STUDY ON *Candida* SPECIES ISOLATED FROM COWS WITH MOLECULAR DETECTION OF SOME VIRULENCE GENE

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ABSTRACT

A total of 400 samples (200 blood samples and 200 vaginal swabs) were collected from cows throughout this study in Al-Basrah province . Clinical specimens should be inoculated on to primary isolation media, like Sabouraud dextrose agar (SDA) ,then the study showed that 90 out of 200 vaginal swabs (45%) were positive for the presence of *Candida* isolates while 100% of the blood samples were negative for *Candida*. All the obtained yeast isolates were subjected to identification by using germ tube test and chlamydo spore,the results showed that 72.22% and 38.88 of yeast were able to produce germ tube and chlamydo spores respectively ,. To differentiate between *Candida* species, CHROMAgar™ Candida Agar was used. The results showed that 38.88 % of isolates were identified as *C.albicans*, 28.88 % were *C.tropicallis*, 24.44% were *C.krusei* and 7.77% were identified as others species. Biochemical tests were done using API 20 AUX strips. By this test the results confirmed that 80% of isolates were *C.albicans*, 8 % were *C. tropicallis* , 4 % were *C.glabrata* , 4% were *C.parpsilosis* and 4 % were *C.famata*. Phenotyping tests showed that 85 % of *C. albicans*could grew on SDA agar with 6.5 % NaCl, and all strains (100 %) were grown at 45˚ C , while 75 % were grown on SDA medium with cycloheximide .By using disc diffusion method for 7 antifungal Agents against 20 *C.albicans* isolates. They were Fluconazole, Clotrimazole, Miconazole and Ketoconazole showed the highest sensitivity to *C.albicans* with a percent of (90%) ,(80%), (80%) and (70%) ,revealed lower susceptibility. Further molecular identification using multiplex polymerase chain reaction (PCR) was done for detection of some virulence genes in the previously identify *C.albicans.*The result showed that 30 % of the isolates having *INT1* gene.

INTRODUCTION

*Candida albicans*is a dimorphic fungus that exists as a commensal of warm-blooded animals . It colonizes mucosal surfaces of the oral and vaginal cavities and the digestive tract and is also able to cause a variety of infections, depending on the nature of the underlying host defect. For several reasons (immunosuppressive treatments, long-term

catheterization, use of broad-spectrum antibiotics and longer survival of immunologically compromised individuals) caused *candida* infection (1).
There are several species of *Candida* including, *C. albicans*, *C. tropicalis*, *C. pseudotropicalis*, *C. krusi*with the *C. albicans*is the most important one responsible for infectious diseases in animals ,such as arthritis in horses and mastitis and abortion in cattle (2).

Vulvovaginal candidiasis (VVC), often referred to as a yeast infection, is a common gynecologic disease, affecting in their life times (3). Vulvovaginal candidiasis is caused by overabundant growth of yeast, belonging to the *Candida* species in the vaginal mucosa (4). *C. albicans*has to cross physical barriers such as epithelial cell layers by active penetration and/or induced endocytosis (5). During the different stages of a *C.albicans*infection and within different host tissues environments, the fungus has to express general as well as stage- and tissue-specific virulence or fitness factors (6,7).The first step for successful colonisation of mucosal surfaces or any other tissue by *C. albicans*is adhesion. Some factors involved in adhesion also have additional roles in tissue invasion by *C. albicans*is the ability to switch between ovoid yeast and filamentous hyphal growth forms. It has been proposed that yeast cells are more suitable for dissemination where as hyphal forms play a key role during invasion (8). *INT1 gene* has been identified as a putative integrin gene in *C. albicans*(9). Genes from the integrin family of mammalian cells are required for binding to the extracellular matrix ligands(9) . The present research aims to study the presence of different species of yeast in cows vaginal and exploring the presence of some virulence gene by multiplex PCR in *C. albicans*and to evaluate the Factors affecting the growth of yeast in order to determine the phenotypic characteristics

MATERIALS AND METHODS

A total of 400 samples were collected from cows (200 blood samples and 200 vaginal swabs) in Al –Basrah province.The vaginal swabs were taken from female cow by sterile cotton swabs with transport media(brain heart infusion broth media) from vagina, and then transferred to the laboratory for culturing and identification . The swabs were directly inoculated onto SDA plates supplied with chlormphenicol, and duplicated culture of each sample were aerobically incubated at 37°C for 48 hours (10).

