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CHARACTERIZATION OF SOME VIRULENCE FACTORS OF *CANDIDA ALBICANS* ISOLATED FROM SUBCLINICAL BOVINE MASTITIS

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Abstract

Mastitis is one of the most economically overwhelming diseases of cattle, It is caused by multi-microbial agents, but in recent years, the fungal agents have been frequently reported as a causative agent of mastitis. The objective of this study was to determine the prevalence of subclinical mastitis in cows caused by *Candida* spp. and particularly *C. albicans* by using conventional and commercial methods, then detection of the main virulence factors of *C. albicans*. One hundred milk samples were collected from Baghdad governorate and the surrounded suburban farms of Abu-Ghraib during November 2018 to March 2019. All milk samples were undertaken to CMT and inoculated on Sabouraud's dextrose agar at 37 and 25ºC for 3-5 days. *Candida* spp. was detected and the *C. albicans* isolates were identified by conventional techniques like Germ tube production test, Chlamydospores production, Urease production and CHROM agar. Also the identification was done by Rapid system: API 20 and Vitek 2 compact system. Four types of the main virulence factors were carried out including Haemolysis, Proteinase, Phospholypase activities and Biofilm formation. The results showed that 84% of samples gave positive (+ve) results for CMT. Sixty three mycotic isolates were detected wherein 15 isolates of *Candida* spp. with (23.8%) and 5 isolates were *C. albicans* with (7.9%, 5/63) of mycotic isolates and (33.33%, 5/15) of *Candida* spp. The results of conventional and commercial methods were identical to the references for *C. albicans* identification. Haemolysis activity and Biofilm formation had been detected in all *C. albicans* isolates (100%), while Proteinase and Phospholypase detected in (40% and 80%) respectively. This indicate that these isolates of *C. albicans* had high ratio of virulence factors which associated with pathogenicity and severity of infection. So, Further studies are required to study another types of virulence factors for *C. albicans* and other types of *Candida* spp. that isolated from bovine mastitis.

Keywords **:** Mastitis, *C. albicans*, virulence factors, conventional methods, Rapid system

Introduction

Mastitis is one of the most common pathologies in dairy cattle due to multi-etiological complex of disease as outcome of interaction of many associated factors such as the host, causative agents and environment (Kibebew 2017; Hussein *et al*., 2018). In spite of the prevalence of mycotic mastitis is low as compared with other mastitic agents, but during the last decade, it has significantly increased and frequently reported as the causative agent of mycotic mastitis in ruminants (Yanuartono *et al*., 2019).

The most repeated yeast species in mastitic cows are *Candida* spp., *Trichosporon* spp., *Cryptococcus* spp. and *Rhodotorula* spp. (Dubie *et al*., 2015) and this problem become increasing in animals due to the wide use of antibiotics in mastitis therapy (Bekele *et al*., 2019) which effect on the antagonist bacterial flora then stimulate yeasts to multiply and colonize the udder and this may be accompanied by reduction in vitamin A that have positive impact on fungi growth (Kalińska *et al*., 2017).

Candida species are the most frequent yeasts among mycotic bovine mastitis (Du *et al.,* 2018) and the *C. albicans* is the predominant *Candida* specie in the examined milk samples worldwide (Mousa *et al*., 2016). This type of *Candida* was isolated from subclinical and healthy milk as well (Pachauri *et al*., 2013) and this may be constituted zoonoses hazard through contamination of milk with these yeasts and possibility transmission to consumers when consumed raw or even processed form (Hasan and Yassein 2018).

Although *C. albicans* considered as normal flora in human (Abharian *et al*., 2018), but it represents opportunistic fungi and can result in health threatening in case of systemic disseminated candidiasis particularly in immunocompromised host due to possess some important virulence factors as mentioned by Dantas *et al*. (2015).

C. albicans has different virulence factors that enhanced it to adhere then invade the tissues resulting in infections. Some of these factors are pseudohyphae formation (Germ tube) for adherence, phenotyping switching, proteinase and phospholipase production for invasion and biofilm formation. These virulence factors associated with pathogenicity of *C. albicans* (Udayalaxmi *et al*., 2014).

There are rare or no reports showed the main virulence factors of *C. albicans* isolated from bovine subclinical mastitis in Baghdad, therefore, the present study aimed to estimate *C. albicans* prevalence in mastitic dairy cows and focused on determination of some virulence factors of *Candida* isolates that diagnosed by using conventional and commercial routs.

Materials and Methods

Collection of milk samples

One hundred milk samples were collected from four quarters of cows that apparently healthy animals from different areas in Baghdad governorate and the surrounded suburban farms of Abu-Ghraib during November 2018 to March 2019. The process of collection included cleaning the teat end with 70% ethyl alcohol and discard of the first 3 steams of milk, Ten ml were collected in sterile test tubes under aseptic condition then transported to the laboratory on ice immediately according to Coles (1986).

