Detection of Phospholipase and Asparty1 Protaines gens in *Candida* Species Isolated From Mammary Candidiasis in Lactating Women

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Abstract

Breast and nipple thrush (BNT) is a yeast infection of the nipple and breast caused by a fungal organism known as *Candida albicans*, a common cause of all thrush infections. It occurs most commonly in breastfeeding mothers. The infection may lead to severe nipple and breast pain, especially during breastfeeding. The current study was conducted at Al-Kut hospital for Gynecology, obstetrics and pediatrics, and Taiba Specialist Centerin Wasit province. (162) milk samples were collected from lactating wommen suspected to have mammary candidiasis infections according to the clinical manifestations diagnosed by the examining physician and patient with symptom , then, the samples were examined by optical microscopy and cell detection which show evidence of the presence of pathogenic yeast. Samples were cultured in sabouraud agar and then in chrom agar to classify the organisms, and they were held for further conformation and to detect the most common virulence factor gen by polymerase chain reaction (PCR) Molecular identification of *Candida spp* in this study, done by conventional PCR by partial amplification gene encoding for large subunit of 18S rRNA gene by specific primer sequences. The yield of the detection *C. albicans* and *C. parapsilosis,* was 37/37(100%) (100%) and 15/15 (100%), respectively. The results of PCR amplification of(SAP1-8)genes in *Candida* isolates (61) were SAP1 gen was present in 61/61(100%) , SAP2 gen was 54/61(96%), SAP3 gen was 45/61(86%), SAP4 gen was 35/61(76%), SAP5 gen was 32/61(66%), SAP6 gen was 45/61(60%), SAP7 gen was 35/61(30%) and SAP8 gen was 21/61(34%). By molecular technique using PCR (PLB1-2 gene) and sequencing, it was seen that Seventeen isolates belong *C. albicans* were positive for PLB1 gene 17/61 (91.6%). Fourteen isolates belong *C. albicans* were positive for PLB1 gene 14/61.

Keywords: *Mammary candidiasis, candida albicans , sap1-8 , lactating women*

Introduction

Candidiasis is a mycotic infection triggered by *Candida* species as a primary or secondary infection. *Candida albicans* is the most widespread Candida infection, accounting for 70 to 80 percent of all cases. *Candida* is an opportunistic yeast pathogen that thrives in patients with predisposing conditions such as immunodeficiencies, such as hepatic infection virus HIV, AIDS, and long-term utilization of broad-spectrum antibiotics and corticosteroids – to name a few(1)**.** Breast and nipple thrush (Mammary Candidiasis) is a yeast infection of the nipple and breast caused by a fungal organism known as *Candida albicans*, a common cause of all thrush infections. It occurs most commonly in breastfeeding mothers. The infection may lead to severe nipple and breast pain, especially during breastfeeding **(2)** Symptomatic mammary candidiasis Characterized by the presence of burning, itching, or shooting pain in the mammilla/areola and in the deep breast, radiating to the shoulder; as well as, areola with flush or white

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coating, dry, or desquamate skin. While, asymptomatic (16), which did not presence mentioned characteristics (3). *Candida* species are implicated in the world's most common opportunistic yeast infection, candidiasis, but *C. albicans* is the most common *Candida* species.Even though *C. albicans* is responsible for 50-90 percent of human candidiasis, it is resent in the commensal flora of more than a billion humans.More than half of the populace is stable. This yeast colonization is advantageous to the host because it not only inhibits the development of other opportunistic pathogenic fungi but also improves the immune system's work (4,5).

Materials and Methods

162 samples were collected through sterile tubes, taking into consideration the neglect of the first drops to prevent contamination also use cotton swaps for the purpose of taking samples from the mouth of children. These samples were collected from the first of October 2020 until the end of February 2021. They were cultured on a medium of sabouraud dextrose agar. After growth, they were examined under a microscope to see the hypha and pseudohypha of *candida spp*. The information for collecting these samples includes name, age, address, presence or absence of symptoms,.

