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Identification of *Cryptococcus neoformans* Isolates by PCR-ITS regions

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Abstract

The aim of the study was molecular detection of *C. neoformans* that isolated from 150 (88 female and 62 male) clinical samples (sputum samples) from pulmonary patients in Baghdad. The diagnoses of *Cryptococcus neoformans* in samples was done by using direct microscopic examination, culture media and PCR Technology. Microscopic examination and cultured revealed that 65 out of 150 (43.33 %) samples were positive and the others samples were Negative. Results of the genetic diagnosis looking for the fungi causing cryptococcosis using primers specific for *ITS* gene which were specially designed for this study revealed that 6 (4 %) of sputum samples were positive. In this study used the PCR technology due to the present of high specificity and sensitivity for the diagnosis of *C. neoformans* added to that which is cheaper and faster than the conventional methods currently used in hospital and laboratories.

Keywords: PCR Yeast, Cryptococcus neoformans, molecular, cryptococcosis.

تشخيص الخميره *Cryptococcus neoformans* بواسطة PCR-ITS regions

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الخلاصة

هدف الدراسه هو الكشف الجزيئي عن الخميره *C. neoformans* الذي تم عزله من 150 (88 اناث و 62 من الذكور) عينة سريرية (عينة بلغم) من مرضى الرئة في بغداد. تم تشخيص الفطر *Cryptococcus neoformans* في العينات باستخدام الفحص المجهري المباشر، والزرع على الاوساطالغذائيه وتكنولوجيا PCR. حيث كانت نتائج الفحص المجهري والزرع 65 عينة من أصل 150 (43.33٪) إيجابية وباقي العينات الأخرى كانت سلبية. وأظهرت نتائج التشخيص الوراثي الذي يبحث عن الفطريات المسببة للمكورات الخبيئة باستخدام البادئات الخاصة بالجين الذي تم تصميمه خصيصا لهذه الدراسة أن 6 (4٪) من عينات البلغم كانت موجبة. تم في هذه الدراسة استخدام تقنية تفاعل البلمرة المتسلسل بسبب امتيازها بدرجة عالية من الخصوصية والحساسية لتشخيص *C. neoformans* اضافةً إلى انها طريقة ارخص وأسرع من الطرق التقليدية المستخدمة حاليا في المستشفيات والمختبرات.

Introduction

Cryptococcus spp. are environmental fungi that have 100 species according to the modern classification [1] but the species that considered pathogenic to human and animal are *C. gattii and C. neoformans* [2]. *Cryptococcus neoformans* is opportunistic fungi. It is encapsulated basidiomycetous yeast caused cryptococcosis, the modern classification for *C. neoformans* is: [3] Kingdom: Mycota, Phylum: Basidiomycota, Subphylum: Basidiomycotina, Class: Tremellomycetes, Order:

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Filobasidiales, Family: Filobasidiaceae, Genus: *Filobasidiella (Cryptococcus)*, Species: *neoformans*. It is yeast form with unicellular cells characterized by its oval or spherical shape, with different diameters ranged from 4-6 mm, and surrounded by polysaccharide capsule which different in size depending on some factors such as temperature and Co2. The complete phase of *C. neoformans* is called *Filobasidiella neoformans* and it considered as an opportunistic fungus particularly in immunocompromised patients causing high percentage of morbidity and mortality, however it can infect immunocompetent but in low percentage [4]. *C. neoformans* is the second commonest opportunistic CNS infection in patients with cancer and mainly associated with lymphoma [5]. The virulence factors of *C. neoformans* include: capsule, melanin, laccase, and amanitol production, in addition ability to survive at 37 Cº, and analysis enzymes that include (proteinase, phospholipase, urease) [6]Many studies in Iraq were done about the *C. neoformans* isolation and identification from environment such as the study of [7] in Basra, 20 samples were collected from pigeon excreta. *C. neoformans* revealed in 66.7% of the examined samples. And the study of [8] showed that out of 250 dried pigeon excreta samples 40 (16%) samples were positive for the presence of *Cryptococcus neoformans* isolates in 9 of the 15 investigated locations. So some studies were isolation the *Cryptococcus neoformans* from burn patients such as the study of [9] in Baghdad the most yeast isolated was *Candida spp*. with (56.94%) followed by *Cryptococcus spp.* with (27.77%). *Cryptococcus neoformans* was recorded the higher percentage 8 (11.11%) follows by another species of *Cryptococcus*. And the study of [10] in Kufa was isolated the *C. neoformans* from from the milk of goat using Multiplex PCR as diagnostic tool. *C. neoformans* rate was 7.24% (5 out of 69).