Detection of Subclinical mastitis by using CMT reagent

All milk samples were undertaken to CMT test by taken 2 ml of each sample and mixed with equal volume of

CMT reagent in plastic paddle and shaking gently in horizontal plane. The reaction was scored within 10-15 sec. of mixing According to Coles (1986).

Isolation of *C. albicans*

Each milk samples were inoculated on duplicate plates of Sabouraud's dextrose agar containing 0.05 mg/ml of chlorumphenicol at 37 and 25ºC for 3-5 days. Subculturing was performed to reach to the pure colonies then examined macroscopically and microscopically according to Washinton *et al.* (2006).

Conventional technique for identification of *C. albicans*

This technique included Germ tube production test, Chlamydospores production by using Corn meal agar, Urease production and Identification by CHROM agar according to (Ellis 1994; Ghelardi *et al*., 2008; Deorukhkar *et al*., 2012; Deorukhkar and Saini 2014 and Neppelenbroek *et al*., 2014).

Identification of *C. albicans* by Rapid system: API 20 and Vitek 2 compact system

The system of API 20 *Candida* includes panels and reagents. Each panel has cavities containing multi-reaction molded into the periphery of plastic tray with dehydrated reactants. This test was performed according to the instructions of the manufacturer company.

The Vitek 2 diagnostic method is depended upon detection of biochemical methods and newly developed substrates to confirm identification of the significant *Candida* spp. following protocols described by manufacturer. This system consists of 64 biochemical tests and the degree of accuracy may reach to the 98 % for yeast detection.

Detection of Virulence Factors of *C. albicans*

C. albicans has many virulence factors, but the present study included detection for some of them which they were:

1. **Heamolytic activity :** The isolates of *Candida* were cultured on the SDA and incubated at 37°C overnight. Yeast suspension was prepared equal to one McFarland turbidity then 10 µl was taken and spotted on SDA containing 7% human blood and 3% glucose. Each plates were incubated at 37°C for 48 hr. Any isolate reveal a clear zone of haemolysis around the colonies were considered as positive for haemolytic activity (Favero *et al*., 2011).
2. **Phospholipase activity :** Yeast suspension was prepared equal to one McFarland turbidity (Approximately 10^8 yeast cell/ ml) then 10 µl was inoculated into wells on the surface of Egg Yolk agar and incubated at 37°C for 48 hr. The diameter of the colonies and the diameter of the zone of opacity were measured (mm) according to (Mahmoudabadi *et al.,* 2010).

   Phospholipase activity PZ= Diameter of colonies (mm)/Diameter of zone of opacity + colonies
3. **Protienase activity :** Ten microliter of yeast suspension was injected into the wells punched on the surface of Bovine Serum Albumin medium incubated at 37°C for 48 hr. The proteinase activity (Pz) was determined in terms of the ratio of the diameter of well to the diameter of the proteolytic zone and when Pz=1 mean no proteinase activity was detected while low Pz means high production of the enzyme according to (Tsang *et al.,* 2007).
4. **Biofilm formation :** A loop full of yeast was cultured into tube containing 2ml of Brain Heart Infusion Broth (BHIB) medium with 0.25%glucose and incubated at 37°C for 24 hr. then all tubes were diluted at ratio of 1:20 by using fresh prepared BHIB, then 200 µl was placed into the sterile 96-well polystyrene microtiter plates which after that covered with lids and incubated at 37°C for 24 hr. This plate was rinsed twice with PBS then inverted to blot. One percent of crystal violet (200 µl) was added to each well with incubation for 15 min. The rinsing was repeated 3 times with PBS then 200 µl of ethanol : acetone mixture (80:20v/v) was added to each well. The OD was recorded by using ELISA reader at 450 nm for each well. This method was described by Melek *et al*. (2012) and the average of OD value was calculated according to Rodrigues *et al.* (2010).

Results and Discussion

California Mastitis Test

The current study showed that from 100 milk samples, 84 samples gave positive (+ve) results for CMT in percent 84% and 16 (16%) samples demonstrated negative (-ve) results for CMT (Table 1).

The result of this study may discuss the badness of management and poor herd udder health due to shedding of pathogens which make this form more prevalent than clinical form by consideration as source of infection to other animals (Yassein and Khalaf, 2014).

Percentage of Mycotic mastitis:

Sixty three mycotic isolates were detected in the present study wherein 15 isolates returned to *Candida* spp. with (23.8%) and 5 isolates were *C. albicans* which constituted (7.9%, 5/63) of mycotic isolates with (33.33%, 5/15) of *Candida* spp. as shown in table (2).

