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Research Paper

CHARACTERIZATION OF VIRULANCE GENES IN CANDIDA ALBICANS STRAINS ISOLATED FROM ORAL LESIONS OF SMALL RUMINANTS

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Candida albicans (*C.albicans*) causes many systematic and opportunistic infection in animals, so this study aimed to investigate some virulence factors in *C.albicans* strains isolated from oral lesions of small ruminates. Eighty oral samples of sheep and goat were collected from local farms at Qena province (40 samples of each species), these samples were collected from apparently healthy and diseased cases for each species (20 samples for each). All samples were subjected to mycological examination. The results showed that *C.albicans* recovered from 20% and 10% of apparently healthy cases of sheep and goat while it recovered from diseased cases of the same species with percentage 50% and 20% respectively. Fourteen isolates out of 21 yeast isolate from sheep samples and six isolates out of 11 yeast isolate from goat samples were identified conventionally as *C.albicans* and confirmed by *ITS1-ITS4* primers. *Als3* gene was detected in 60% and 50% of *C.albicans* isolates of diseased sheep and goat respectively. While *Hwp1* gene was detected in one isolate of diseased goat samples only. *C.albicans* isolates were shown a high sensitivity to Clove oil, rather than other herbal oils.

Keywords: Sheep, Goat, C.albicans, ITS, Als3, Hwp1, Herbal oils

INTRODUCTION

Candida species are considered a normal flora and commonly seen in gastrointestinal tract, mucosa, and skin of animals and human but it behaves as opportunistic yeast in immuno-compromized hosts (Jones *et al.*, 1997). The transmission of mycotic infection in animals occurs by the contaminated environmental sources that often contains the yeast including air, soil, feeds (Hazen and Howell, 2003). *C.albicans* is a dominant reason of mucosal disease which indicate that this yeast has a distinctive virulence factors differ from other species of *Candida*. *Als3* gene (agglutinin-like sequence 3) is a one of eight members of *ALS* family and consider a specific virulence gene in

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*C.albican*s, also it's responsible for adherence to host cells, cell invasion and biofilm formation (Liu and Filler, 2011). Hyphal wall protein1 (*Hwp1*) another adhesion gene (Staab *et al*., 2004), which increases the synthesis of protein with oxygen and iron limitation (Sosinska *et al*., 2008). Safe and unconventional treatment was needed to conquer severe fungal infections. Many essential herbal oils have antioxidant activity and antifungal activity than synthetic ones, therefore they potentially act as antimycotic agents (Chaieb *et al*., 2001).

Therefore, our aim in this study was investigation the pathogencity of *C.albicans* by detection some virulence genes like *Als3* and *Hwp1* genes in *C.albicans* strains isolated from sheep and goat and evaluation the sensitivity of these strains to different herbals as an antifungal agent.

MATERIALS AND METHODS

Samples

A total of eighty oral samples of sheep and goat were collected from local farms in Qena governorate (40 samples of each species of animals) during winter season 2016. These samples were collected from oral cavity of diseased and apparently healthy cases (20 samples of each). Diseased cases suffering from oral lesion, loss in appetite and decrease of weight. Oral swaps were collected and amerced in tubes containing Sabouraud dextrose broth and transferred to laboratory as rapid as possible where incubated at 37 ^{o}C.

Isolation and Identification of *C.albicans*

A loopful of each broth tube were streaked onto plates of Sabouraud dextrose agar (oxoid, England), containing 0.05 mg of chloramphenicol per ml, the plates were incubated at 37 °C and examined after 48 hours for the presence of yeast colonies (Srujana *et al*., 2011). The isolated colonies were identified as recommended by Ellis *et al.* (2007) and Barnet (2000) as followed: macroscopic examination, germ test tube, chlamydospore formation in corn-meal, microscopic examination and carbohydrate assimilation test.

DNA Extraction

DNA extraction protocol was performed according to Moeller *et al*. (1992) with slight modification as following: the collected *C.albicans* isolates were cultured in 10 ml tube containing 5 ml of PDA broth after 24 hour incubation, cells were collected in Eppendorf tube by centrifugation at 16,000 g for 15 s and discarding the liquid. Cells mixed well with 0.7 ml 2X Cetyl Trimethyl Ammonium Bromide (CTAB) buffer and 0.4 gm glass beads. They were incubated at 65 °C for 30 min then 0.7 ml of chloroform was added and mixed briefly after centrifugation at 15,000 g for 10 min. The supernatant was transferred into a new tube mixed 0.6 ml isopropanol chilled to -20 °C followed by centrifugation at 15,000 g for 5 min. The supernatant was discarding and remaining pellets were washed twice with 1 ml ethanol 70% followed by drying under vacuum and thereafter dissolved in 0.1 ml TAE buffer (Tris-acetate EDTA). The DNA concentration was evaluated by agarose gel electrophoresis.