Materials and Methods

Culture Media

Sabroud dextrose agar (Oxiod, England) (SDA) was prepared by dissolving 65 gm of medium in 1000 ml of distilled water. After autoclaving at 15 Ibis/sq. inch pressure / 121 °C for 15 minutes the medium temperature was lowered up to 50 C.

Collection of specimens

In this study 150 clinical sputum samples were collected from pulmonary patients (male and female) from (National Center for Thoracic and respiratory Diseases of medical city and Al Yarmok hospital in Baghdad) from August 2017 to March 2018.

Processing of specimens

The specimens was cultured directly on SDA, spreading by loop, and incubation at 37±1 C° for three days with daily observation [11].

Yeast Identification

The isolated yeasts were identified depending on morphology characters of fungus on culture medium and microscopic examination [12].

Culture examination

After centrifugation of sputum samples, the remains are easily cultured on SDA media. Colonies of *Cryptococcus neoformans* should be appearing on culture media within 1 to 5 days. Diagnosis is typically made by identifying the morphology characters of colonies which are white to cream in color, mucus, and silky [13].

Microscopic examination

By adding drop of (Indian ink) to the suspension of yeast that present on clean slide, and examination under microscope to observe the present or absent of capsule [14].

Identification of *Cryptococcus neoformans* by PCR and gene sequence:

After identification the yeast *C. neoformanas* through routine method depend on morphology of colony and microscopic characters. It was molecular diagnosed by PCR technique to confirm the diagnosised. The result showed that all isolates that were identified as yeast were diagnosed accurately if the length of DNA of yeast 550bp.The molecular diagnosed by PCR to support and confirm the diagnosis based on the characteristics of the demonstrated technical excellence of precision [15].

Genomic DNA Extraction

DNA was extracted by ZR Fungal/Bacterial DNA MiniPrep™ according to manufactures protocol:

1- Gel Dissociation
2- DNA Binding
3- Wash
4- DNA Elution

In DNA extraction used ZR Fungal/Bacterial DNA MiniPrep™ kit, the componantsone of the kit showed in table

Table 1- ZR Fungal/Bacterial DNA MiniPrep™ kit componants.

Item	Volume
ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm)1	50
Lysis Solution	40 ml
Fungal/Bacterial DNA Binding Buffer2	100 ml
DNA Pre-Wash Buffer3	15 ml
Fungal/Bacterial DNA Wash Buffer	50 ml
DNA Elution Buffer	10 ml
Zymo-Spin™ IV Spin Filters (Orange Tops)	50
Zymo-Spin™ IIC Columns	50
Collection Tubes	150
Instruction Manual	1

Detection of Gene ITS by Using PCR

Detection of *ITS* gene was conducted primers for amplification. A forward primer *ITS1* F: 5′-TCCGTAGGTGAACCTGCGG -3′) and a reverse primer (*ITS4*R:5′ TCCTCCGCTTATTGATATGC-3′) (17).

PCR condition

The PCR amplification was performed in a total volume of 25µl containing 1.5µl DNA, 5 µl Taq Pol PCR PreMix ,1µl of each primer (10 pmol) then distilled water was added into tube to a total volume of 25µl.

PCR program

The thermal cycling conditions were done as follows: Denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45s, 52°C for 1 min and 72 °C for 1min with final elongation steps at 72 °C for 7 min using a thermal Cycler (Gene Amp, PCR system 9700; Applied Biosystem).

Agarose gel electrophoresis of DNA

The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by exposure to ultraviolet light (302nm) after red stain staining

Preparation of sample

Three µl of the processor loading buffer has been mixed with 5 µl of the supposed DNA to be electrophorese (loading dye), after the mixing process, the process of loading DNA to holes to the holes of the gel performed. An Electric current of 7 v\c2 has been exposed for 1-2 h till the tincture has reached to the other side of the gel. The gel has been tested by a source of the UV with 336 nm after put the gel in pool contain on 30µl Red safe Nucleic acid staining solution and 500 ml from distilled water.

Sequencing and Sequence Alignment

The PCR products were separated on a 2% agarose gel electrophoresis and visualized by exposure to ultra violate light (302 nm) after ethidium bromide. Sequencing of gene was performed by national instrumentation center for environmental management (nicem) online at

(http://nicem.snu.ac.kr/main/?en_skin=index.html), biotechnology lab, machine is DNA sequencer 3730XL, Applied Biosystem), Homology search was conducted using Basic Local Alignment Search Tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm.nih.gov) and BioEdit program.

Results and Discussion

Isolation and Identification of *Cryptococcus neoformans* from clinical specimens

Result showed that a total of 150 sputum samples from suspected patient with pulmonary diseases showing fungus the cultural and microscopic examination illustrated that ten samples were belonged to the yeast *Cryptococcus* species; six were *C. neoformans* 4 male, 2 female at rate (9.23%) and four were *C. gattii* (from 2male. 2 female) at rate (6.15%), while other positive samples showed yeasts not related to *Cryptococcus* species at rate (84.61%).

