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DNA Sequencing Detection of *Candida albicans* CALB1 Gene Isolated from Oral Candidiasis Patients in Al-Najaf Governorate

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Abstract: **Background:** In Iraq, there is a scarcity of data concerning fungal species isolated from different clinical cases. Consequently, this study aims to fill these gaps in the existing knowledge., **Aim**: This research highlights the significance of fungi as causative agents in humans and provides a genetic and molecular identification profile to accurately pinpoint the pathogens responsible for oral candidiasis. It also provides a genetic characterization of these pathogens, comparing them with standard isolates recorded in global databases like NCBI. **Method:** Oral swabs total specimens were 50, collected from Al-Najaf province during period from January 2023 to May 2023. Identification was according chromagar medium then VITEK2 technique results were completed with PCR, using PCR technique and DNA sequencing of ITS1 and ITS2 regions. **Results:** 50 specimens some of them are negative 35%, positive 65%, *Candida albicans* 21 % other *Candida* spp 62% without growth 17%. There is no significant association between genders in oral candidiasis and yeast in all groups of ages. There is significant association between type of disease and yeast at ($p \leq 0.01$). No notable correlation was observed between age and yeast ($p \leq 0.05$). The sequences obtained from each sample were compared to the NCBI database using the online NCBI BLAST tool. In oral candidiasis the *Candida albicans* specific gene CALB1 was found in strains *C. albicans OT5/1* strain, *C.albicans OT2* strain *C. albicans OT1/1*, strain *C. albicans cn152* and strain *C. albicans cn150* with 98 %. **Conclusion**: Concluded some *Candida albicans* have the capacity to produce CALB1 gene, and DNA sequencing is the best method for identification of fungi compared with chromagar medium test and PCR analysis.

Keywords: C. albicans, Oral candidiasis, Apportunistic, Sequence technique.

Introduction

Currently, there is limited data in Iraq on fungal species isolated from different clinical cases. This study is designed to address this gap. It underscores the role of fungi as causative agents in humans and offers a genetic and molecular identification profile to accurately identify the pathogens involved (Suleyman & Alangaden, 2016). Candida albicans is linked to a significant number of symptomatic infections each year, which range from superficial (e.g., skin and mucous membranes) to invasive infections of internal organs. These infections predominantly affect

immunocompromised individuals and typically originate in the gastrointestinal tract. Therefore, it is important to fill the knowledge gap about Candida spp. colonization, their commensal behavior, and their transition to pathogenic states. Interestingly, Candida spp. may also have host-beneficial roles (Romo & Kumamoto, 2020). Species of Candida have the ability to cause various diseases in both humans and animals (Pfaller et al, 2006).

Candida is act as normal flora in the human body but may behave as a pathogen when the host immune system is depressed and attack the body and colonize the mucous membranes and resulting in Candidiasis (Mohandas and Ballal, 2011)

Materials and methods

Identification of *Candida* spp. isolates

The yeast isolates were examined for their shape, size, color, edges, and general appearance on SDA media after being incubated for 24-48 hours. Chromo agar was used to assist in diagnosing *Candida* species based on their color. Single cells from the yeast growth on SDA were picked with a loop, cultured, and incubated for 24-48 hours at 37°C (Horvath et al., 2003). Colonies were then removed, placed on a slide, and stained with lacto phenol cotton blue or Gram stain for examination under a light microscope (Kayser et al., 2005).

Growth at 45C°

This test was employed to distinguish *C. albicans* from other species. *Candida* isolates were inoculated into test tubes containing SDB and incubated at 42°C-45°C for 48-72 hours. *C. albicans* exhibited robust growth at these temperatures, while other species showed minimal or no growth (Kim et al., 2002).

Identification of *Candida* spp. by VITEK technique

The isolates were cultured on SDA agar for 24-48 hours and subsequently analyzed using VITEK 2 technology. The VITEK 2 Compact is an automated system for identifying microbes, utilizing barcode cards to enhance traceability and decrease transcription errors. Results are available within 18 hours of preparation (Kord et al., 2020; Dos Reis et al., 2022).

Detection of Candida species using the PCR method.

Primers preparation.

Table (1): Primers pair used in this experiment were Universal primers.