According to the present study, the percentage of yeasts including subclinical mastitis increased in comparison to studies carried out by Sartori *et al*. (2014), Eldesoukey *et al*. (2016) and Kalinska *et al*. (2017). This investigation may be return to the season of the samples collection especially during winter and spring when the yeast isolation is abundant, this opinion is in line with Akdouche *et al*. (2014). In addition, there were previous studied showed the *Candida* spp. are the most common pathogens responsible for mycotic bovine mastitis causing impacts ranging from reduction in milk production to disagalactia as reported by (Sukumar and James 2012; Stanojevic and Krnjajic 2018).

Identification of *C. albicans* by conventional methods

After isolation of *C. albicans* from 5 milk samples of subclinical bovine mastitis and studying the features of this yeast macroscopically which represented by production creamy, smooth, pasty and convex colonies for 2-3 days post inoculation on Sabouraud dextrose agar at 37˚C that may become wrinkled with long incubation period. While microscopical examination revealed presence of pseudohyphae with cluster of budding cells by using Lactophenol Cotton Blue stain and when these isolates stained with Gram stain, they showed Gram positive large yeast cells, these morphological features may indicate that these isolates were *C. albicans* . Then to confirm the results,

all these isolates were cultured on selective media such as Corn Meal Agar that also known as Dalmau plate method to enhancement of blastoconidia and chlamydospore formation.

Germ tube test which is called Reynolds- Braude phenomenon gave positive result for these isolates that may be considered as the main test for identification of *C. albicans* and in most laboratories when the result of this exam is positive, there are no need to further tests to identify *C. albicans.*

There was another selective and deferential media that used for detection *Candida* spp. which it is CHROMagar *Candida* system that depending on color alteration of the colonies of these yeasts. In this study, the *C. albicans* produced leaf-green colored colonies as in figure (1).

On the other hand, there was no changing in the color of Urease test when inoculated with *C. albicans* isolates and the test remained in yellow color (Table 3).

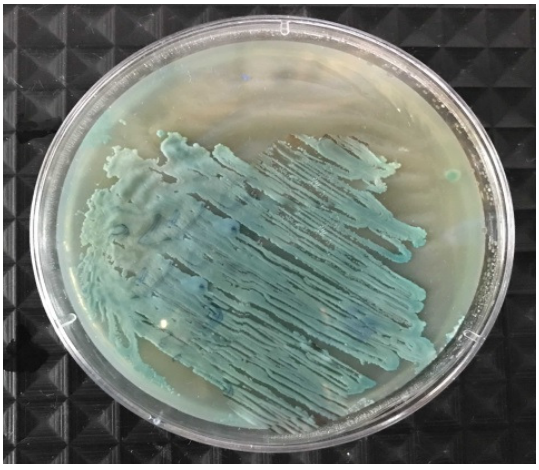

Fig. 1 : Morphology of *C. albicans* on CHROMagar shows leaf-green colored colonies

Many studies that carried out in College of Veterinary Medicine in Baghdad aimed to isolate bacterial as causative agents of mastitis and could be isolated *Candida* spp. by the way, but there are no specific research dealing with isolation of *Candida albicans* from subclinical mastitis in cows with investigation the most virulence factors of this yeast, therefore, In the current study, the *C. albicans* that constituted 33.33% of *Candida* spp. were identified by conventional methods. All the results of conventional methods were identical with the findings of Deorukhkar and Saini (2014) that performed to identify *Candida* spp. although these methods are exhausting of time, less accuracy and misdiagnosis may be occurred.

Result of Rapid commercial Kit based system

All five isolates that identified as positive with the biochemical tests were subjected to API 20 and Vitek 2 system to confirm the diagnosis of these tests. The results of API 20 *Candida* were recorded and compared with standard of the manufacturer as shown in figure (2).

Fig. 2: API 20 System results for *C. albicans*

Final identification for the isolates was performed with Vitek 2 compact system, the results were compared to the database of reaction for each taxon, and a numerical probability calculation was done depending on 64 biochemical tests (BioMerieux, USA). The five isolates of *C. albicans* were identified as positive isolates with the probability of the positive results of vitek 2 system were between 93- 97%.

These results demonstrated coincidence with routine methods as focused by Posteraro *et al*. (2015). Karbicak *et al*. (2016) evaluated of common commercial systems for identification of yeast isolates in microbiology laboratories. Moreover, Deorukhkar and Roushani (2018) mentioned the accurate identification of infected strain of *Candida* is essential for selection of appropriate prophylactic and therapeutic antifungal drugs, Although commercial systems are rapid and high cost, conventional techniques remain the of species identification of *Candida* isolates in most clinical microbiology laboratories.