Molecular detection

Primer (17)

Fungal genomic DNA Extraction

Using the EZ-10 Spin Column Fungal Genomic DNA Mini- Preps Package, fungal genomic DNA from *Candida albicans* isolates was isolated and performed according to company instructions as follows:

· The sample was moved to a sterile 1.5 ml micro centrifuge tube for a 100mg yeast fungi culture.

· 180 µl of Universal Digestion Buffer and 20 µl of Proteinase K were applied to the sample and thoroughly combined with the vortex. It was then incubated for 60 minutes at 56 ° C.

· 100 µl Universal Buffer PF was applied and combined at -20 °C for 5 minutes by inverting and incubating. Centrifuge for 1000 rpm then

At room temperature for 5 minutes. The supernatant was then moved into a new 1.5 ml micro centrifuge tube.

· 200 By vortexing, Uniform Buffer BD was introduced and thoroughly combined.

· By vortexing, 200µl ethanol (96-100 percent) was applied and thoroughly blended.

· The column of EZ-10 was put in a 2 ml collection tube and all the mixture (including any precipitates) was moved to the column. It was then centrifuged for 1 minute at 12000rpm. And the collection tube of 2 ml holding the flow-through was discarded and the column was put in a fresh collection tube of 2 ml.

· The DNA filter column was added with 500 µl of Universal PW Solution, then centrifuged for 1 minute at 10000rpm. The flow-through was discarded and the column in the 2 ml collection tube was placed back:

v Add to each column 500 µl of Universal Wash Solution. It was then centrifuged for 1 minute at 12000rpm. The flow-through was discarded and the column in the 2 ml collection tube was inserted back in.

v To dry the column matrix, all the tubes were centrifuged again for 3 minutes at 12000 rpm.

v A clean 1.5 ml microcentrifuge tube was moved to the dry DNA filter column and 50µl of pre-heated TE elution buffer was applied to the middle of the column matrix.

v To ensure the elution buffer was absorbed by the matrix, the tubes were let stand for at least 5 minutes. The filtered DNA was then eluted at 10000 rpm for 30 second

Genomic DNA investigation

Using the Nanodrop spectrophotometer (THERMO, USA), which tests and measures the purity of DNA by reading the absorption at (260 /280 nm), the extracted genomic DNA was tested as follows:

· After opening the Nanodrop program, the required application was picked (Nucleic acid, DNA).

· A dry wipe was taken and the measuring pedestals were washed a few times. 2µl of ddH2O is then carefully piped onto the top of the lower measurement pedestal.

· The sampling arm was lowered and the Nanodrop was blanked and then cleaned off the pedestals by pressing OK.

· The pedestals are then washed and 1µl of DNA sample is piped for calculation.

Preparation of PCR master mix reaction

Table (1) PCR master Mix for sap1-8, PLB1 and PLB2

PCR Master mix	Volume
DNA template	5µL
F. primer 10pmol	2µL
R. primer 10pmol	2µL
PCR water	13µL
Total volume	22µL
Total volume	20µL

PCR Thermocycler conditions

Table(2) PCR thermocycler system of *candida spp*. and SAP 1-8, PLB1and PLB2

PCR cycle	repeat	Temp.	Time
Initial denaturation	1	95°C	**5min**
Denaturation	30	94°C	**30sec.**
Annealing		62°C	**30sec**
Extension		72°C	**1min**
Final extension	1	72°C	**5min**
Hold	**-**	**4°C**	Forever

Gel electrophoresis of PCR product

The following steps were tested for PCR products of each gene using the agarose gel electrophoresis method:

· 1% Agarose gel was prepared using 1X TBE and dissolved for 15 minutes in a water bath at 100 ˚C, after which 45˚C was left to cool.

· In the agarose gel solution, 3μL of ethidium bromide stain was then applied.

