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Molecular Detection of Virulence Factor Genes in *Candida parapsilosis* Isolated from Subclinical Mastitis Goats and Antifungal Susceptibility in Mosul Province

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Abstract

THIS STUDY aimed to isolate and diagnose *Candida parapsilosis*. The utilization of molecular technologies for identification and virulence gene detection leads to the discovery of Subclinical Mastitis in goats. According to the California Mastitis Take, fifty goat milk samples with subclinical mastitis appearances were collected from specific areas of Mosul province. They were examined using rapid diagnostic tests, which were confirmed by a polymerase chain reaction (PCR) assay. *Candida parapasilosis* was identified in 20/50 (40%) of the mastitis milk samples. In relation to molecular techniques and the consequences of PCR amplification of the 18S rRNA gene for identification of *C. parapasilosis*, the gene was present in 20 samples. Moreover, the Metallo-aminopeptidase gene was present in 20/20 (100%), and the alkaline phosphatase sequence gene was present in 15/20 (75%). However, alpha-glucosidase was present in 10/20 (50%) and sterol esterase genes in 8/20 (40%). Consequence, Antifungal sensitivity testing results showed that fluconazole and amphotericine B provided the most exceptional senitivity against *C. parapasilosis* isolates, followed by ketoconazole, itraconazole, and nystatin.

The outcome of the experiment indicates that goat milk contains many species of the Candida genus, with a significant prevalence of *C. parapasilosis*. This particular species may act as an opportunistic pathogen in mastitis.

Keywords: *C. parapasilosis*, molecular techniques, Subclinical Mastitis, goats, Antifungal Susceptibility.

Introduction

Indeed, goats were the first animals to be domesticated in recorded history, and humans have consistently consumed their meat and dairy products in major quantities. Goat milk and dairy products have experienced a surge in demand in developing nations due to their suitable physicochemical qualities [1, 2]. An investigation has indicated that goat milk is more easily digested and causes fewer allergic reactions compared to cow's milk. Furthermore, Goat dairy products' nutritional and dietetic qualities have led to their classification as functional foods [1, 3, 4]. Actually, research has demonstrated that goat milk contains a lower amount of lactose [3]. Also, there is a higher concentration of medium-chain triglycerides and mono- and polyunsaturated fatty acids [5]. Moreover, a greater proportion of milk protein and essential amino acids [6].

An inflammatory disorder of the mammary gland called clinical mastitis is typically identified by a veterinarian examination using visual inspection and palpation. The essential cause of this condition is bacterial infection, although it can also occur due to organ injury. Additionally, there are many types of subclinical mastitis (SCM) that are induced by coagulase-negative Staphylococci. When compared to the clinical variants, these forms are around six times more common [7]. Nevertheless, they lack the typical mammary signs of the aforementioned clinical form. In addition, these cases are linked to reduced milk production, elevated bacterial levels, and diminished antioxidant content [8].

It has been determined that higher somatic cell count (SCC) levels in cow's milk are related to SCM. The diagnostic importance of EU regulations in establishing the permissible

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thresholds for this parameter underscores its significance [9]. While, there is no discernible relationship between clinical and subclinical mastitis and the somatic cell count (SCC) value in goat milk, as indicated by previous studies [10-12]. In accordance with the way goats produce milk, goat milk contains higher percentages of somatic cells than cow milk [13, 14]. Furthermore, it should be noted that the shedding of cytoplasmic particles from the apical area of secretory cells in milk can lead to an erroneous inclusion of these particles in the count of somatic cells [15, 16].

The stage of animal lactation and the number of lactations also directly impact the SCC value. Actually, the SCC naturally increases as the lactation stage advances and is elevated in goats with a higher number of pregnancies. Therefore, the diagnosis of mastitis in goats is determined by assessing the clinical signs of the mammary gland and/or doing bacteriological testing [16]. Dairy goat farmers are facing severe economic losses from intramammary infections [17]. Contamination of milk by fungi, often represented by potentially pathogenic organisms, can pose a risk to consumers [18].

Prior to 2005, there was originally a division of C. parapsilosis into three agencies, I through III. Nevertheless, in a similar vein, sufficient variations revealed by genetic studies have resulted in the division of the industry into closely related, superior species: *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis* [19]. However, few clinical microbiology laboratories identify between those species, especially given that industrial systems are insufficient to do so. Despite this, C. parapsilosis is responsible for the majority of clinical illnesses. Furthermore, not many studies have made this distinction, but it is hoped that further basic research would take each species individually into account [20].

