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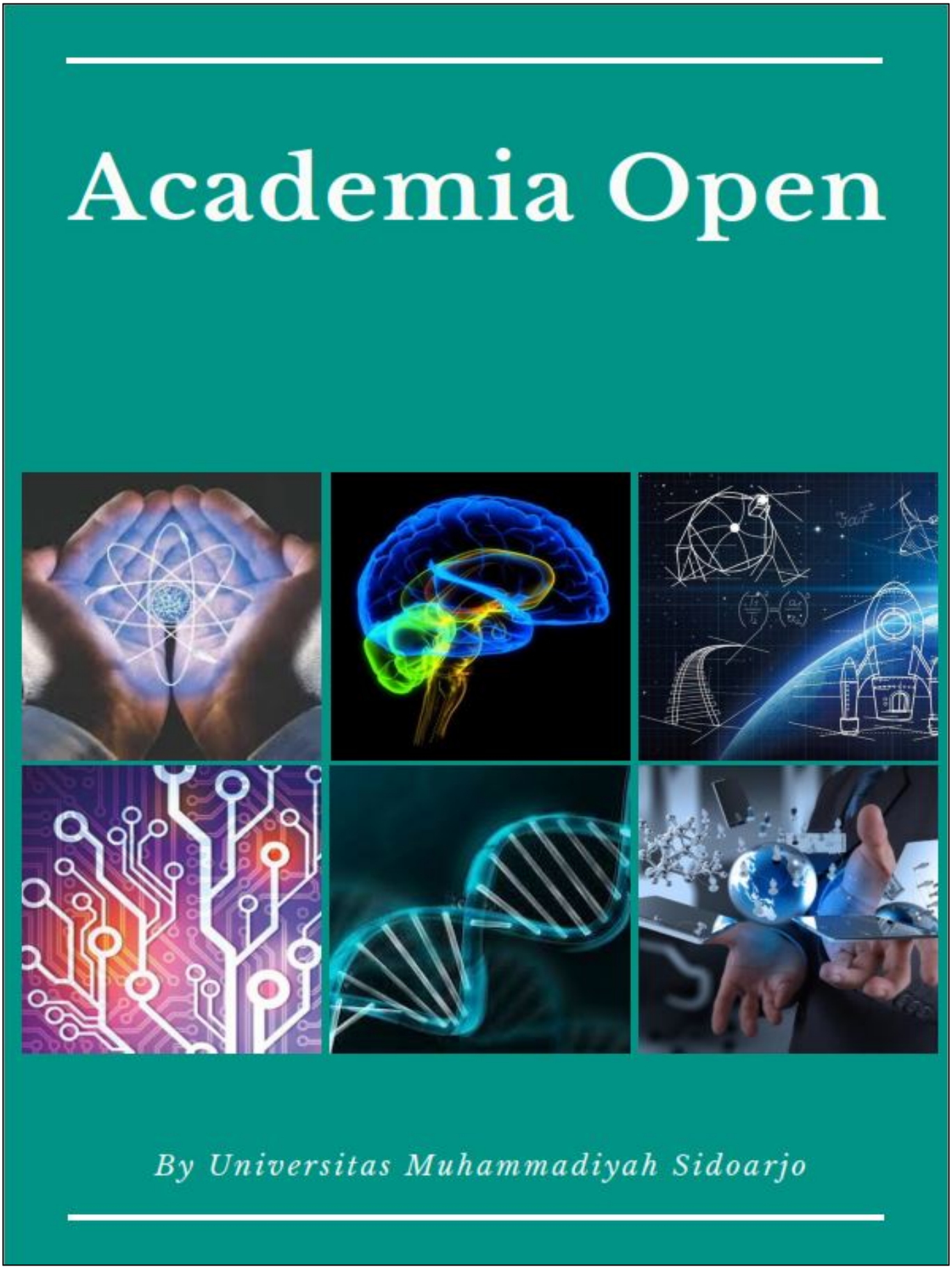