Universal primers	Sequence	Specific primers	Sequence	Reference
ITS1	(5'TCCGTAGGTG AACCTGCGG-3')	CA3	(5'GGTTTGCTTGAA AGACGGTAG-3')	Tarini *et al.*, 2010.
ITS2	(5'GCTGCGTTCTT CATCGATGC-3')	CA4	(5'AGTTTGAAGAT ATACGTGGTAG-3').	

Culturing of the isolates

The fungal strains were streaked onto Sabouraud dextrose agar (SDA) plates and cultivated at 37°C for twenty four and forty eight hours to cultivate *Candida* species. A single colony from the SDA plate was then transferred to Sabouraud dextrose broth (SDB) and incubated for 24 and 48 hours prior to DNA extraction.

DNA extraction

Total DNA was extracted from the culture broth by transferring 1.5 milliliters into Eppendorf tubes. The samples underwent centrifugation at 4,300×g for five minutes, and the supernatant was subsequently removed. Then, 200 µl of TE buffer was introduced, the mixture was vortexed thoroughly, boiled for 10 minutes, and immediately placed on ice for 1 minute. Afterward, The

sample underwent a second centrifugation at 6,700×g for 10 minutes, and the supernatant was retrieved to be used as the DNA template (Ali, 2022).

PCR

The PCR assay was conducted to amplify ITS1 and ITS2 sequence for identification of *Candida* spp. Table (2).

Table (2): PCR Mixture Component Used in the Reaction

PCR Master mix	Volume
DNA template	5µl
Forward primer (10pmol)	1.5µl
Reveres primer (10pmol)	1.5µl
Deionized water	12µl
Total volume	**20µl**

PCR Conditions

PCR amplification system was used with the following program.

Table (3): Cycling parameters of Genes Amplification for *Candida* spp.

PCR step	Temperature	Time	Repeat
Initial Denaturation	95C°	3 min.	1
Denaturation	95C°	30 sec.	37 cycle
Annealing	57C°	30 sec.	
Extension	72C°	30 sec.	
Final extension	72C°	5 min.	1
Hold	4C°	Forever	-

Analysis of PCR products

DNA content was assessed Electrophoresis using agarose gel, with detection performed using a UV transilluminator (Sambrook & Russell, 2001). The PCR products were analyzed following the agarose gel electrophoresis protocol (Stephenson, 2003).

Identification of Virulence Factors of *Candida* spp. by PCR Technique

Recently, the identification of Candida has increasingly relied on molecular methods (Kadry et al., 2018). Candida species, particularly Candida albicans, may possess several virulence factors, including the specific gene CALB1, which is critical for pathogenicity (Eldesouky et al., 2016).

Table (4): Primers designed for C. albicans and virulence factor target genes, including their sequences and DNA sizes

Target genes	Primer sequences	DNA sizes
CALB1	Forward 5'-TTTATCAACTTGTCACACCAGA-3' Reverse 5'-ATCCCGCCTTACCACTACCG-3'	273 bp
Reference	Eldesouky, *et.al*,2016.	

PCR conditions

PCR amplification system was used with the following program.

Table (5): Cycling Parameters of Gene´s Amplification for *Candida.*

PCR step	Temp	Time	Repeat
Initial Denaturation	94C◦	5min	1
Denaturation	94C◦	1min	35 cycle
Annealing	52C◦	1min	

Extension	72C◦	1min	
Final extension	72C◦	5min	1
Hold	4C◦	Forever	-

DNA sequencing

The PCR products of Candida were sent to Macrogene Lab in Korea for DNA sequencing. The purified PCR products were prepared using a PCR purification kit as per the manufacturer's guidelines. Sequencing results for Candida albicans were then analyzed using Bio Edit software for multiple sequence alignment. The sequencing was performed using the Sanger method. (Hawksworth *et al.,* 2016).