· After fixing the comb in the correct position, the agarose gel solution was poured onto the tray and allowed to solidify for 15 minutes at room temperature. , then the comb was gently removed from the tray and 10μl of PCR product was applied to each comb well and 5ul (100bp Ladder) in one well.

· In the electrophoresis chamber, the gel tray was fixed and filled with a 1X TBE buffer. The electrical current was then carried out for 1 hour at 100 volts and 80 AM.

· Using ultraviolet trans illuminators, PCR goods have been visualized.

Statistical Analysis

The statistical kit for social sciences (SPSS) version 21 for Windows Applications and Microsoft Excel 2010 was used to statistically enter and interpret all data derived from the current analysis. The Chi-square test and one direction variance analysis (ANOVA) with the least important differences were used to determine the relationship between the analyzed variables.

Results and Discussion

Candida spp Diagnosed by PCR

Conventional PCR Screening for 18S rRNA Gene of *Candida albicans* and *Candida parapsilosis*

Molecular identification of *Candida spp* in this study, relied conventional PCR for the amplification of a partial gene of 18S rRNA by specific primer sequences. This gene was present in 37/37(100%) of *C. albicans* with a PCR product size of 88 bp , and in 15/15(100%) of *C. parapsilosis* with PCR product size of 98 bp,

Conventional PCR Screening for Aspartyl Proteinase (SAP1-8) Genes

The results of PCR amplification of these genes in *Candida* isolates (61) were SAP1 gen was present in 61/61(100%) , SAP2 gen was 54/61(96%), SAP3 gen was 45/61(86%), SAP4 gen was 35/61(76%), SAP5 gen was 32/61(66%), SAP6 gen was 45/61(60%), SAP7 gen was 35/61(30%) and SAP8 gen was 21/61(34%) with a PCR product different sizes , as shown in Table and (1) the distribution (SAP1-8) gen on mammary candidiasis causative pathogen Table (3,4).

Table(3) Prevalence of Aspartyl Proteinase (SAP1-8) Genes in *Candida spp.*

Gen	**SAP1**	**SAP2**	**SAP3**	**SAP4**	**SAP5**	**SAP6**	**SAP7**	**SAP8**
No.	61	54	45	35	32	45	35	21
Percentage (%)	100	96	86	76	66	60	30	34
X2	25.64							
P value	0*							

* Highly significant difference (P <0.01)

Table (4) the distribution (SAP1-8) gen on mammary candidiasis causative pathogen

Candida spp	**SAP1**	**SAP2**	**SAP3**	**SAP4**	**SAP5**	**SAP6**	**SAP7**	**SAP8**	**X2**	**Pvalue**
C.albicans	100%	93.3%	83%	66.6%	56.6%	50%	26%	43%	96.96	0***
C.tropicalis	71.4%	57.1%	14.2%	42.8%	28.5%	28.5%	0%	0%	8.45	0.294
C.parpsilosis	61.5%	38.4%	23%	7.6%	30%	15.3%	7.6%	0%	16.13	0.04*
X2	12.57	15.93	19.9	17.77	3.44	4.92	1.97	----		
P value	0.002**	0**	0**	0**	0.179	0.085	0.160	----		

Conventional PCR Screening for Aspartyl Proteinase (SAP1-8) Genes

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Table(5) Prevalence of Aspartyl Proteinase (SAP1-8) Genes in *Candida spp.*

Gen	**SAP1**	**SAP2**	**SAP3**	**SAP4**	**SAP5**	**SAP6**	**SAP7**	**SAP8**
No.	61	54	45	35	32	45	35	21
Percentage (%)	100	96	86	76	66	60	30	34
X2	25.64							
P value	0*							

* Highly significant difference (P <0.01)

Table (6) the distribution (SAP1-8) gen on mammary candidiasis causative pathogen