Widely dispersed throughout nature, *C. parapsilosis* frequently isolates itself from a range of non-human sources, including soil, aquatic environments, domestic animals, insects, and insects [21]. Adhesion and hyphae formation, which cause tissue invasions, are necessary stages of candidal infection. Additionally involved in adhesion, tissue injury, and invasion are the secreted aspartyl proteinases (SAP family), and phospholipase (PLB) aids in the breakdown of phospholipids, which are crucial components of the cell wall [22]. The components that determine the virulence of the candidal disorder include the ability to adhere to the host surface, the capacity to change morphology between filamentous growth and yeast, the ability to form biofilms, and the secretion of extracellular hydrolytic enzymes like lipases, phospholipases, or secreted aspartyl proteinases. Nevertheless, conflicting information exists about whether phospholipases are present in scientific isolates or not [23].

Further investigation into the presence of Candida, both in the milk of clinically healthy and ill goats, and their virulence factors is desired in order to ascertain their correlation with the amelioration of fungal mastitis in this animal species. The investigation also records the existence of unique species within the genus. Unique species of Candida are able to attach, colonies, and infiltrate host tissues by a variety of enzymatic activities, most often proteases, hemolysins, and phospholipases [24-26]. Furthermore, the development of biofilms facilitates Candida infection and resistance to antibiotics. [27,28]. The goal of this study molecular detection of virulence factor genes in *Candida parapsilosis* isolated from subclinical mastitis goats and antifungal susceptibility in Mosul Province.

Material and Methods

Ethical approve

This project has obtained ethical approval under licence code UM.VET.2023.051.

Location of study

This study has been carried out in the laboratory of the Department of Biology—College Education for Pure Science—University of Al-Hamdaniya, in Mosul Province, for the duration of November 2023 to January 2024.

Sample Collection

Fifty samples of goat milk with subclinical mastitis, with the aid of the California mastitis, have been collected from distinct areas of Mosul province during the period from November 2023 up to January 2024. Mastitis used to be identified with versions in udder and milk. The classic symptoms of clinical mastitis were sudden onset, changes in the composition and appearance of milk, decreased milk production, and the presence of the inflammation-causing signs and symptoms in contaminated mammary glands, which included pain, swelling, warmth, and abnormal milk appearance (**Figure 1**).

Previously collected milk samples, the udder, teat orifices, and milker's hands were thoroughly cleansed with tap water and a disinfecting cleaning

solution, then disinfected with 70% ethyl alcohol. After discarding the first streams of milk to prevent contamination, two to five ml of milk were collected in sterile tubes. Specimens were then labelled with serial numbers and kept cool in a container at 4°C during the journey to the laboratory.

Isolation and identification of *Candida parapsilosis*

Inoculating milk samples onto Sabouraud's Dextrose agar supplemented with 0.05 mg/ml chloramphenicol, the samples were incubated at 37°C for a duration of 24 hours, lasting up to one week. Following incubation, assessments of macroscopic and microscopic morphology were performed in order to classify the organisms at the genus level. This study utilized conventional PCR to identify Candida parapsilosis at the molecular level by amplifying a segment of the 18S rRNA gene using a specific primer [18,29].

Molecular Detection Primers

PCR primers were designed in this study using NCBI-Genbank database and primer 3 plus online (Metallo-aminopeptidase, alkaline phosphatase, alpha glucosidase and sterol esterase. Whereas the diagnostic primer is composed of a particular sense corresponding to the sequences, a partial gene of 18S rRNA is in accordance with a reference (30). These primers were provided by Macrogen Company from Korea, as shown in **Table (1).**

Fungal DNA Extraction

Employing the Fungi/Yeast Genomic DNA Extraction Mini Kit, as previously documented [31]. The sequence of steps used to isolate fungal genomic DNA from the isolates was as specified by the manufacturer.

PCR amplification

The PCR master mix reaction was previously coordinated using the Maxime PCR premix reagent i-Taq protocol. Once formulated as per the manufacturer's guidelines, the master mix comprised of three microliters of template DNA, one microliter each of forward and reverse primers (10 pmol), and twenty microliters of nuclease-free water. **Table 2** outlines the previous configuration of the PCR workstation for 30 cycles. Following one hour of agarose gel electrophoresis at 100V, the PCR products underwent visualisation and photography of the DNA bands using a gel documentation system.