Results of Virulence factors

The present study showed that all *C. albicans* isolates had haemolytic activity with (100%) when grown on SDA with blood. While the Phospholipase activity was determined in 40% (2/5) of *C. albicans* isolates as shown in table (4). This study got mean of Pz value was 0.59 among the isolates that produced phospholipase. In concerning of proteinase activity, the results revealed that 80% (4/5) of isolates had activity for protein hydrolysis and the mean of Pz value was 0.58 as shown in table (5). Additionaly, The biofilm was formed in all isolates of *C. albicans* with (100%) but classified into weak, moderate and strong (Table 6, Figure 3).

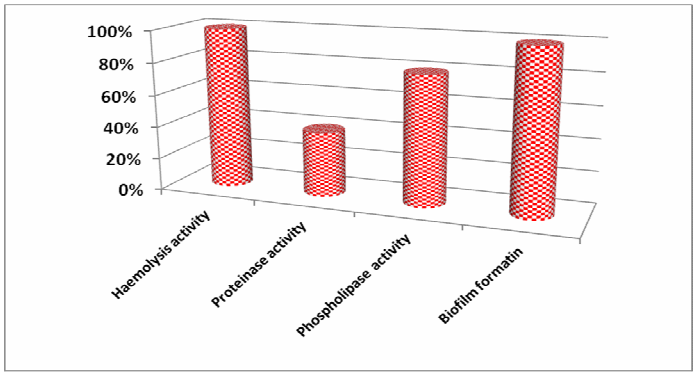

Fig. 3: Illustrate the comparison between virulence factors among isolated *C. albicans*.

This study included four main types of virulence factors that retuned to *C. albicans* and suggested that the ability of this yeast to produce these factors may be a reflection of the pathogenic potential of the isolates.

The severity of infection is correlated with the virulence factors of pathogen which responsible for site and stage of infection, type of infection and the nature of the host response. Once the contact is occurred, enzymes enhance the adherence by damaging cell membranes and releasing the extracellular proteins, then permitting the *C. albicans* to enter the host causing infection as explained by Lahkar *et al*. (2017).

The investigations of the present study showed that all *C. albicans* isolates produced beta haemolysis on SDA containing blood human with (100%). This may indicate that these isolates need to source of iron which obtained it from haemoglobin for growth. This fact was pointed by Favero *et*

al. (2011). The percent of this study was near to the results of Udayalaxmi *et al*. (2014) when found that *C. albicans* isolated from genitourinary tract produced haemolysis activity with 97.5%.

Also, Eighty percent of *C. albicans* isolates had proteinase activity. This high ratio responsible for induction of damage in the surface proteins like albumin and keratin then degrades the protective IgA and facilitates the tissue invasion. Other study was performed by Lahkar *et al.* (2017) recorded similar percent (81.1%) of *C. albicans* isolated from oropharyngeal samples in HIV- infected patients.

Whereas the *C. albicans* isolates demonstrated (40%) phospholipase enzymes activity which correlated with the cell membrane damage, adhesion and penetration which constitute the major mechanism sharing to fungal virulence as mentioned by Mohandas and Ballal (2011)

Many researches were performed to detect the capacity of *Candida* spp. for biofilm production in cases of chronic periodontitis (Machado *et al*., 2011), from urine and vaginal samples (Udayalaxmi *et al*., 2014), from HIV- infected patients (Lahkar *et al*., 2017) and from Peritoneal, Pleural and cerebral spinal fluids (Schin *et al*., 2014). All these studies were performed on samples of human patients, while in case of mastitic animals there are rare or even no information of the capacity of *Candida* spp. producer biofilm as the one main of virulence factor to study the pathogenicity of *Candida* spp. in udder.

Biofilms are represented microbial communities which are attached and elevated in a matrix of exopolymeric materials and considered as pointer for development of the infection. The current study showed all isolates of *C. albicans* had the ability to produce Biofilm (100%) which play a pivotal role in pathogenicity of candidiasis through evading host immune system, resisting antifungal therapy and withstanding the competitive pressure from other pathogens as mentioned by Lahkar *et al*. (2017).

Conclusion

The present research found that the mastitis remains warranting serious attention for its control and preventions due to considered as most economically overwhelming disease, and the *C. albicans* that isolated from subclinical bovine mastitis constituted 33.33% of all *Candida* spp. isolates and revealed high ratio of virulence factors that associated with the pathogenicity and severity of infection, So, Further studies are required to study another types of virulence factors for *C. albicans* and other types of *Candida* spp. that isolated from bovine mastitis.

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