Molecular Characterization of *C.albicans* Isolates by Using *ITS1*/*ITS4* Primers

The PCR design was performed to amplify the internal transcribed spacers of the ribosomal DNA by using *ITS1*/*ITS4* primers (White *et al*., 1990; and Nada *et al*., 2014) (Table 1). PCR

amplifications were carried out in a total volume of 50 µl by mixing 2 µl of DNA with 0.5 µM of each primer, 150 µM of dNTP, 1 U of Taq DNA polymerase (Promega), and PCR reaction buffer.The mixture were subjected to 35 cycles in a hybrid thermal cycler (Bioneer, Korea) with the following incubations: Denaturation at 94 °C for 30 sec, primer annealing at 59 °C for 1 min and extension at 72 °C for 45 sec. Ten µl from the amplified DNA were analyzed in 1.5% agarose gel with 1X TBE buffer stained with ethidium bromide and visualized by illumination with UV light.

Molecular Detection of *Als3* and *Hwp1 Genes* in *C.albicans* Isolates

PCR amplification technique was carried out in a total volume of 25 µL of reaction mixture for each primer, *Als3* primer (Hoyer *et al*., 1995) and *Hwp1* primer (Nas *et al*., 2008) (Table 1). The reaction mixture consisted of 5 µL of 10X reaction buffer without MgCl2 (Fermentas, USA), 10 mM of each deoxynucleoside triphosphate at 0.5 µL (Fermentas, USA), 25 mM of $MgCl_2$ at 2.5 µL, 1 U of Taq polymerase (Fermentas, USA), 1.5 µL of primers, and approximately 10 ng of template DNA at 4 µL, and was then brought to a final volume of 25 µL with distilled water. The *Als3* mixture was subjected to 35 cycles of the following incubations: Denaturation at 94 °C for 30 sec, primer annealing at 59 °C for 1 min and extension at 72 °C for 45 sec. For *Hwp1* primers, PCR reaction was conducted as follows: 1 cycle of 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 1 min, and 72 °C for 2 min. A final extension cycle was performed at 72 °C for 5 min. 10 µl from the amplified DNA were analysed in 1.5% agarose gel with 1X TBE stained with ethidium bromide stained DNA amplicons were seen using a gel imaging system (Dolphin-View, USA).

Preparation of Different Concentrations of Herbal Oils

The following oils were purchased from markets of herbs in crude forms ready for use as clove, garlic and onion oils from Al-Ahram pharmaceutical comp., Egypt. Each crude oil were dissolved in DMSO (Sigma) and sterilized by 0.45 µm Millipore filters (Nalgene, UK) to obtain different concentrations. Different concentrations were prepared from herbal oils ranging from oils 2-10% and shaken strongly (Hili *et al.,*1997).

Antifungal Screening of the Different Herbal Oils by Using Well Diffusion Assay

Well diffusion assay was performed according to Omoya and Akharaiyi (2010) as following: hundred micro liters of *C.albicans* spore suspension (10^5/ml) was added to sterile plate contained 20 ml of Sabouraud dextrose agar (SDA), and then mixed well. With 6 ml cork borer wells were bored on the surface of agar. 100 µl of each oil concentration were added to wells in triplicate. 100 µl of fluconazole (Sedico pharmaceutical comp. Egypt) were added to wells as a positive control. The plates were incubated at (37 °C for 24 hrs), after that the inhibitory zones were measured around the wells in the plates.

Statistical Analysis

Analysis of variance (ANOVA) was used to analyze the results (Mean±S.E).