Upon culturing on Sabauroud dextrose agar, colonies of *Cryptococcus neoformans* appeared spherical with white to cream color, mucous and smooth within a two days when incubated at 37±1C° Microscopic examination illustrated Globule to ovoid, single or multiple budding, yeast-like cells. It usually is surrounded by distinct, wide gelatinous capsules are present

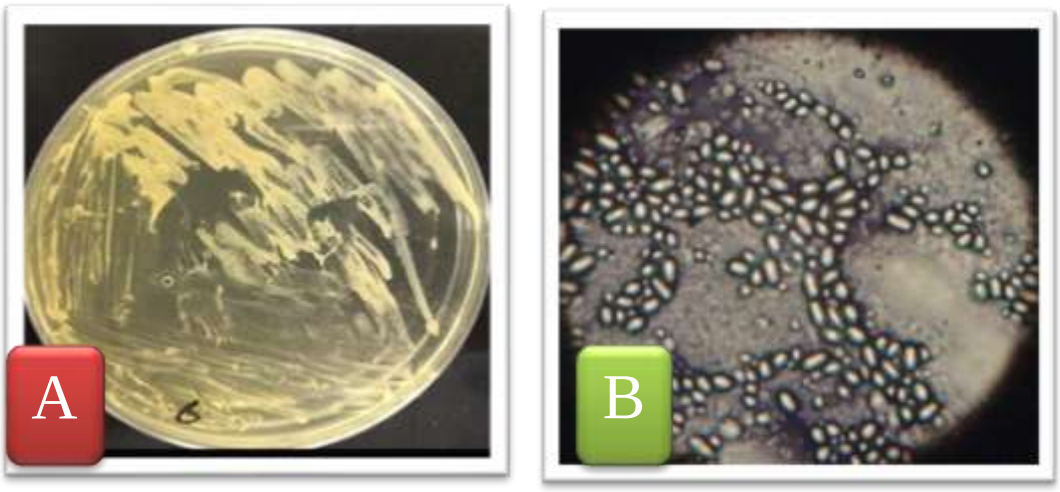

Figure 1-[A] *Cryptococcus neoformans* grown on SDA medium at 37°C after 2 days of incubation, colonies appear mucous because of the presence of a polysaccharide capsule, [B] Microscopic feature of *Cryptococcus neoformans* stained with India ink (40X), exhibiting thick polysaccharide capsule

Genomic DNA Identification

PCR assay was used for the identification of *ITS* gene using specific primer.

Extraction of Genomic DNA from *Cryptococcus neoformans* isolates

Genomic DNA was extracted successfully from *Cryptococcus neoformans* isolates using ZR Fungal DNA MiniPrepextraction kit. After the activation of (6) isolates from clinical samples, the colonies scrape method developing well on the SDA medium. After the completion of the extraction process should measure the concentration and purity of samples, Figure-2 shows the DNA bundles extracted