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Table Of Contents

Academia Open

Vol. 10 No. 2 (2025): December
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Molecular Detection of Virulence Genes in Candida albicans Isolated from Cow Milk: Deteksi Molekuler Gen Virulensi pada Candida albicans yang Diisolasi dari Susu Sapi

Deteksi Molekuler Gen Virulensi pada Candida albicans yang Diisolasi dari Susu Sapi

Aseel Razak Al-Rekaabi, arazaq@uowasit.edu.iq, ()
College of Science, University of Wasit, Iraq

Mahdi Saber Al-Deresawi , Mahdisaber@gmail.com, ()
College of Science, University of Wasit, Iraq

(1) Corresponding author

Abstract

General Background: Milk and its derivatives are essential nutritional sources, but they can serve as vehicles for pathogenic microorganisms, including opportunistic yeasts like *Candida albicans*, which pose significant health risks. **Specific Background:** *C. albicans* is a commensal organism capable of transitioning into a pathogenic state, aided by virulence genes such as *ALS1* and *PLB1*, which enhance adhesion and biofilm formation. **Knowledge Gap:** Despite its clinical relevance, limited molecular data exist on *C. albicans* virulence factors in raw cow milk from rural regions, where hygienic practices may be suboptimal. **Aims:** This study aimed to isolate *C. albicans* from cow milk and detect key virulence genes (*ALS1* and *PLB1*) using PCR analysis. **Results:** Of 50 milk samples, 28% were positive for *C. albicans*; 92% of isolates formed biofilms, while *ALS1* and *PLB1* were detected in 85% and 100% of isolates, respectively. **Novelty:** This research provides the first molecular evidence of virulent *C. albicans* in cow milk from rural Wasit Province, emphasizing its zoonotic potential. **Implications:** The findings highlight the need for improved dairy hygiene, routine monitoring, and antifungal strategies targeting virulence factors to enhance milk safety and public health.

Highlight :

- A total of 28% of cow milk samples were found to contain *Candida albicans*.
- The virulence genes PLB1 (100%) and ALS1 (85%) were successfully identified using PCR.
- The findings indicate that *C. albicans* in milk can pose health risks to consumers if not handled hygienically.

Keywords : Candida Albicans, CALB1 Gene, ALS1 Gene, PLB1 Gene, PCR

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Introduction

Lately, more and more people and animals seem to be getting yeast infections. It's become a global thing. Since animals can pass these infections to humans, it would really help doctors to know which antifungal treatments work best. Testing milk samples for yeast and seeing how likely they are to cause disease could make a big difference in how we handle these cases [1]. The causes of yeast infections in dairy animals are numerous because, aside from poor machine-based or manual-based cleaning techniques with regard to milk contamination, there is also the indiscriminate and repetitive use of antibiotics by veterinarians and livestock owners. Besides, intra-mammary application of contaminated drugs, as well as utilizing non-sterile injectors and cannulas, contributes to the injection[2]. The Candida albicans and its spores specifically raise public health concerns since they can withstand pasteurization temperatures and thus may be a threat to people with a habit of milk and dairy products consumption[3]. Most human infections brought on by fungal pathogens are caused by Candida species. According to Lamoth et al. [4], The most frequent source of opportunistic infections is members of these species. Candida is a common fungus that inhabits human mucosa and certain environmental reservoirs. Temperature adaptation, adhesion and invasion, nutrient acquisition, immune evasion, and drug tolerance are the primary virulence traits used by albicans. Some virulence factors that Candida albicans exploits include: a: temperature adaptation; b: adhesion and invasion; c: nutrient acquisition; d: immune evasion; and e: drug tolerance [5]. Chwo et al. [6] note that most instances of human colonization in the mouth, vagina, and gut tend to take place during infancy and this is mainly due to breastfeeding or vaginal birth. The capacity of C. albicans to switch between the yeast and hyphal phases to get access to mucosal layers and produce invasive disease can be considered a part of its pathological cycle of life. The yeast is the commonly thought of commensal form, as it is able to colonize superficial commensal sites [7]. Conversely, hyphal form is generally regarded as the invasive form of the fungus and through this, C. albicans can infiltrate deep-seated tissues and breach host barriers [8]. The morphological state of C. albicans can be altered by many environmental parameters, including host temperature, pH, availability of food, detection of quorum, etc [9]. The other virulence mechanisms of C. albicans that appear critical to pathogenicity are adhesion and synthesis of hydrolytic enzymes. Phospholipase B1 (PLB1), being one of the major virulence factors of this organism, causes damage to the cell membrane by hydrolyzing its membrane lipids. As reported up to now, in animals cases, it is only PLB [10]. In C. albicans the ALS protein is a protein Agglutinin-like Seguence, which allows to adhere itself to the host surface, and encodes large cell surface glycoproteins. Isolates of C. albicans have so far been identified to possess nine ALS proteins [11]. The objective of the current research was to molecular identification of C. albicans in Cow milk and discovery the existence of ALS1 and PLB1genes that were expressed as virulence factors in C albicans.