RESULTS

According to phenotypic identification and molecular characterization, *C.albicans* isolates were recovered from the oral swaps of the diseased sheep and goats with percentages of

Table 1: Primer Sequences

Primers	Sequence 5´-3´	bp
ITS1	F-5TCCGTAGGTGAACCTGCG3´	535
ITS4	R-5TCCTCCGCTTATTGATATGC3´	
Als3	F-5ATGACACCATGTCAAGTTCAGA3´	342
	R-5´CACACCAAATTGGAGGTGATT3´	
Hwp1	F-5ATGACTCCAGCTGGTTC3´	572
	R-5TAGATCAAGAATGCAGC3´	

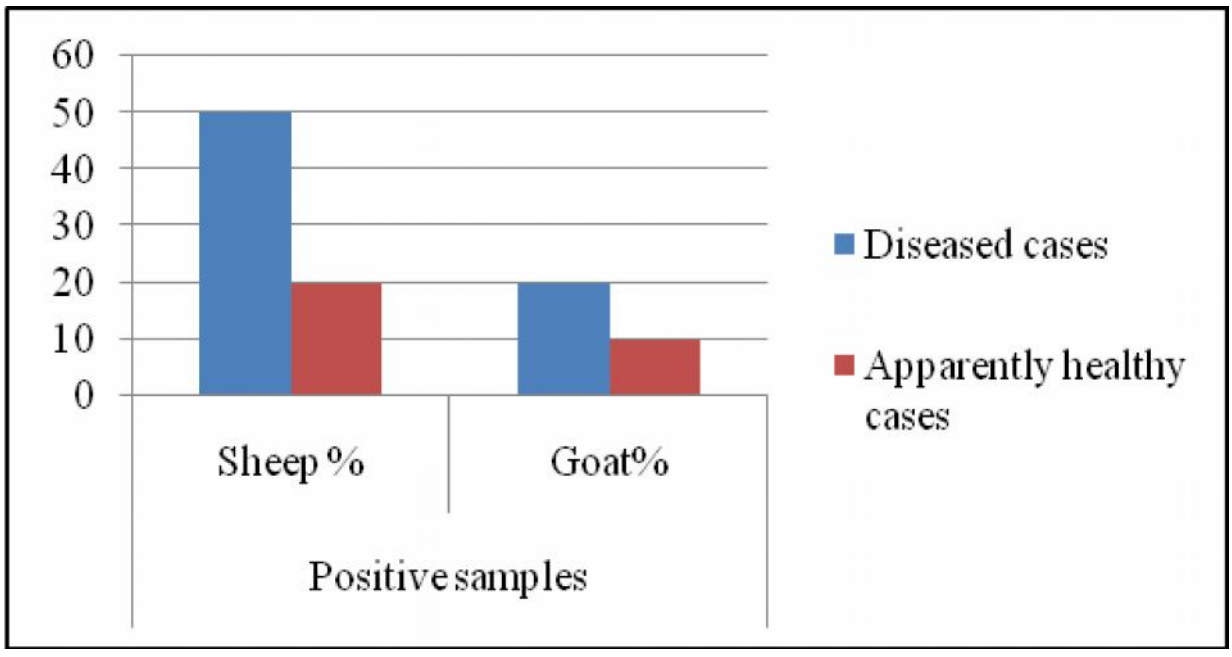
50% (10/20) and 20% (4/20), respectively while they were recovered from 20% (4/20) and 10% (2/20) of apparently healthy sheep and goat samples, respectively (Figure 1).

The data illustrated in Table 2 and Figure 2 showed fourteen isolates out of 21 yeast isolates from sheep samples and 6 isolates out of 11 yeast isolates from goat samples were identified conventionally as *C.albicans*. The *C.albicans* strains were confirmed by using *ITS1/ITS4* primers at (535 bp).

In the current study *Als3* gene were detected in *C.albicans* isolates of diseased sheep and goat with a percentage 60% and 50% respectively. While *Hwp1* gene were detected in 25% (1/4) of *C.albicans* strains isolated from diseased goat samples only (Table 2 and Figures 3 and 4).

Natural herbal oils were evaluated as antifungal agents for controlling *C.albicans* infection including clove, garlic and onion oils, These herbal oils were tested on *C.albicans* isolates which contained *Als3* and *Hwp1* genes, these isolates resisted most of herbal oils at different concentrations (2-10%) while the sensitive antifungal effect of clove oil was showed at concentration10% (23±0.21) (Table 3).

Figure 1: Incidence of C.albicans Isolated from Sheep and Goats



Note: *C.albican* strains recovered from diseased cases of sheep and goat at percentage 50% and 20% while it recovered from 20% and10% of apparently healthy cases of each species.

