infected the meninges, and this explains why low blood sugar level at fungal meningitis may return shortage of sugar in the CSF To the lack of brain tissue's ability to transfer sugar as a result of influences that spoke microorganisms in brain membranes (32).

A true normal range cannot be given for CSF glucose. As a general rule, CSF glucose is about two thirds of the serum glucose measured during the preceding two to four hours in a normal adult. This ratio decreases with increasing serum glucose levels. CSF glucose levels generally do not go above 300 mg per dL (16.7 mmol per L) regardless of serum levels. Glucose in the CSF of neonates varies much more than in adults, and the CSF-to-serum ratio is generally higher than in adults(30).

CNS infections can cause lowered CSF glucose levels, although glucose levels are usually normal in viral infections . Normal glucose levels do not rule out infection, because up to 50 percent of patients who have bacterial meningitis will have normal CSF glucose levels(33).

Chemical meningitis, inflammatory conditions, subarachnoid hemorrhage, and hypoglycemia also cause hypoglycorrhachia (low glucose level in CSF). Elevated levels of glucose in the blood is the only cause of having an elevated CSF glucose level. There is no pathologic process that causes CSF glucose levels to be elevated (34).

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2 - Protein concentration.

the results showed the same above-mentioned table that the rate of protein concentration was between 28-400 mg / dl . The reason for increasing protein concentration in CSF may be due To increase vascular permeability of the blood-brain barrier and liquid-rich albumin leakage from capillaries and veins, (35).

CSF protein concentration is one of the most sensitive indicators of pathology within the CNS. Newborn patients have up to 150 mg /dl (1.5 g per L) of protein. The adult range of 18 to 58 mg / dl(0.18 to 0.58 g per L) is reached between six and 12 months of age. The physician should know what the normal reference range is for his or her laboratory, because the measurement is somewhat technique-dependent. Elevated CSF protein is seen in infections, intracranial hemorrhages, multiple sclerosis, fungal meningitis , and a variety of inflammatory conditions (36).

3 - WBCs count

The results in the table (2) shown that the number of white blood cells between 500-1000 cells / mm^3 in patients with fungal meningitis The lymphocytes were predominance , this results may be due to the occurrence of inflammation as a result of entering the microorganisms membrane-inflammatory as it is the defense case by the host (37). or due to neuronal cell death leading to edema, hemorrhage, and necrosis, patients can have increased CSF red blood cells and CSF white blood cells with a lymphocytic pleocytosis (38).

The WBC count seen in normal CSF is comprised of approximately 70 percent lymphocytes and 30 percent monocytes. The cell differential alone cannot differentiate between bacterial and nonbacterial meningitis.

Lymphocytosis is seen in viral, fungal, and tuberculosis infections of the CNS, although a predominance of PMNs may be present in the early stages of these infections. CSF in bacterial meningitis is typically dominated by the presence of PMNs. However, more than 90 percent of fungal meningitis cases will show a lymphocytic predominance, especially early in the clinical course and when there are more than 1,000 WBCs per mm^3).(39).

Elevated numbers of white blood cells in the CSF are diagnostic for meningitis or encephalitis, although this finding alone cannot determine the cause of the CNS inflammatory response. While these general guidelines may be helpful to broadly characterize CSF findings in many cases, several studies have demonstrated that no single laboratory finding, including the CSF WBC count, can accurately categorize the cause of CSF pleocytosis in all patients (40).

Table (2) the number of white blood cells and chemical changes in the means of the spinal cord in patients with Fungal meningitis.

No .of patients	Protein level Mg/dl	Control Mg/dl	Sugar level Mg/dl	control Mg/dl	WBC cell/ml^3	control cell/ml^3	Prevalent WBC
32	100-300	15–45	15-38	90	500-1100	500	Lymphocytes

Detection of *sap* gene in *Candida albicans*

Results showed 25(89.28%) of *C.albicans* isolates had *Sap* gene , which is one of the virulence factors in yeast *C. albicanes*. Figure (3). Results of this study are consistent with his findings (12) which indicated that 30% from C. albicans isolates, which were isolated from clinical infections has this gene. So sap gene found in other types of Candida, a *C. tropicals* , *C. Parapsibsis* and *C. dubliniensis* (41). So ,CT value in positive samples was ranged between (30-30).