Material and Methods

2.1. Samples Collection:

Fifty samples were taken from cow's milk in several rural Wasit Province locations. The morphological and microscopic characteristics of the isolates were used to identify them. Blood Agar, MacConkey Agar, and Sabouraud Dextrose Agar (SDA) were plated with 10 ml of milk and incubated for two weeks at 37◦C. The phenotypic identification of yeasts was followed by the genotypic identification of specific species. The API-Candida (API C) and API-ID32C assays (bioMerieux) were used to identify the Candida species.

2.2. Biofilm Formation:

Biofilm formation ability of Candida albicans isolates was evaluated in accordance with the adherence of tubes: assessing biofilms produced by Candida albicans isolates as it is described by Christensen et al. [12]. The C. albicans was cultured in Sabouraud Dextrose Agar (SDA), after a loopful was inoculated into test tubes full of 10 mL of tryptic soy broth (TSB), containing glucose. These tubes were left to incubate at 37 Celsius degrees, 24 hrs. The contents were discarded after a period of incubation, and the tubes were washed three times by phosphate buffered saline (PBS, pH 7.2). This was done by decontaminating the tubes in a container that had a disinfectant. In order to observe the biofilm formation, tubes were stained by the inner surface with 1% crystal violet during three hours. After staining, the tubes were washed out, turned upside down and allowed to dry in the air. The presence of the visible nature of stained film layers attached to the inner surface of the tubes was regarded as biofilm formation.

2.3. Genomic DNA Extraction:

Following the manufacturer's instructions, genomic DNA was extracted using the Geneaid yeast DNA Isolation Kit (GEY100). Separation with ethidium bromide in TBE buffer on a 1% agarose gel confirmed DNA isolation. After 40 minutes of electrophoresis at 80 volts, the gels were examined at 320–380 nm using a transilluminator (Vilber lourmat: Japan).

2.4. Detection of Virulence Genes:

2.4.1. Primers Preparation:

Lyophilized primers were supplied by Macrogen Company / Korea. Primers dissolved in free DNase/RNase water to yield a final concentration of 100 pmol/µl as stock solution. To create a 10µM concentration as work primer, 10 pmol/µl was resuspended in 90 µl of demonized water to reach a final concentration of 10µM as work solution.