Figure 2: The Amplification Profile of PCR Targeting the ITS1-ITS4 Region, Lane 1: Ladder (100 bp), Lane 2: Positive Control, Positive Isolates for C.albicans Lane 3, 4, 5, 6, 7, 8, 9, 11 and 12 Band Size (535 bp) and lane 10: Negative Control

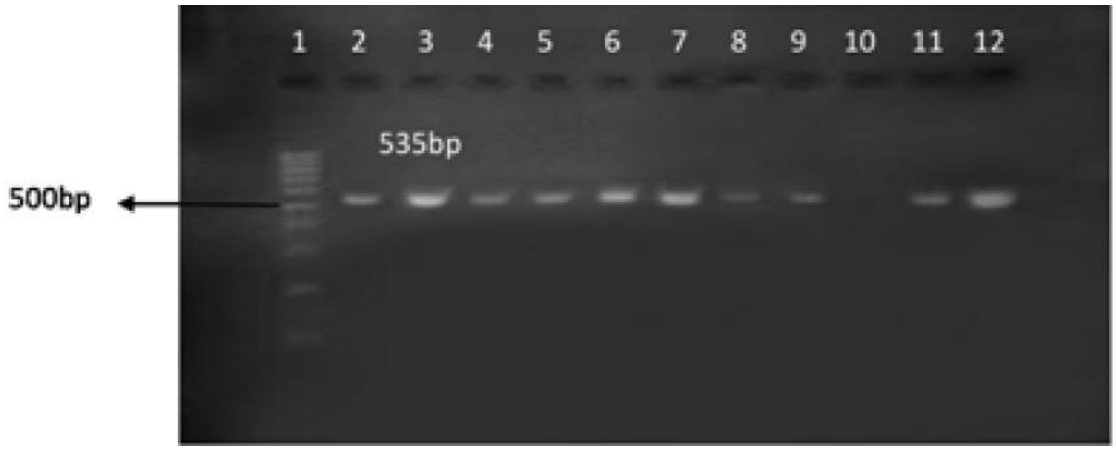


Table 2: ALS3 and HWP1 Genes in Confirmed Isolates of C.albicans by ITS

***Animal* spp.**		**No. of *C.albicans* Isolates Confirmed by *ITS***	**Positive Isolates for *Als3***	**Positive Isolates for *Hwp1***
Sheep	Apparently healthy cases	4	0	0
	Diseased cases	10	6 (60%)	0
Goat	Apparently healthy cases	2	0	0
	Diseased cases	4	2 (50%)	1 (25%)

Table 3: Inhibition Zone of Herbal Oil Against C.albicans Isolates by Using Well Diffusion Technique

***Yeast* spp.**	**Herbal Oils**									**Positive Control**
	Clove Oil			**Onion Oil**			**Garlic Oil**			**Fluconazole**
C.albicans	6%	8%	10%	6%	8%	10%	6%	8%	10%	(100 µl)
	R	13±0.2	23±0.21	R	9± 0.5	10±0.1	R	R	R	13±0.2

DISCUSSION

C.albicans is a opportunistic yeast and the predominant *candida spp*. that causes several infections extending from oral to systemic infection specially in immunocompromized hosts (Monod and Borg-von, 2002). The result illustrated in Figure 1 showed that *C.albicans* recovered from diseased cases of sheep and goats with a high percentage than apparently healthy cases. These results supported by Hassan *et al*. (2012) and Hassan *et al*. (2010) who isolated *C.albicans* from different sources of diseased sheep and goats by a high incidence than apparently healthy cases, also Gudlaugsson *et al*. (2003) found that the pathogenic effect of *Candida spp*. depended on the impairing of immune system, which

Figure 3: Amplification Profiles of the PCR Targeting the Als3 Gene, Lane 1: Ladder (1 kb), Lane 2: Positive Control, Positive Isolates for Als3 Lane 3, 4, 5, 6, 7, 8, 9 and 10 Band Size (342 bp) and Lane 11: Negative Control