Results and discussion

Some of *Candida* is not identified by VITEK2 system, this is consistent with Al-Dossary and Al-Shamahy, (2018), the reason may be due to new types, or it may be a combination of more than one type and it is difficult to identify (Mondelli *et al*., 2012). 50 specimens some of them are negative 35%, positive 65%, *Candida albicans* 21 % other *Candida* spp 62% without growth 17%. There is no significant association between genders in oral candidiasis and yeast in all groups of ages. There is significant association between type of disease and yeast at ($p \leq 0.01$). There is no significant association between age and yeast at ($p \leq 0.05$). NCBI BLAST online software was utilized to compare the sequences from each sample against the NCBI database. In oral candidiasis the *Candida albicans* specific gene CALB1 was found in strains *C. albicans OT5/1* strain, *C.albicans OT2* strain *C. albicans OT1/1*, strain *C. albicans cn152* and strain *C. albicans cn150* with 98 %.Candidiasis may have an association with a variety of malignancies (Domingues *et al*., 2009). The reasons that lead to increasing *Candida* infections such as diabetics or excessive intake of antibiotics, sugars, and this affect immune system during a chemotherapy treatment in cancer patients on the normal body criteria such as osmolality, the PH, and others, these changing in conditions are conducive to the growth of opportunistic organisms and allow them to grow, multiply and transcript the genes of virulence factors and other factors that lead to the settlement of the disease, then the microbes, weakening the immunity and paved the way to the opportunity to multiply as one of the body's infectious agents and delaying its immune defense, and other causes that inactivate immunity by killing immune cells and replace beneficial natural bacteria.

Oral candidiasis is very existing, but in very few cases it leads to death. It may be caused by the patient not receiving treatment, or because his illness is related to many other conditions, or because of the difficulty of feeding and that is caused by candida sores, due to pain, this agreed with Davies *et al*., (2010), who said that oral yeast carriage is common in patients with advanced cancer. In addition, chemotherapy, radiotherapy, and other treatments for cancer patients can lead to encouragement of inflammation in the body, exaggeration of the mucous membrane of oral cavity, and making it more vulnerable to microbes and any injury, especially challenging ones, including *Candida,* this is consistent with Abdullah *et al*., (2021).

In states of candidiasis so as a result of immunity inhibition, all normal flora may be opportunistic, some of these opportunistic fungi microbes, which is characterized by *Candida* species, are yeast like fungi, eukaryotic opportunistic organisms that be a flora on the mucosa of the digestive tract as well as genital tract (Lim *et al*., 2012). All these results were analyzed according (Glantz,2005). A p-value of ≤ 0.05 was regarded as statistically significant.

Culturing of the isolates

Colony's texture was smooth, glistening, or dry, colored creamy to yellow. Figure (1) explain these properties, this concurs with (Ali, 2022).

Figure (1): The *Candida albicans* identification on SDA medium after 48 h of growthand 37C.

The growth of yeast is colored according to genus and species, this colony color difference is used to demonstrate the colors of yeast and its identification, these colors' appearance agreed with (Ataa,2020; Ali, ,2022). Figure (2) explain these properties.

Figure (2): The *Candida albicans* identification by chromagar medium after 48 h of growthand 37C.

Identification Information				Analysis Time:		17.78 hours		Status:		Final					
Selected Organism				87% Probability Bionumber:		Candida albicans 6102766065317771									
ID Analysis Messages															
Biochemical Details															
3	LysA	-	4	IMLTa	+	5	LeuA	+	7	ARG	+	10	ERYa	-	12	GLYLa	-
13	TyrA	-	14	BNAG	-	15	ARBa	-	18	AMYa	-	19	dGALa	+	20	GENa	-
21	dGLUa	+	23	LACa	+	24	MAdGa	+	26	dCELa	-	27	GGT	+	28	dMALa	+
29	dRAFa	-	30	NAGA1	+	32	dMNEa	+	33	dMELa	-	34	dMLZa	-	38	ISBEa	-
39	IRHAa	-	40	XLTa	+	42	dSORa	+	44	SACa	+	45	URE	-	46	AGLU	+
47	dTURa	+	48	dTREa	+	49	NO3a	-	51	IARAa	+	52	dGATa	-	53	ESC	-
54	IGLTa	+	55	dXYLa	+	56	LATa	+	58	ACEa	+	59	CITa	+	60	GRTas	+
61	IPROa	+	62	2KGa	+	63	NAGa	+	64	dGNTa	+						

Figure (3):The *Candida albicans* identification by VITEK2 system.

Figure (4): The identification of *Candida albicans* specific gene CALB1 273 base pair, by using PCR technique, L: ladder.