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[Table 1. is here]

2.4.2. Polymerase Chain Reaction:

The specific primers CALB1 was designed by Eldesouky et al.,[10] for the Internal Transcribed Spacer (ITS) region of C. albicans and ALS1 primer described by Inci et al (2013)(Table-1). PCR was performed for each gene in 25 µl reaction mixture (5 µl target DNA, 12.5 µl PCR master mix (2X) (2xEasyTaq® PCR SuperMix/Chania), 1 µl each . primer (10 Mm), 5.5 µl ddH2O). The run was carried out by Veriti Thermocyclar/ Applied Biosystems/USA. Thermal cycler steps and conditions for each gene showed in table-2

[Table 2. is here]

2.4.3. Gel Electrophoresis:

PCR products (15 µl) were migrated in Gel electrophoresis system (Major Science: Japan). 1% agarose gel (Promega/ USA) were melted in 50 ml of 1X TBE (Promega/ USA) buffer and run at 80 volt for 1hr. The PCR products and the DNA ladder (100-1500bp) were visualized by Transilluminator (Vilber lourmat: Japan) at 320-380 nm. Sterile bidistile water as negative control.

Results

3.1. Isolation and Identification of C. albicans

With the use of striped tests that assessed API-Candida and APII-D32C bioMerieux, isolates were categorized into seven distinct species based on their phenotypic characteristics. There were 14 (28%) Candida albicans in 50 milk samples. The following is the identification of the other isolates as Candida spp. Three isolates of Candida guilliermondii, four isolates of Candida famata, two isolates of Candida krusei, and two isolates of Candida parapsilosis

3.2. Biofilm results:

Production of Biofilm was detected to be positive in 13(92%) C. albicans isolates and 1 isolate as negative (Table- 3).

[Table 3. is here]

3.3. Molecular Identification of Virulence Factors:

The genomic DNA which extracted from C. albicans showed an integrity bands with concentration about 6µg/ml, figure-1. The isolates identified by conventional PCR techniques. The isolates identified by conventional methods as C. albicans were confirmed by using specific gene (CALB1) encoded ITS region. The results showed 100% (table-3) the pattern of CALB1 products (figure-2). ALS1 a virulence factor also was detected in (85%) of these isolates (table-3), the PCR product illustrated in figure -3. PLB1 gene was recorded 1n 100% of C .albicans isolates (table-3; figure-3).

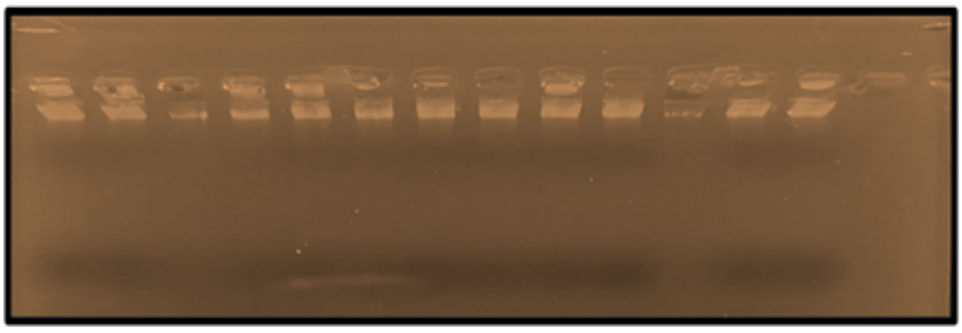

Figure 1. Gel electrophoresis of genomic DNA extraction .1% Agarose gel at 100 volt for 30 min. then visualized under U.V after staining with Ethidium Bromide.

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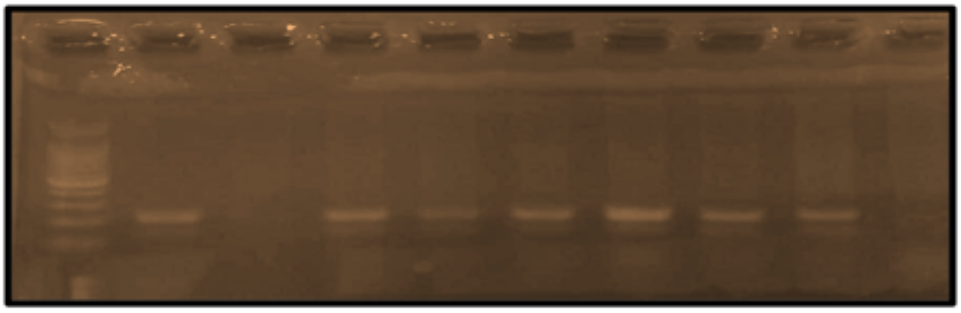