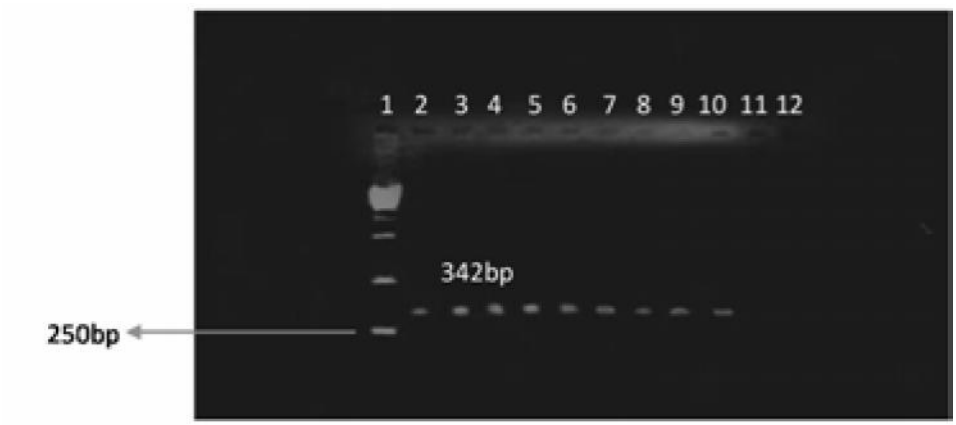


Figure 4: Amplification Profiles of the PCR Targeting the Hwp1 Gene, Lane 1: Ladder (100 bp), Lane 2: Positive Control, Positive Isolates for Hwp1 Lane 5 Band Size (572 bp) and Lane 10: Negative Control

facilitated their colonization and adhesion. Donskey (2004) published that *Candida* infection could be resulted from polluted water, food, manure and silage, so reach to the animals and birds during eating and drinking (Slavikova and Vadkertiova, 2003).

PCR allows accurate identification of *C.albicans* DNA. Different types of primers have been proposed for the identification of *C.albicans*, the most common types of genes targeted by primers were Intergenic spacer regions (*ITS*). These genes were similar in all species but the lengths of the *ITS* regions depended on the species (Peman and Zaragoza, 2010), so analysis of the complete *ITS* region gave a good results in molecular identification of *Candida* spp. In the current study, the presence of *C.albicans* DNA was confirmed in 14 isolates from sheep samples and 6 isolates from goat samples by using *ITS1*/*ITS4*, the PCR product size of amplified *ITS* region was characterized at 535 bp which represented the PCR sizes of

C.albicans as revealed in Figure 2, the same results are recorded by Nada *et al*. (2014). Using of *ITS* primer in identification of *Candida* spp has been reported previously by Pinto *et al*. (2004) and Ciardo *et al*. (2006).

In the present study the data postulated in Table 2 and Figures 3 and 4 revealed that *Als3* and *Hwp1*were detected in *C.albicans* isolates of diseased cases of sheep and goat while these genes not detected in strains of healthy cases. These results agree with Green *et al*. (2004) and Nas *et al.* (2008) who detected *Als* genes and *Hwp1* in *C.albicans* strains isolated from diseased cases suffering from oral lesions and vaginal candidiasis. Also detection of *Als3* and *Hwp1* in *C.albicans* strains will help to recognize the roles of these genes in colonization and disease (Hoyer, 2001).

Antifungal with chemical nature didn't treat the yeast infections completely due to multidrug resistant strains of fungus which has led to a search for alternatives therapeutic agents like herbal oils have antifungal properties (Viviani *et al.,* 2003). In current study, *C.albicans* isolates, which contained on *Als3* and *Hwp1* genes, they have been tested for their sensitivity against different herbal oils. *C.albicans* strains showed a high sensitivity to Clove oil at concentrations 10% (23±0.21 mm) (Table 3), these results supported by NCCLS (2008) and The Rosco Criteria (2011) who found that any inhibition zone lesser than 14 mm was resistance, 15-18 mm was intermediate and more than 19 mm was sensitive. The strong antifungal effect of clove oil back to the molecules of this oil which altered the morphogenesis of yeast and they affected on virulence factors responsible for adhesiveness and the morphological transition of *C.albicans* to hyphal form (He *et al*., 2007). The resistance of these strains to other types of herbal oils may be due to many factors, for instance, *fungal* spp., oil concentration and oil components (Adam *et al*., 1998).

CONCLUSION

Finally we concluded that *C.albicans* strains were isolated from apparently healthy and diseased cases of sheep and goats while virulence genes were detected only in *C.albicans* strains isolated from diseased cases, these results proved that *Als3* and *Hwp1* genes play an important role in pathogencity of *C.albicans* also we found that *C.albicans* strains showing a high sensitivity to clove oil than other oils.

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