The blood samples have been collected from the same 200 female cows .About 5 ml. of venous blood samples from cows were directly collected by sterile tube with anticoagulant ,and transmitted to the laboratory for culturing . The blood samples were centrifuged at 3000 rpm for 15 minutes.These concentrates were inoculated into screw-Capped tubes containing brain heart infusion broth and incubated at 37°C for 1-2 day,

then a 0.5 ml of the broth was inoculated onto the surface of SDA with chlormphenicol. The inocula were evenly spread with sterile L-shape spreader and incubated at 30°Cand 37°C for 2 weeks (10,11). . All inoculated plates were incubated at 37°C under aerobic condition and examined after 24 and 48 h. Plates that did not show any growth after 48 h. were re-incubated for one week(12).

Yeast Identification

Candida species and other yeasts colonies were diagnosed depending on cultural characteristics on SDA that include shape ,color and size to determine the morphology of the yeast cells. The following tests were used for the identification of the isolated yeast, Germ tube test (13);Dalmau plate technique (14);Growth on CHROMagar™*candida*(15)and API 20C AUX system(BioMerieux-France) were also performed according to the manufacturer's directions.
Other tests were done on *Candida* isolates to determine its ability for growth on different conditions like, growth on SDA with cycloheximide (14),growth at 45° C (16) and growth on SDA with 6.5% NaCl (17).

Antifungal susceptibility test:

Twenty isolates of *Candida species* were tested for antifungal susceptibility by sub-culturing on SDA and incubated at 37°C for 24h. A suspension of overnight cultures of *C. albicans* were prepared in sterile distal water.Turbidity was adjusted to 0.5 McFarland standard density resulting in an inoculum containing 1.5×10^6 CFU/ml. About 25µl of suspension inoculated on SDA plates and rolled on the surface of the agar medium. Plates were dried for 15min at room temperature in laminar hood and then antifungal disks were placed on the inoculated agar with a forceps. The plates were incubated at 37°C for 24h, then the zone of inhibition diameters were measured then the antimicrobial breakpoints and interpretation were done according to Salehi et al. (18).

Molecular study:

Extraction of DNA from *Candida albicans*:

The genomic DNA from *C.albicans* was extracted according to EZ-1O Column Yeast Genomic DNA Yeast Purification Kit (Bio Basic INC.-Canada)The extracted genomic DNA was subjected to electrophoresis in(1%) agarose gels containing ethidum bromide .

PCR amplification:

For the detection of the virulence gene *INT1* by multiplex PCR ,the specific primer were designed according to Bunce et al(19).The PCR amplification was done in PCR laboratories in College of Veterinary Medicine, Research central unit. The mixture and conditions of the multiplex PCR reaction INT1 gene were applied according to Gale et al (20).The PCR of amplification mixture were transferred to preheated thermocycler and the program of amplification was started . The results of PCR were detected after the amplification process. *INT1* has been identified as a putative integrin gene in *C. albicans*(9). Genes from the integrin family of mammalian cells are required for binding to the extracellular matrix ligands,INT1 gene have sequence(Forward)-A A G T A T TT G GG A G A A G GG A AA G GG, (Reverse) – AAAATGGGCATTAAGGAAAAGAGC,and 310 bp.

RESULTS AND DISCUSSION

Primary isolation of Candida species from naturally infected cows displayed that 90 out of 200 45% vaginal swabs were positive culture, while non of the blood samples were positive for *Candida* as shown in table (١). Similar results were obtained by other workers (21,22).On the other hand ,the results of vaginal swabs were positive culture the present study were disagreed with that of.(23).These variations may be either due to how we take sample collections or geographical situations.

Results of the present study indicated that the overall identification of *Candida* species isolates from blood samples were 0/200 (0%). This result was is nearly agreed with (23) . They isolated *Candida* species from blood samples in cow in Egypt with incidence of 00.06 % .*Candida spp*. are rarely seen in peripheral blood smear. Timely diagnosis of candidemia has proven to be difficult as blood culture often require 2 to 3 days of incubation (24).