Susceptibility for Some Antifungal Drugs

C. parapsilosis isolates were subjected to susceptibility testing by standard methods for the antifungal test used in this study [32, 33]. A standard reference procedure has been described by the National Committee for Clinical Laboratory Standards [34]. Antifungal susceptibility was determined by using five antifungal discs (Amphotericine B, Fluconazole, Ketoconazole, Nystatine, and Itraconazole) according to guidelines recommended by the [32], corresponding to the drugs considered routine testing and reporting on yeast.

Results

Out of 50 milk samples, 20 (or 40%) had Candida isolates obtained from goats with subclinical mastitis. Based on cultural, morphological, and molecular identification, the highest percentage was *C. parapasilosis* the 20/50 (40%). According to molecular techniques, regarding the results of PCR amplification of 18S rRNA gene for identification of *C. parapasilosis*, this gene were present 20 samples. Metallo-aminopeptidase gene was present in 20/20 (100%) and alkaline phosphatase sequence gene was present in 15/20 (75%). while alpha glucosidase was present in 10/20 (50%) and sterol esterase genes 8/20 (40%), were produced distinct bands corresponding to molecular size approximately 507 bp, 538 bp ,598 bp, 550 bp and 489 bp which exhibited 18S rRNA gene, Metallo-aminopeptidase, alkaline phosphatase, alpha glucosidase and sterol esterase genes, respectively. Show **Figures (2, 3, 4, 5, and 6).**

Antifungal Profile for *C. parapasilosis*

Antifungal profile for *C. parapasilosis* isolates from goat milk with mastitis according to antibiotic disc diffusion method. The sensitivity test was applied for all 20 isolates of *C. parapasilosis*. These isolates were tested for their sensitivity to itraconazole, fluconazole nystatine, ketoconazole and amphotericin B. The results were interpreted by measuring the inhibition zones around the disc and compared with break points of interpreted according to the manufacturer's instructions (DHN PAN Krakow, Poland). Zones diameters of ≥ 18 mm indicated the susceptibility (S), and that of ≤ 14 mm indicated the resistance (R) to each of nystatine, fluconazole, ketoconazole and itraconazole. The zones S were ≥ 16 mm and R ≥ 12 mm, for amphotericin B (33). These zones were translated in term of sensitive (S) and resistance (R), **Table (3).**

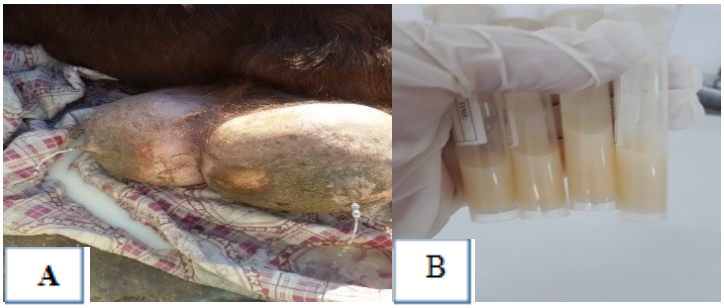

Fig. 1. Goats mastitis: A- Changes in the udder included swelling, warmth, B- Watery milk and clot

TABLE 1. PCR identification gene primers with their nucleotide sequence and product size for *Candida parapsilosis*.

Primers		Sequence(5'-3')	PCR product size bp
18S rRNA gene	F	CTGCGGAAGGATCATTACAGA	507
	R	TCCTCCGCTTATTGATATGCTT	
Metallo-aminopeptidase	F	GCAACCACCCAAATGGAACC	538
	R	GATGGGCCAATTCGTGCATC	
Alkaline phosphatase	F	GGGGCCACTGCATTTTCTTG	598
	R	CATTGTGGTGTGAAGCGTGG	
Alpha glucosidase	F	ATGCTACTCATGCCGATGGG	550
	R	GTATCAACGCCGGCCAATTC	
Sterol esterase	F	TGTGCCTCGAGAACCATACG	489
	R	CTCTGGAGTCCACCTTGCAG	

TABLE 2. PCR program setting for *Candida parapasilosis*.