DNA sequencing

The PCR products were dispatched to Macrogen Lab, where both forward and reverse strands of the amplified PCR products were compared with reference sequences in the NCBI GenBank. The strains were retrieved online and compared with the NCBI database using BLAST software. Multiple sequence alignments were performed using BioEdit software, The sequences were submitted to NCBI in FASTA format using Sequin software. NCBI BLAST online tool was utilized to match the sequences from the samples against the NCBI database. For oral candidiasis, the Candida albicans specific gene CALB1 was detected in strains *C. albicans* OT5/1, C. albicans OT2, C. albicans OT1/1, C. albicans cn152, and C. albicans cn150 with 98% similarity. Detailed results are presented in Table 1 and Figures 1-10.

Table (1): DNA sequences alignments of CALB1 gene of isolated (*C.albicans*) from oral candidiasis patients, in comparing with database obtained from NCBI website.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Candida albicans isolate cn152 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and inter...	Candida albicans	409	409	41%	4e-109	97.50%	474	OQ363167.1
Candida albicans isolate cn150 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and inter...	Candida albicans	409	409	41%	4e-109	97.50%	476	OQ363165.1
Candida albicans isolate cn149 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and inter...	Candida albicans	409	409	41%	4e-109	97.50%	481	OQ363164.1
Candida albicans isolate cn146 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and inter...	Candida albicans	409	409	41%	4e-109	97.50%	484	OQ363161.1
Candida albicans isolate cn145 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and inter...	Candida albicans	409	409	41%	4e-109	97.50%	472	OQ363160.1
Candida albicans isolate cn136 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and inter...	Candida albicans	409	409	41%	4e-109	97.50%	453	OQ363153.1
Candida albicans isolate cn133 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and inter...	Candida albicans	409	409	41%	4e-109	97.50%	492	OQ363151.1
Candida albicans isolate cn128 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and inter...	Candida albicans	409	409	41%	4e-109	97.50%	452	OQ363147.1
Candida albicans isolate cn108 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and inter...	Candida albicans	409	409	41%	4e-109	97.50%	462	OQ145119.1
Candida albicans isolate cn100 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and inter...	Candida albicans	409	409	41%	4e-109	97.50%	448	OQ134735.1
Candida albicans isolate cn96 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and intern...	Candida albicans	409	409	41%	4e-109	97.50%	444	OQ134731.1
Candida albicans isolate cn94 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and intern...	Candida albicans	409	409	41%	4e-109	97.50%	465	OQ134730.1
Candida albicans isolate cn89 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and intern...	Candida albicans	409	409	41%	4e-109	97.50%	421	OQ134725.1
Candida albicans isolate cn88 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and intern...	Candida albicans	409	409	41%	4e-109	97.50%	465	OQ134724.1
Candida albicans isolate cn70 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and intern...	Candida albicans	409	409	41%	4e-109	97.50%	465	OQ134713.1
Candida albicans isolate cn63 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and intern...	Candida albicans	409	409	41%	4e-109	97.50%	439	OQ134709.1
Candida albicans isolate cn59 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and intern...	Candida albicans	409	409	41%	4e-109	97.50%	465	OQ067223.1

Candida albicans isolate OT5/1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

Sequence ID: OR058928.1 Length: 538 Number of Matches: 1

See 1 more title(s) See all Identical Proteins(IPG)

Range 1: 169 to 404 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
407 bits(220)	3e-112	231/236(98%)	2/236(0%)	Plus/Plus

```
Query  2    AAATCTTTC-AC-ACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA  59
            ||| ||||| || ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  169  AAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGA  228

Query  60   TACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTGAACGCACATTGCGCCC  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  229  TACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTGAACGCACATTGCGCCC  288

Query  120  TCTGGTATTCCGGAGGGCATGCCTGTTTGAGCGTCGTTTCTCCCTCAAACCGCTGGGTTT  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  289  TCTGGTATTCCGGAGGGCATGCCTGTTTGAGCGTCGTTTCTCCCTCAAACCGCTGGGTTT  348

Query  180  GGTGTTGAGCAATACGACTTGGGTTTGCTTGAAAGACGGTAGTGGAACGGCGGGAT  235
            ||||||||||||||||||||||||||||||||||||||||||||| | ||||||||
Sbjct  349  GGTGTTGAGCAATACGACTTGGGTTTGCTTGAAAGACGGTAGTGGTAAGGCGGGAT  404
```

Figure (5) : DNA sequences alignments of CALB1gene of isolated (*C.albicans OT5/1*) from oral candidiasis patients, in comparing with database obtained from NCBI website