Candida spp	SAP1	SAP2	SAP3	SAP4	SAP5	SAP6	SAP7	SAP8	X2	Pvalue
C.albicans	100%	93.3%	83%	66.6%	56.6%	50%	26%	43%	96.96	0***
C.tropicalis	71.4%	57.1%	14.2%	42.8%	28.5%	28.5%	0%	0%	8.45	0.294
C.parpsilosis	61.5%	38.4%	23%	7.6%	30%	15.3%	7.6%	0%	16.13	0.04*
X2	12.57	15.93	19.9	17.77	3.44	4.92	1.97	----		
P value	0.002**	0**	0**	0**	0.179	0.085	0.160	----		

Conventional PCR Screening for Aspartyl Proteinase (PLB1-2) Gene

Seventeen isolates belong *C. albicans* were positive for PLB1 gene 17/61 (91.6%), PCR product of this gene was 161 bp. Fourteen isolates belong *C. albicans* were positive for PLB1 gene 14/61 (93.3%), PCR product of this gene was 171 bp, The result of Conventional PCR Screening for 18S rRNA Gene of *Candida albicans* and *Candida parapsilosis* are incompatible with a study done by (3), applied rapid diagnostic tests and nested PCR method, and he obtained 46 (17.7%) positive samples for *Candida* spp out of 260 women milk samples. by nested PCR technique 6 different species of *Candida* were identified, *C. tropicalis* was the predominant one (26.1%), followed by *C. parapsilosis* (21.7%), *C. kefyr* and *C. krusei* (17.4%) for each, *C. rugosa* (13%), and *C. glabrata (*4.4%). Rapid identification and molecular biology based tests have begun to be used more easily and effectively than conventional tests in the identification of fungal pathogens. Diagnostic polymerase chain reaction (PCR) method has been widely applied in laboratories for identification of many fungal species due to their speed, high sensitivity, and specificity (13). The percentage of *C. albicans* obtained by PCR in this study agrees also with a study conducted in Nigeria (100%) using molecular techniques in yeast recognition PCR and sequencing of the domains D1 and D2 of the 26S rRNA genes (17). The result of Present Aspartyl Proteinase (SAP1-8) Genes in candida spp.in the current study is agree with (14) who report the present of gen (ASP1-8) were 20 (100%) , 20 (100%) ,16 (80%) ,12(60%), 15 (75%), 11(55%), 6(30%) , 2(10%) respectively.

The present results and regarding PLB1 and PLB2 genes for *C.albicans* were agree with those obtained in a study done by (15,16,17) who report nineteen isolates out of 20 (95 %) were positive for PLB1 gene, eight isolates out of 20 (40%) belong were positive for PLB2 gene. Aspartyl Proteinase (SAP) is one of virulence factor of *candida* spp role of this gen has mentioned (6,7). That exemplified how SAP proteins influence adherence. The Sap family is capable of filling any niche in *C.allbicans*(8,9). And the precise mechanisms by which Sap proteins trigger the adherence process are unknown. At the moment, two hypotheses are the most common(10,11,12). In the first case, *C. albicans* proteinases on host cells could act as ligands for surface moieties that aren't normally mediated by enzymes. *C. albicans* modifies target proteins or ligands on the fungal surface or in host cells in the second case (i.e. epithelial cells protei), using Sap proteins as active enzymes to modify surface hydrophobicity or lead to conformation changes, thus promoting greater fungal adhesion Which will contribute to an increase in the amount of virulence properties if Sap ns acted directly as *C.albicans* adhesions.Proteinases are one of the most flexible and multifunctional gene groups of virulence exhibited by *C. albicans*, and they already exhibit tissue degradation, invasion, and host defense evasion. *C.albicans'* Influence Transforming into hyphae can be considered a pathogenic determinant in the early stages of superficial tissue invasion, since hyphae may help *C. albicans* bind to and penetrate host tissues. As mentioned earlier.

Conclusions

C.albicans is the most common species that causes mammary candidiasis.

Conflict of Interest: None

Funding: Self

Ethical Clearance: Not required

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