Figure 2. Electrophoresis of PCR of CALB1 gene on agarose gel stained with ethidium bromide. For 1 hour, electrophoresis was done on a 2% agarose gel with a 80 volt. Lane M is a (100 bp) ladder, Lane 2,4,5,6,7,8 was a positive (273 bp) and lane 9 Negative controls

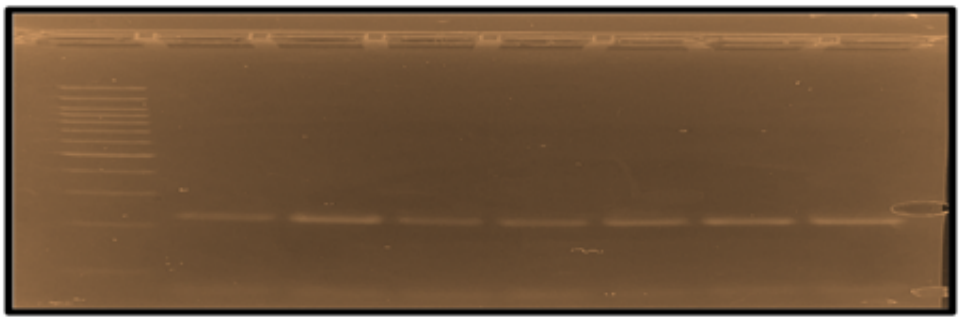

Figure 3. Electrophoresis of PCR of ALS1 gene on agarose gel stained with ethidium bromide. For 1 hour, electrophoresis was done on a 2% agarose gel with a 80 volt. Lane M is a (100 bp) ladder, Lane 2,3,4,5,6,7,8,9 was a positive (318 bp) .

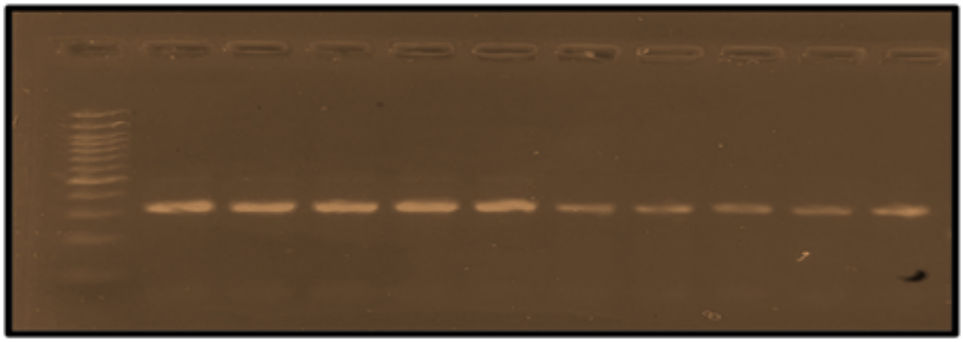

Figure 4. Electrophoresis of PCR of PLB1 gene on agarose gel stained with ethidium bromide. For 1 hour, electrophoresis was done on a 2% agarose gel with a 80 volt. current. Lane M is a (100 bp) ladder, Lane 2,4,5,6,7 was a positive (216 bp).