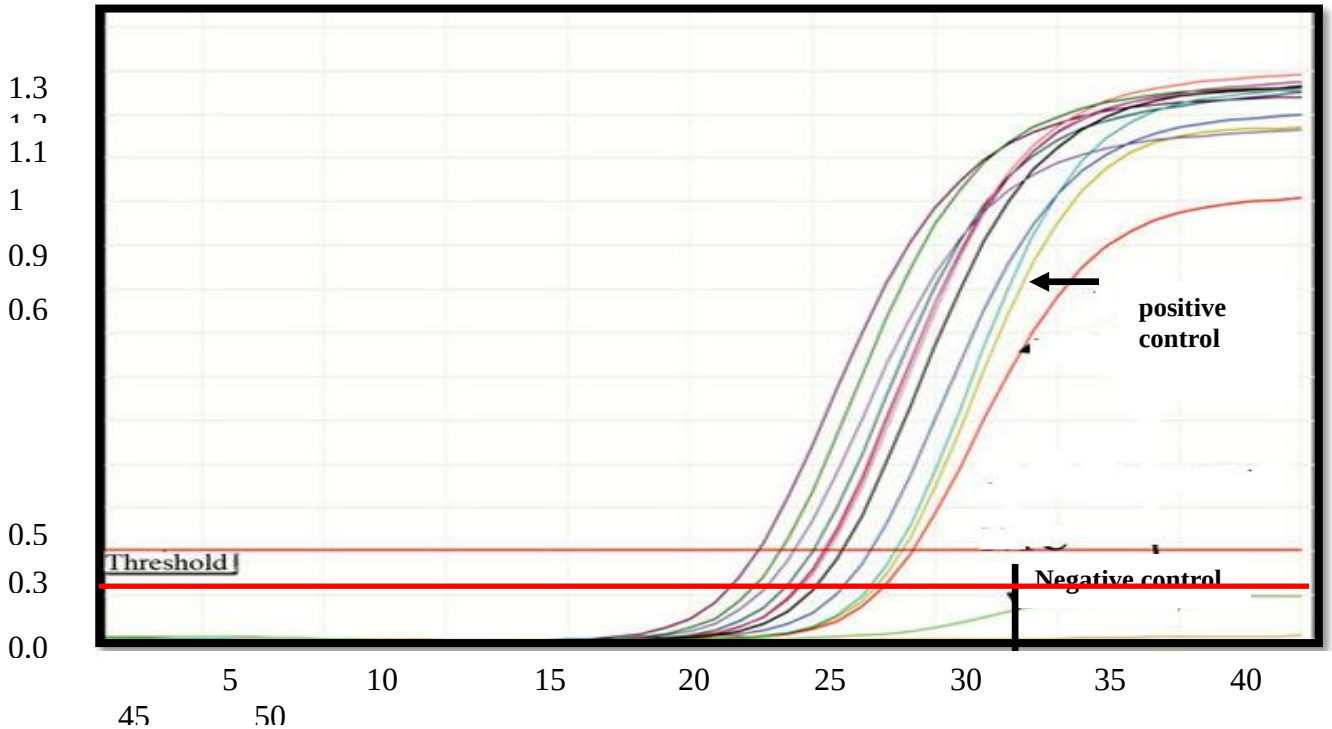

Figure (3) Amplification plot in Real-Time PCR for *sap* gene in *Candida albicans*

In the severely immunocompromised host, *C. albicans* may also cause deep seated or even life-threatening systemic infections. In order to colonize, infect and evade host defense mechanisms, *C. albicans* possesses a repertoire of virulence attributes. In particular, the secreted aspartic proteinases (*Saps*), encoded by the *SAP* gene family with ten members, appear to play a major role in *C. albicans* virulence (42 ;43).

The presence of a *SA*P gene family in *C. albicans* clearly provides the fungus with an efficient and provide proteolytic system that may prove vital to its success as an opportunistic pathogen. Furthermore, *Sap* production is a highly regulated and tightly controlled process, which appears to be a central factor in many aspects of *C. albicans* virulence and is indicative of the multiple functions this gene family possesses. These include the simple role of digesting molecules for nutrient acquisition, the contribution to host tissue invasion by digesting or distorting host cell membranes, the degradation of host surface molecules b enhance adhesion, and the digestion of cells and molecules of the host immune system to avoid or to resist antimicrobial attack. (44; 45).

Antifungal sensitivity

The MICs obtained by broth micro dilution are summarized in Table (3*). C. albicans* showed the lowest MICs (1 µg/ml) for amphotericin B. and higher MICs for fluconazole (4 - 32 µg/ml) and Nystatin (8-32 µg/ml) While *C.neoformans* showed low MICs for all antifungal agents used in this study ranged from (0.06-0.5 µg/ml) for amphotericin B and (0.25 - 1 µg/ml) for fluconazole and (1-4 µg/ml) for nystatin .These results agreement with results that found by (46;47)

Table (3) antifungal sensitivity of *C.albicans* and *C.neoformans*

Yeast	Antifungal / MICs(µg/ml)		
	Amphotericin B	Floconazole	Nystatin
C. albicans	1	0.25 - 32	8-32
C. neoformans	0.06-0.5	0.25 - 1	1-4

Failures of drug treatment in fungal infections combined with improvements in performances and standardization of antifungal susceptibility testing have drawn attention to the problem of antifungal resistance and its underlying mechanisms (48) Resistance of Candida species and *Cryptococcus neoformans* to fluconazole(5FC) develops during monotherapy. Acquired resistance results from a failure to metabolize 5FC to 5FUTP and 5FdUMP, or from the loss of feedback control of pyrimidine biosynthesis. Resistance to AmB is unusual, *C. albicans* is the most susceptible to AmB resistance (49). Acquired resistance to AmB is often associated with alteration of membrane lipids, especially ergosterol. Concomitant with the widespread use of fluconazole there have been increasing reports of fluconazole resistance in *Candida* species and *C. neoformans*. Fluconazole resistance was mostly associated with prior use of fluconazole as intermittent therapy or prophylactic continuous treatment for recurrent disease (50). In contrast to Failure to accumulate azole antifungal has been identified as a cause of resistance in several post-treatment *C. albicans, C. glabrata* and *C. krusei* isolates. In azole-resistant *C. albicans* isolates from AIDS patients with oropharyngeal candidiasis, multidrug efflux transporters of the ATP-binding cassette (ABC) super family and of the class of major facilitators (MF) have been shown to be responsible for the low level of accumulation of azole antifungal agents (51).

Conclusion

Fungal Meningitis which caused by *Candida albicans* which have SAP gene that consider as virulence factors and Resistant to antifungal agents and *Cryptococcus neoformans* is a debilitating and potentially deadly disease that affects patients with both intact and impaired immune systems. early diagnosis and treatment is essential for optimal outcomes. Patients with Fungal Meningitis have multisystem disturbances and require a well-organized and executed plan of care, and neuroscience nurses play an integral role in influencing the outcomes for these complex patients .

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