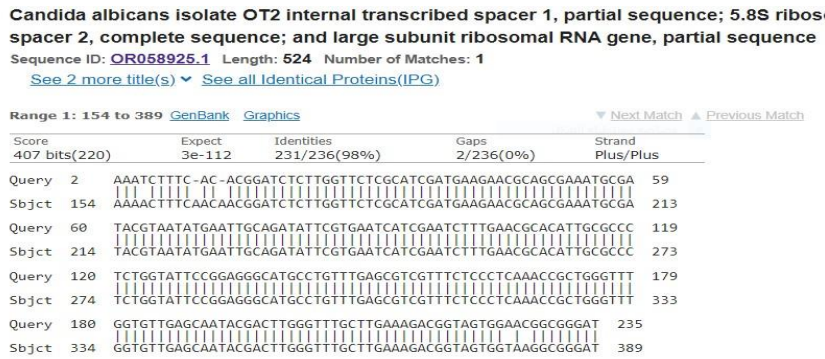

Figure (6) : DNA sequences alignments of CALB1gene of isolated (*C.albicans OT2*) from oral candidiasis patients, in comparing with database obtained from NCBI website

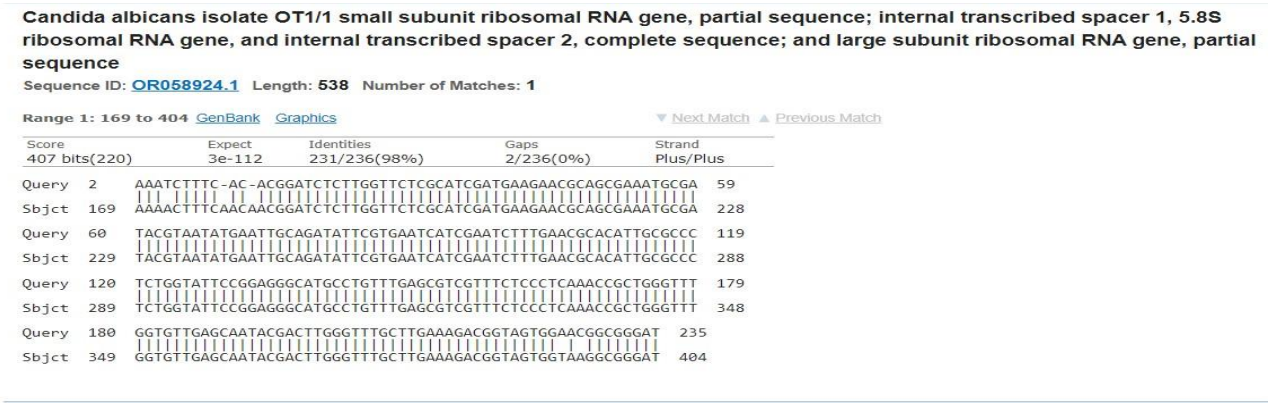

Figure (7) : DNA sequences alignments of CALB1gene of isolated (*C.albicans OT1/1*) from oral candidiasis patients, in comparing with database obtained from NCBI website

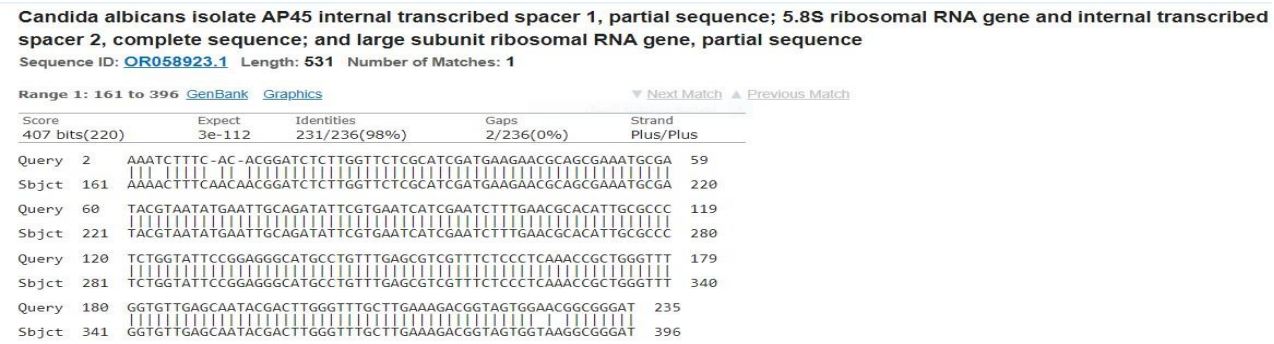

Figure (8) : DNA sequences alignments of CALB1gene of isolated (*C.albicans AP45*) from oral candidiasis patients, in comparing with database obtained from NCBI website