Step		*C. parapasilosis*					
	18S rRNA gene	Metallo-aminopeptidase	Alkaline phosphatase	Alpha glucosidase	Sterol esterase	Time	
Initial denaturation	95°C	95°C	95°C	95°C	95°C	2 min	30 Cycle
Denaturation	95°C	95°C	95°C	95°C	95°C	30 sec	
Annealing	57.0	64.9°C	64.9°C	64.8°C	64.8°C	30 sec	
Extension	72°C	72°C	72°C	72°C	72°C	50-60 sec	
Final Extension	72°C	72°C	72°C	72°C	72°C	5 min	
Hold	4°C	4°C	4°C	4°C	4°C	10 min	

TABLE 3. Antifungal susceptibility and resistance profile for *Candida parapsilosis*

Type of antifungal	*Candida parapsilosis* (n=20)			
	S	%	R	%
Fluconazole	10	50	10	50
Nystatine	5	25	15	75
Amphotericine B	15	75	5	25
Ketoconazole	9	45	11	55
Itraconazole	10	50	10	50

R= Resistance, S= Sensitive

Discussion

According to the findings, most species of C. parapasilosis were the principal etiological agents of goat mastitis in Mosul province in 2024. The isolated colonies of Candida spp. were described as cream-colored, mucous, and smooth at 37 °C, which developed into white, wrinkle-creamy colonies after one-week incubation with yeast odor. All white creamy colonies were identified by molecular methodologies, which were utilized in accordance with the manufacturer's instructions. Out of the 50 milk samples taken from goats with subclinical mastitis, twenty (40%) Candida isolates were recovered. These isolates were identified based on cultural, morphological characteristics and molecular identification was carried out the highest percentage was *C. parapasilosis*. According to molecular techniques, concerning the results of PCR amplification of the 18S rRNA gene for identification of *C. parapasilosis*, this gene was present in 20/20 isolates. Metallo-aminopeptidase gene was present in 20/20 (100%), and alkaline phosphatase sequence gene was present in 15/20 (75%). while alpha-glucosidase was present in 10/20 (50%) and sterol esterase genes in 8/20 (40%). Yeast identification was done using traditional procedures, which are standard diagnostic methods in our laboratory. Despite their lower sensitivity and longer time requirements compared to molecular approaches, they are frequently regarded as the gold standard for Candida identification. Milk from both clinically healthy goats and other ruminants, with or without mastitis, is primarily composed of non-*C. albicans* species. [34]. Ruminant mammary glands are known to harbor a rich range of bacteria; however, less is known about their fungal microbiome. Studies have found many Candida species in dairy goats [34]. Candida species can attach, colonise, and penetrate host tissues by a number of enzymatic activities, most notably proteases, hemolysins, and phospholipases.

Moreover, biofilm development promotes Candida infection and resistance to antimicrobials [35, 36]. Our findings study *C. parapasilosis* accounted only (40%) these higher than study in Mexico (34) who percentage isolates *Candida parapsilosis* (0.45%). Also the present study higher isolates than study in Iraq [20] who isolates *C. parapasilosis* from goats milk 4 (21%) identified by used Vitek 2 identification method. Some forms of Candida, like other microbes, have adapted to become more pathogenic by using a variety of tactics. Abiotic surfaces of medical devices and prosthetics, as well as the host's mucosal epithelium, are major entry points for *C. parapsilosis*, which is responsible for its virulence. Biofilm production and, by extension, host harm, depend on this capability [37].

Candida species differ in their ability to colonies mucosal surfaces or inert materials [38]. Clinical isolates of *C. parapsilosis* show very high interspecies diversity in adhesion ability compared to other Candida species. Since C. parapsilosis mucocutaneous isolates are stickier, the site of isolation may affect adherence. Regarding molecular techniques in our study, concerning the results PCR amplification virulence gene of Metallo-aminopeptidase gene was present in 20/20 (100%) and alkaline phosphatase sequence gene was present in 15/20 (75%). while alpha glucosidase was present in 10/20 (50%) and sterol esterase genes 8/20 (40%), Different strains of yeast produce different virulence factors in vitro, and these factors may vary depending on the infected area's anatomy or the yeast's role in a disease. The findings of a recent study conducted in Mosul province revealed 75% susceptibility of *C. parapsilosis* to Amphotericine B and 50% fluconazole and traconazole. Antifungal susceptibility testing is a fantastic technique that can help forecast the failure of antifungal treatment, identify clinical response, and assist in the effective selection of antifungal medications. Susceptibility to antifungals result in this study agreement with study in Iraq [20] who isolated *C. parapsilosis* sensitive to each fluconazole as 3 (75%) and ketoconazole 4 (100%). It is crucial to test Candida species for antifungal susceptibility and track the evolution of resistant isolates in order to provide clinicians with information that will enable an appropriate treatment outcome [39]. Mastitis is associated with substantial decreases in both milk production and quality. The diagnosis of goat pathology is predominantly based on the evaluation of clinical signs and/or bacteriological analysis. Therefore, it is strongly advised to create novel analytical tools for the rapid and non-intrusive detection of mastitis in goats