Germ tube and chlamydospore formation are the characteristic feature of *C.albicans* isolates table (1). While other *Candida* species and yeasts do not able to form it.The result of the present study showed that 72.22% isolates were able to produce germ-tubes. The germ tube production test have the advantage to be simple and efficient in the economical and fast identification of *C.albicans*(25).

Instead of germ tube test, chlamydo spore is another identification character for identifying of *C. albicans* rather than other species. Chlamydo spores are produced on cornmeal agar at 22 to 25℃ within 48 to 72 hr. They were spherical, thick-wall, and usually produced on suppurating cells that occur along pseudohyphae or at the tip of hyphae. It is known that *C. albicans* tends to form a single chlamydo spore on the tip of

an elongated suspensor cell (26). The result of current study showed that 38.88 % of *C.albicans* were able to form chlamydo spore on corn meal agar with tween 80.

Based on the colony color and morphology (rough/smooth) that developed on CHROMAgar™ Candida agar , 35 isolates of vaginal swabs samples appeared with green color were identified as *C. albicans* , 26 isolates of vaginal swabs samples appeared with blue –darkblue color were identified as *C. tropicalis*, while, the dry, irregular, pink colonies were identified as *C. krusei*(22 isolates). In contrast, the colonies which appeared with beige, yellow or brown (7 isolates) were identified as *C. glabrata*, *C. kefyr*, and *C. parapsilosis* ,as shown in table(2).Further assays were performed to complete the identification at the species level

The isolates were subjected to API 20 C AUX system for conformation the species identification as shown in table (3) .According to these standard procedures of isolation and identification ,the present study revealed that the overall identification of *C.albicans* were 20/25 (80%), the biochemical identification of isolated *Candida albicans*. was performed in order to confirm the species of isolated organism. There was a series of biochemical tests in API *Candida* test strip . This API *Candida* system is specific designed for *Candidasp*. isolated in clinical microbiology laboratories(27) .Thus ,the physiological or biochemical identification of *Candida sp*. Can be determined easily and rapidly via this API *Candida* system (28).

The result of the present study showed that 20 out of 25 (80%) isolates were confirmed as *C.albicans.* This result was in agreement with that of Liguor et al. (29) and Mohammed (30). They used API system and confirmed that (87.3%) and (63.6 %)were *C.albicans*, respectively.

Among 20 of API20C AUX *Candida albicans* positive isolates , 17 isolates showed the ability to grow on SDA with 6.5 % NaCl, while 3 isolates failed to grow on this medium. As well as, without all the 20 isolates revealed the ability to grow at 45˚C, but 15 isolates were able to grow on SDA with cycloheximide.

Growth at 45°C was initially considered to be a useful test for differentiation between *C. dubliniensis* (not grow) from *C. albicans*(grow) in the original description of this species (31).

Considering the growth on SDA with 6.5% NaCl, it was observed that 17 out of 20(85 %) isolates grow on this medium (table 4).This result was in agreement with that of Najem(32) who recorded (81.25%) of *C.albicans* isolates were able to tolerate this concentration of NaCl.

By using disc diffusion agar method , 20 isolates of *Candida albicans* from cows were tested for their antifungal susceptibility against 7 antifungals. *Candida albicans* showed high sensitivity to Fluconazole ,clotrimazole and ketoconazole with a percentage of (85%) ,(85%) and (70%),respectively. Whereas, the sensitivity of *C.albicans* for miconazole ,amphotericin B, iltraconazole and ketoconazole with a percentage of (65%) ,(10%) , (0%)and (70%) ,respectively (Table5). This result was in agreement with that of Seifi et al.(33) who reported that (66.7%) and (85.7%) of *C.albicans* were susceptible to clotrimazole and miconazole ,and with that of Mohammed (34) who showed that (66.6%) of *C.albicans* were susceptible to fluconazole. Salehi*et al*., (18) who reported that (77.35%)and (24.52%) of *C.albicans* were susceptible to clotrimazole and ketoconazole ,whereas (85.1%) of *C.albicans* were resistant to fluconazole.On the other hand ,the result of the antifungal susceptibility of the present study was in disagreement with that of Chaudhary*et al.*(35) who found that (8%), (40 %) and (23%)of *C.albicans* were susceptible to fluconazole ,clotrimazole and iltraconazole, respectively.
Out of ten isolate of *Candida albicans*,only three (30%) isolates were positive for *INT1 gene* (table 6, figure 1). Early diagnosis of invasive fungal infections such as candidiasis is necessary to help clinicians in better treatment decisions and increase the patients' chance of survival. The ability of molecular biology methods to detect fungal pathogens is far superior to that of traditional phenotyping methods (36,37).*C.albicans INT1gene*was originally cloned by virtue of its limited homology to vertebrate leukocyte integrins, adhesion proteins that bind the extracellular matrix and induce morphologic changes in response to extracellular signals (38). In *C.albicans,INT1* is a virulence factor that contributes to the ability of the pathogen to adhere to epithelial cells (39).