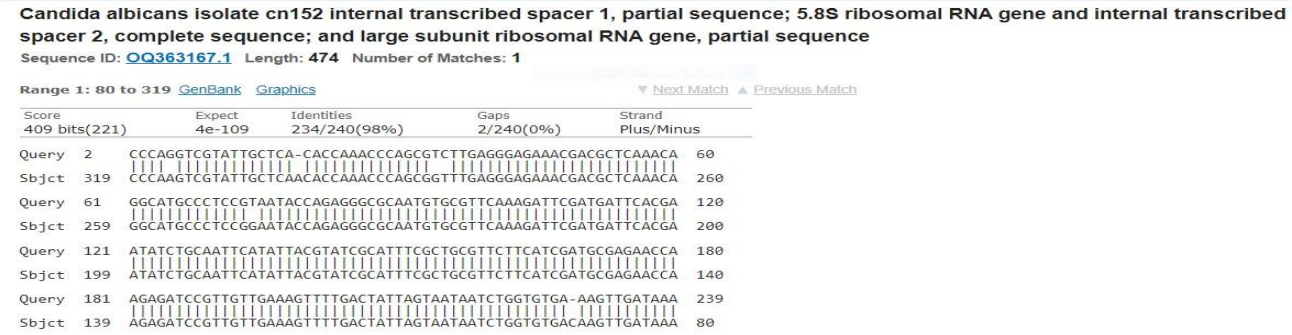

Figure (9) : DNA sequences alignments of CALB1gene of isolated (*C.albicans cn152*) from oral candidiasis patients, in comparing with database obtained from NCBI website

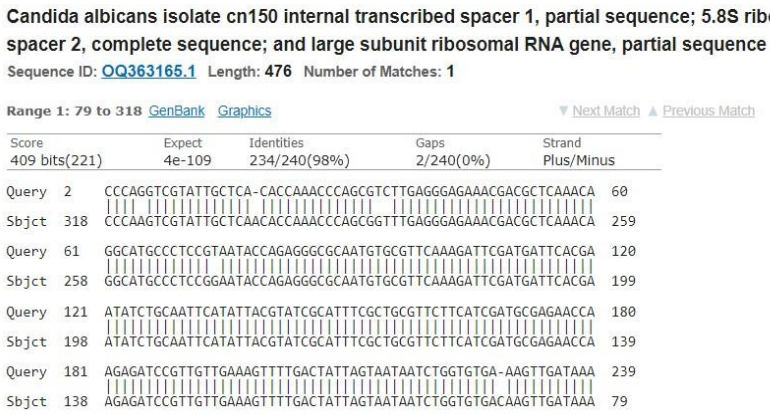

Figure (10): DNA sequences alignments of CALB1gene of isolated (*C.albicans cn150*) from oral candidiasis patients, in comparing with database obtained from NCBI website

Conclusion

Concluded some of *Candida* spp. have the capacity to produce CALB1 gene. Molecular techniques like PCR and Sequence are faster and more accurate than routinely methods in identification of microbes.