Conclusion

The diagnosis of mastitis in goats follows a similar approach as that used for cows and

other animals. Subclinical mastitis in goats can be identified with somatic cell count monitoring. However, it is important to interpret the results carefully because of the higher occurrence of epithelial cell shedding and the presence of cytoplasmic aggregates in the milk. Nevertheless, identifying and treating mastitis in goats at an early stage offers the best opportunity for a favourable outcome when treatment is necessary. To effectively avoid mastitis, it is crucial to implement proper animal care practices, maintain cleanliness, employ effective milking procedures such as post-milking teat dipping, administer medication during the non-lactating period, and remove animals that are chronically infected.

Recommendation

Every breastfeeding goat should get a CMT monthly. That will allow for the earlier detection of goats with subclinical illnesses as well as the identification of goats that may be causing high cell counts.

Conflict of interest

The authors have no conflict interest to be announced.

Author Contributions

Writing original draft, Hawraa F.H. Al-Abedi, review and editing, Israa Ibrahim Khalil.

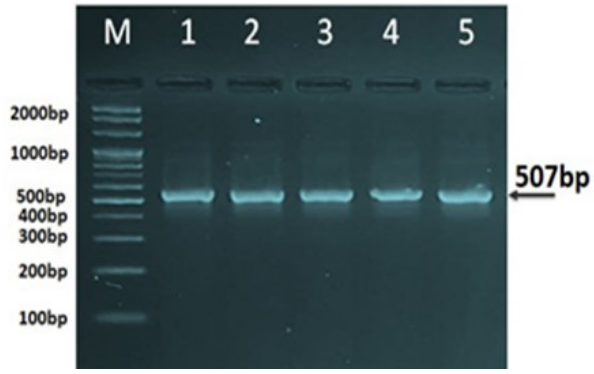

Fig. 2. the agarose electrophoresis product of the 18S rRNA gene by PCR. Lane NTC represents the negative control. Lane 1 represents the molecular dimension marker at 2000bp. Lanes 1-5 represent 18S rRNA genes with 507bp.

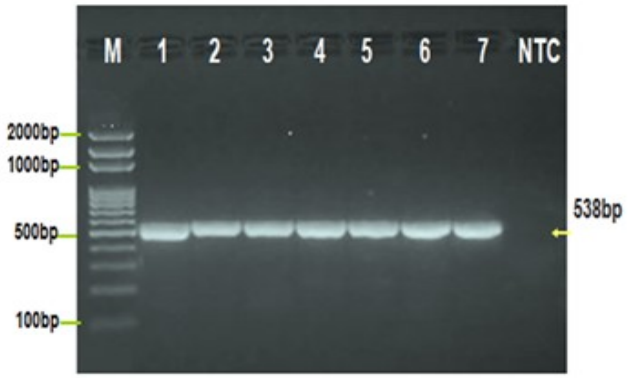

Fig. 3. Metallo-aminopeptidase gene PCR product. With respect to agarose electrophoresis. Lane 1: Molecular dimension marker (2000bp); Lanes 1-7: Negative control (lane NTC); and Lane 1: Metallo-aminopeptidase gene measuring 538bp.

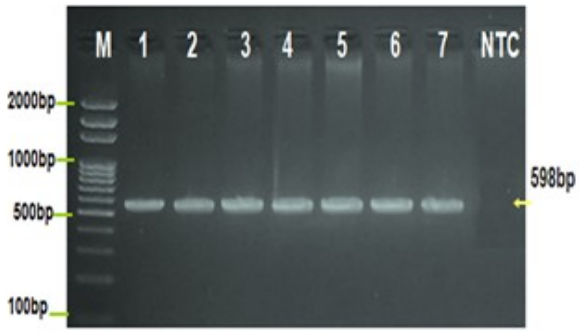

Fig. 4. Alkaline phosphatase gene PCR product. on the electrophoresis of agarose. Lanes 1–7 contain the alkaline phosphatase gene (598 bp); Lane NTC (negative control) is the molecular dimension marker (2000 bp).