Discussion

As an opportunistic yeast pathogen, Candida is frequently thought to infect udder skin, milker's hands, milking machinery, drugs, sanitary products, and other equipment [14]. The number of mammary gland infections brought on by Candida species has increased recently, despite the fact that the species' distribution varies throughout several nations. Candida strains were initially identified phenotypically in our tests using API-Candida (APIC) and API-ID32C (BioMerieux), and then verified using genotypic techniques. Species differences were confirmed by comparing the sizes of the PCR products. However, isolates that were classified as a single species based only on morphological traits had genotypic differences. Some strains required nearly a week to grow on the medium, but the majority of colonies emerged 48 to 72 hours after the milk samples were plated. We believe that the delayed growth may have been caused by anomalous conditions that were previously present in the milk (such as an acidic pH or a lack of proteins), which made it more difficult for the fungi to flourish in a different environment. This finding implies that if growth observation is stopped too soon, fungal growth may be

underestimated. According to this study, of 50 milk samples, 28 percent contained Candida albicans, 4 percent contained Candida krusei, 4 percent contained isolates of Candida parapsilosis, 8 percent contained Candida famata, and 6 percent contained isolates of Candida guilliermondii. All of the milk samples included Candida species, according to Gogoi et al. [15]. The species that were found were Candida krusei (77.50%), Candida albicans (13.75%), Candida tropicalis (6.25%), and Candida glabrata (2.50%).Although Morrill et al. [16] discovered that human milk contained Candida albicans, a yeast that caused localized breast infections (mammary Candidiasis), the comparison between these studies shows that Candida albicans is more common from other species, possibly due to bovine mastitis that has been linked to several yeast species, including Cryptococcus neoformans and Candida albicans [17,2,10]. The phenotypic characterization of Candida albicans may be difficult due to its physical and biochemical resemblance to other Candida species [18]. Consequently, over the past years, Candida albicans has been diagnosed through molecular procedures. In this study, PCR was the most correct method of diagnosis, especially in this study. In (14) samples of milk, Candida albicans isolates showed 100 percent having PLB1 and 85 percent having ALS. Candida albicans isolates had 92 percent positive result in the present study, 92 percent in filament staining as well as in the biofilm production. According to Flanagan et al, [19], the PLB1 and ALS1 genes portray the most crucial virulence markers among the C. albicans. Phenotype screening in Petri dish and molecular identification through application of suitable CALB-specific primers suggests the presence of 14 isolates of C. albicans. These proteins, termed as ALS proteins, participate in adhesion and the formation of biofilm and eight gene mediating this gene family has been identified [11]. Specifically, ALS1 was demonstrated to be very vital as far as confirming initial adhesion and early biofilm maturation of infections were concerned [20]. The C. albicans isolates were found to be negative against formation of biofilm but that of other family of proteins linked to ALS or other pathway related to adhesion may have contributed to it in the research study under consideration.

It was also found that C. albicans belonged to the normal flora with earlier findings available that showed C. albicans in healthy animals [21]. The correlation of biofilm formation and ALS gene expression has been already showed [22]. In addition, all C. albicans isolates in the current work were positive to the PLB1 gene and this finding is consistent with the previous studies that had documented the universal presence of PLB1 in isolates grown in bovine milk [10,14].

To enhance the safety of milk produced in smallholder units, there is a need to encourage education of farmers with regard to hygienic milking practices, judicious use of antimicrobials, and measures on surveillance programs of antimicrobial residues. Also the recent study of new antifungal agents which are antifungal agents which will target the virulence factors, including PLB1 and ALS1, could be an encouraging approach of treating Candida infections with Candida albicans and not causing antifungal resistance..

Conclusions

The presence of the PLB1 gene validated the findings of Eldesouky et al.,[10], and this study enhanced the ALS1 gene, which is essential for attaching to the host and generating biofilms. The results of this investigation provide credence to the theory that Candida albicans is present in milk. When people drink milk contaminated by this yeast or its toxins, their health is at stake. Suitable management procedures, especially in relation to the milking process, and hygienic methods employed at different stages of milk production. Understanding the pathogenicity mechanisms used by Candida albicans during infection is essential for developing new antifungal therapies and diagnostics. Traditionally, antifungal drugs were designed to either destroy the pathogenic bacteria or have fungicidal properties. We recommend further research on this gene since several virulence factors, including as dimorphism, adhesin and invasion expression, protease secretion, and others, have been suggested as suitable targets [23].

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Vol. 10 No. 2 (2025): December
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