The existence of integrin-like protein in *C. albicans* has been reported recently and the gene α*INT1* was cloned and its DNA sequence was reported (9,20). It was clarified that *INT1 gene* was unique to *C. albicans*. The species-specific DNA sequences within the *INT1* gene are attractive targets for PCR-based detection methods. Primers complementary to species-specific DNA sequences would be suitable for the detection of *C. albicans*(40).

Table(1) : Percentage of positive growth of different samples from cows

Sample	Total Sample No.	Positive yeast culture(%)	Germ tubetest(%)	Chlamydospore Test (%)
Blood sample	200	0(0.0)	0(0.0)	0(0.0)
Vaginal swab	200	90(45)	65(72.22)	35 (38.88)

Table (2) : Number and percentage of *Candida species* isolates onCHROMAgar™ *Candida* Agar media

Type of *Candida*	*C.albicans*	*C.tropicals*	*C.krusei*	*Others*	Total
No.	35	26	22	7	90
Percentage	38.88	28.88	24.44	7.77	100

Table (3) : Number and percentage of *Candida species* isolates identified by API 20 C AUX (25 isolates) .

Species	No. of isolates	%
C.albican	2o	80
C. tropicalis	2	8
C. glabrata	1	4
C.parpsilosis	1	4
C. famata	1	4

Table (4) : Phenotypic differentiation between *C.albicans* (20 isolates)

Test / Result	SDA with 6.5 % Nacl	Growth at 45˚C	Growth with cycloheximide
Positive	17 (85 %)	20 (100 %)	15 (75 %)
Negative	3 (15 %)	0 (0 %)	5 (25 %)

Table (5) :Susceptibility of *Candida albican* isolates against seven antifungal drugs

Antifungal	Susceptibility	Dose dependent	Resistance
Fluconazole	17 (85%)	0 (0 %)	3 (15 %)
Clotrimazole	17 (85 %)	3 (15 %)	0 (0 %)
Miconazole	13 (65 %)	7 (35 %)	0 (0 %)
Ketonazole	14 (70 %)	3 (15 %)	3 (15 %)
Econazole	2 (10 %)	4 (20 %)	14 (70 %)
Amphotericin B	2 (10 %)	0 (0 %)	18 (90 %)
Iltrconazole	0 (0 %)	2 (10 %)	18 (90 %)

Table (6) : Number and percentage of positive isolates of *Candida albicans* for *INT1 gene* in cows

Type of gene	Total No. of Samples	Number of positive samples	% of total positive
INT1 gene	10	3	30 %