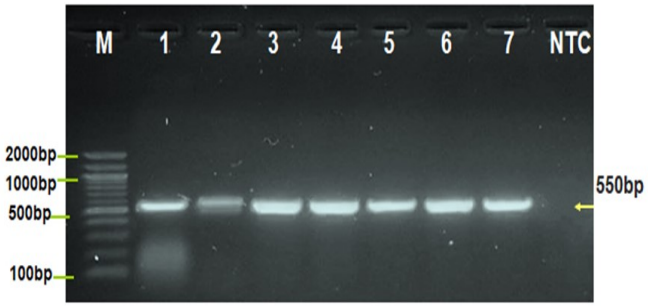

Fig. 5. PCR product of alpha glucosidase gene. On agarose electrophoresis. Lane 1: Molecular dimension marker (2000bp); Lanes 1-7: alpha-glucosidase gene with 550 bp; Lane NTC (negative control).

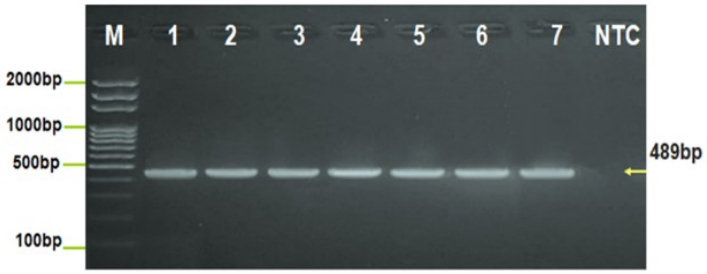

Fig. 6. PCR product of the sterol esterase gene. Using agarose electrophoresis. Lane 1: Molecular dimension marker (2000bp); Lanes 1–7: sterol esterase gene (489 bp); Lane NTC (negative control).

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الكشف الجزيئي لجينات عوامل الفوعة في داء المبيضات المعزولة من التهاب الضرع تحت السريري في الماعز والحساسية المضادة للفطريات في محافظة الموصل

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الملخص

هدفت هذه الدراسة إلى عزل وتشخيص داء المبيضات *Candida parapsilosis*. التي تسبب التهاب الضرع تحت السريري في الماعز باستخدام التقنيات الجزيئية لتحديد وكشف جينات الفوعة. تم جمع خمسين عينة من حليب الماعز المصاب بالتهاب الضرع تحت السريري الذي تم الكشف عنه باجراء اختبار كاليفورنيا لالتهاب الضرع من مناطق مختلفة من محافظة الموصل وفحصها باستخدام اختبارات التشخيص السريع وتأكيدها بواسطة مقايسة تفاعل البوليميراز المتسلسل (PCR). تم التعرف على داء المبيضات في 50/20 (40٪) من عينات حليب التهاب الضرع. بناءً على التقنيات الجزيئية، فيما يتعلق بنتائج تضخيم PCR لجين rRNA18 ، كان هذا الجين موجودًا في 20 عينة، وكان جين Metallo-aminopeptidase موجودًا في 20/20 (100٪) وكان جين تسلسل الفوسفاتيز القلوي موجودًا في 20/15 (75%). بينما كان ألفا جلوكوزيداز موجودًا في 20/10 (50٪) وجينات استيريز الستيرول 20/8 (40٪). أظهرت نتائج اختبار الحساسية للمضادات الفطرية أن الفلوكونازول والأمفوتريسين ب أعطوا أفضل حساسية ضد عزلات *C. parapasilosis*، يليها الكيتوكونازول والإيتراكونازول والنيستاتين.

الاستنتاجات: يحتوي حليب الماعز على عدة أنواع من جنس المبيضات، وكانت نسبة عزل عالية للنوع *C. parapasilosis* التي يمكن أن تلعب دوراً كمسببات أمراض انتهازية في التهاب الضرع.

الكلمات المفتاحية: داء الباراباسيلوس، التقنيات الجزيئية، التهاب الضرع تحت السريري، الماعز، الحساسية المضادة للفطريات.