References

1. **Abdullah, A.T., Jaafar, F. N., Saleh, A. A. (2021).** Evaluation of the efficacy of extracts of Matricaria chamomilla flowers on some types of pathogenic fungi isolated from different clinical cases. Basrah: Master Thesis, 1- 224. (in Arabic).
2. **Ali,Y.K.(2022).**Molecular study of fungal isolates associated withdiabetic foot ulcer patients in Al-Najaf province and evaluate inhibition activity of some natural products Faculty of Science/ University of Kufa.
3. **Al-Dossary, O. A. E. I., and Al-Shamahy, H. A. (2018).** Oral Candida albicans colonization in dental prosthesis patients and individuals with natural Teeth, Sana'a City, Yemen. Biomedical Journal, 11(2), 1-5.
4. **Ataa,K.A.(2020).**Detectionof morphogenesis gene in *C. albicans* isolated from different clinical caces. MSc, Faculty of Science/ University of Kufa.
5. **Davies,A.N.; Brailsford,S.; Broadley ,K.and Beighton,D.(2010).**Oral yeast carriage in patients with advanced cancer. Oral Microbiol Immunol. 17:79 .
6. **Domingues,F.M.; Grumach, A.S.; Duarte, A.J.and De-Moraes,V. D**. **(2009).**Esophageal cancer associated with chronic mucocutaneous candidiasis. Could chronic candidiasis lead to esophageal cancer? *Med Mycol*.47:201-5.
7. **Eldesouky, I.; Mohamed, N.; Khalaf, D.; Salama, A.; Elsify,A.; Ombarak , R.; El- Ballal, S.; Effat, M. and Shabrawy, M.A.L. (2016).** *Candida mastitis* in dairy cattle with molecular detection of *Candida albicans*.Kafkas Universitesi Veteriner Fakültesi Dergisi, 22, 461-464.
8. **Dos Reis,C.M. ;de, P.A, Ramos, G.L.; Pereira, R.D.C.L.; Vallim, D.C. and de Oliveira Costa, L.E. (2022).** Evaluation of VITEK® 2 and MALDI-TOF/MS automated methodologies in the identification of atypical *Listeria* spp. isolated from food in different regions of Brazil. *Journal of Microbiological Methods*, p.106434.
9. **Glantz, S.A.(2005).** Primer of biostatics. 5th ed. P. 208-310. MacGraw, USA.
10. **Horvath,L.L.**; Murray, D.R.; C.K. and Doooley, D.P.(2003).Direct isolation of *Candida spp.* from blood cultures on the chromogenic medium chromagar *Candida* .J.Clin Microbiol .41:2629-32.
11. **Hawksworth, D.L.; Hibbett, D.S.; Kirk, P.M. and Lucking, R. (2016).** Proposals to permit DNA sequence data to serve as types of names of fungi. *Taxon* 65:899-900.

12. **Kadry, A.A.; El-Ganiny,A.M.and El-Baz,A.M.(2018).** Relationship between Sap prevalence and biofilm formation among resistant clinical isolates of *Candida albicans*.*African Health Science*,18,1166-1174.Research, 64, 211- 215.
13. **Kayser, F.H.; Bienz, K.A. ; Eckert, J. and Zinkernagel, R.M.(2005**).Medical Microbiology. Thieme Stuttgart, New York. 229-233; 348-374.
14. **Kim**, D.;Shin , W.; Lee , K.; Kim , K. and Park , J. (2002) . Rapid differentiation of Candida albicans from other Candida species using it unique germ tube formation at 39 C0 . Yeast.19 : 957 – 962.
15. **Kord, M.; Salehi, M.; Khodavaisy, S.; Hashemi, S. J.; Ghazvini, R. D.; Rezaei, S. and Arastehfar, A. (2020).** Epidemiology of yeast species causing bloodstream infection in Tehran, Iran (2015-2017); superiority of 21-plex PCR over the Vitek 2 system for yeast identification. *Journal of medical microbiology*, 69(5), 712.
16. **Lim,Y.; Rosli, R.; Seow, F. and Chong ,P.(2012).** *Candida* and invasive candidiasis: back to basic. *Eur .J. Clin .Microbiol. Infect . Dis*. 31(1):21-31.
17. **Mondelli, A. L.; Niéro-Melo, L.; Bagagli, E.; Camargo, C. H.; Bruder Nascimento, A.; Sugizaki, M. and Villas Boas, P. J. F. (2012).** *Candida* spp. manual identification (reference method) and automated identification (Vitek system platform). *Journal of Venomous Animals and Toxins including Tropical Diseases*, 18, 335-339.
18. **SambrookJ.and Russell, D.W. (2001).** Molecular cloning:a laboratory manual, Vol(2) 3rd ed .Cold spring harbor laboratory press, cold spring harbor.
19. **Suleyman, G.** and Alangaden, G.J. (2016). Fungal infections: epidemiology, infection control, and prevention. Infect Dis Clin North Am. 30(4):1023-52.
20. **Stephenson, F.H.** (2003). Calculations in molecular Biology and Biotechnology. Academic Press, Animprint of Elsevier, USA .