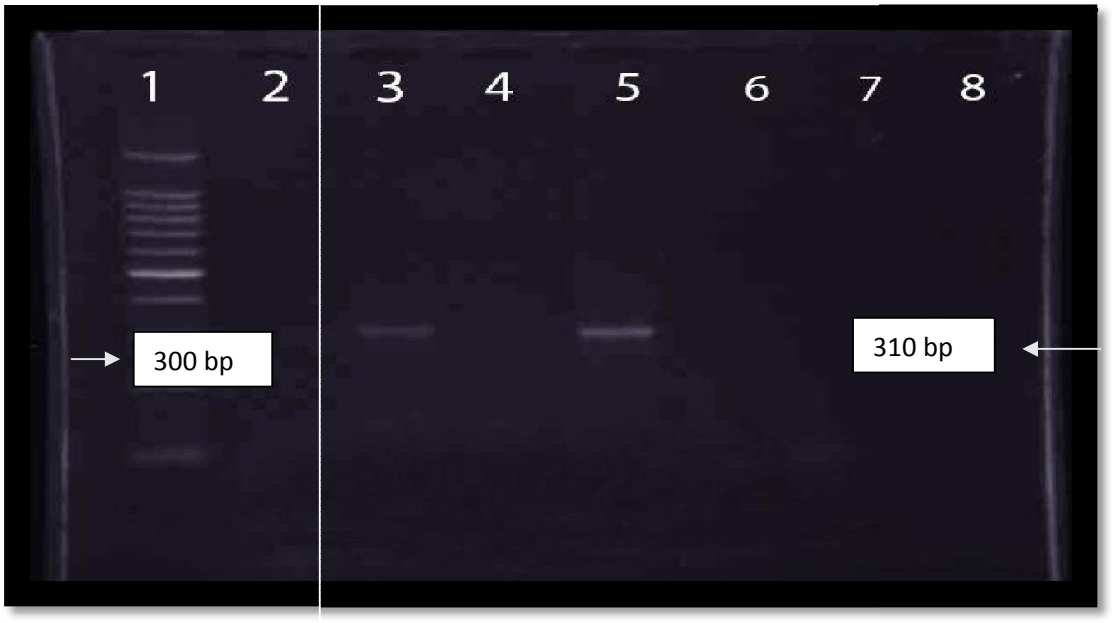

Figure 1: Multiplex PCR amplification products showing presence of *INT1* gene in *C.albicans* isolates. Lane 1= DNA marker; Lane 3,5,8, *INT1 gene* positive; 2,4,6,7, *INT1 gene* negative.

دراسة لخمائر المبيضات المعزولة من الابقار مع التحري الجزيئي لبعض جينات الضراوة فيها

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الخلاصة

في هذه الدراسة ، جمعت ٤٠٠ عينة من الأبقار (والتي تضمنت ٢٠٠ عينة من دم الأبقار ، ٢٠٠ مسحة من مهبل الأبقار) في محافظة البصرة .وتم زراعة العينات باستخدام الوسط الغذائي SDA . وبينت الدراسة ان من بين ٢٠٠مسحة ظهرت ٩٠ منها عينات موجبة بنسبة (٤٥%)لعزل الخمائر بينما كانت نتيجة العزل سالبه ١٠٠%بالنسبة لعينات الدم .اظهرت العزلات ايجابية لاختبار ال germ tubeبنسبة72.22 % وقدرته على تكوين الابواغ الكلاميديةchlamydospores كانت38.88 % . ايضا تم التفريق بين الانواع بالاعتماد على استخدام وسط chromagar candida للتفريق بين انواع ال *Candida spp*.وكانت النتيجة 38.88 % من ال *Candida albicans* و 28.88 من ال *C.tropicalis*و 24.44% من *C. krusei*و٧.٧ % من الانواع الاخرى . وفي الاختبارات المظهرية(Phenotypin) والذي تم اجرائه على عينات *C.albicans* .اظهرت النتائج انه ٨٥ % من *C.albicans* اظهرت قدرتها في النمو على وسط SDA مع اضافة ٦.٥ من NaCl وكانت كل العينات قادرة على النمو في درجة حرارة ٤٥ °C بينما ٧٥ % من عينات *C.albicans* كانت قادرة على النمو في وسط SDA المضاف لها مادة .Cycloheximide اجري اختبار Biochemical باستخدام API 20 AUX وتم اثبات انه ٨٠ % من العينات كانت *C.albicans* و ٨ % من العينات كانت *C.tropicalis* و ٤ % كانت *C. glabrata* و ٤ % كانت *C.parpsilosis* و ٤ % *C.famata*

باستخدام طريقة الانتشار بالاكار استخدمت سبعة انواع من المضادات الفطرية ضد ٢٠ عزلة من *C.albicans* . *هذه الانواع هي* Fluconazole, Clotrimazole, Miconazol,Ketoconazole

والتي اظهرت اعلى حساسية % 70 and % 80 , 80% , % 90.

وتم اجراء تفاعل البلمرة المتسسل PCR للكشف عن وجود عوامل الضراوة لل.*C.albicans* وقد اظهرت نتائج هذا الفحصان ٣٠%من عزلاتCandida albicans كانت موجبة للجين*INT1*

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