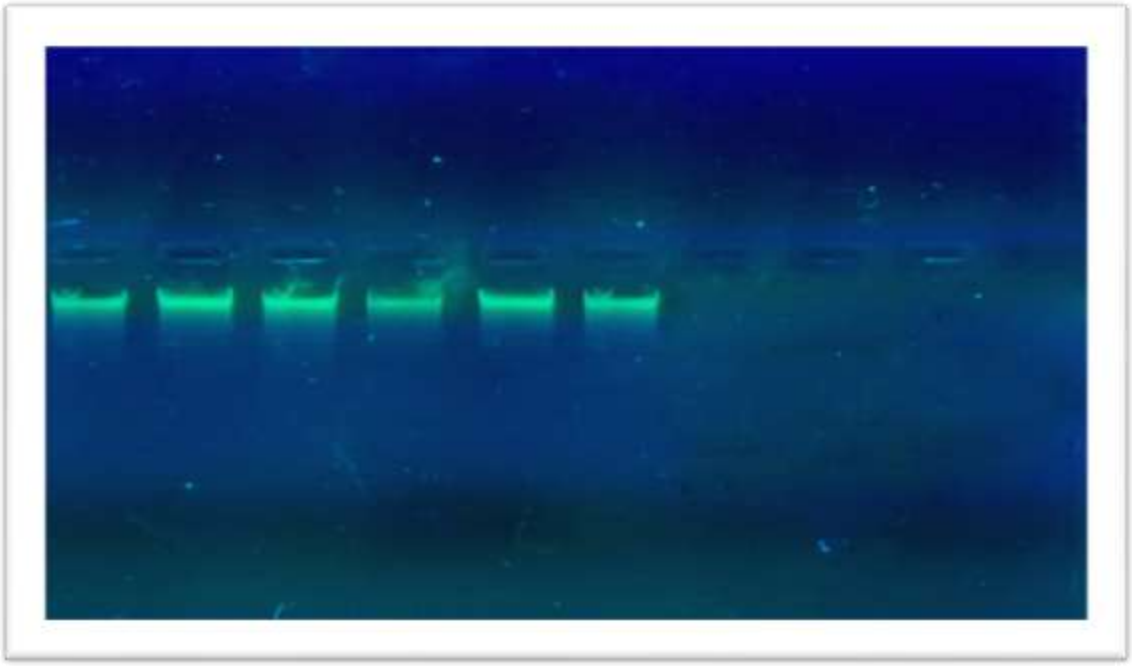

Figure 2-Agarose gel electrophoresis of the total genomic DNA for *Cryptococcus species* isolates. Fragments were detected by electrophoresis on a 1% agarose gel visualized under U.V. light after staining with ethidium bromide lan 1-6 at 5 vol /cm for 1: 5 houre.

Detection of *ITS* Gene in *Cryptococcus neoformans*

The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by exposure to ultraviolet light (302nm) after red stain staining

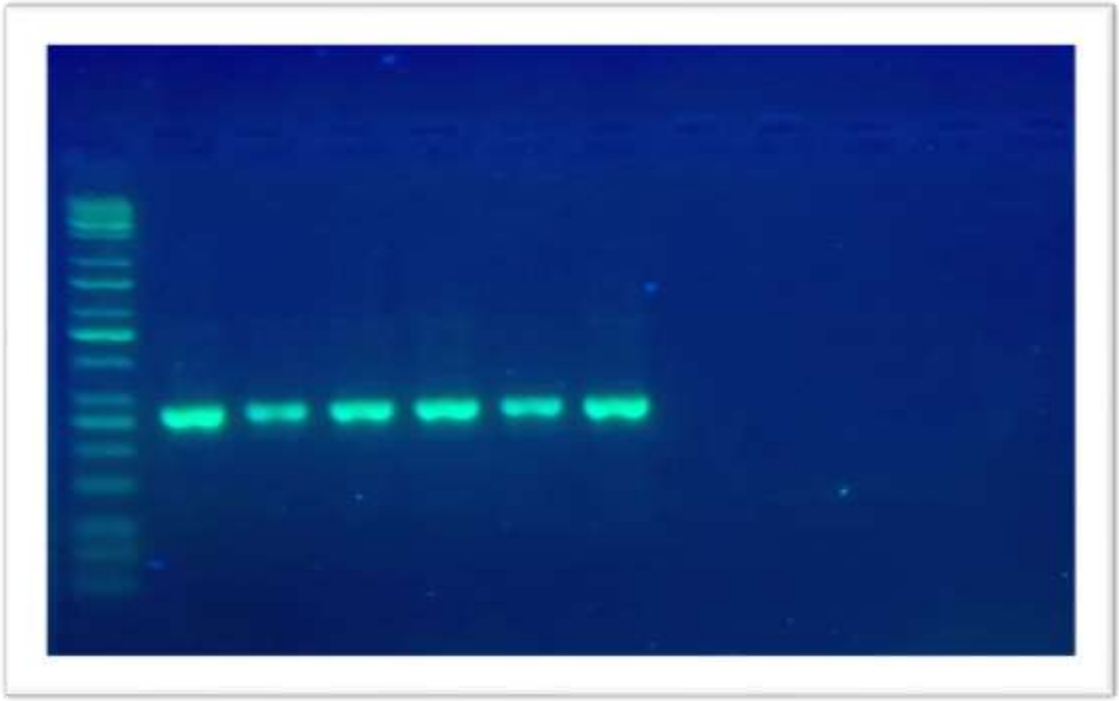

Figure 3-PCR product for *ITS* primer. The product was electrophoresis on 1.5% agarose at 5 volt/cm^2. 1x TBE buffer for 1:30 hours. N: DNA ladder (100), line (1-6) PCR product of band size 550 bp, visualized under U.V light

Sequencing and alignment of NCBI

The results indicated that a yield of single band of the desired product of *ITS* regions of *Cryptococcus neoformans* was obtained from 6 samples and sent for sequencing related to molecular weight 550bp. Six PCR product samples were sent for sequence analysis; of *Cryptococcus neoformans* isolated from clinical and 25 µl (10 pmol) from the forward primer. The samples were treated with AB13730XL Applied Biosystems machine in national instrumentation centre for environmental management NICM/USA company online. The result of the sequence was analysed by blast in the National Centre Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm.nih.gov) and BioEdit program to detect polymorphism and mutation in *ITS regions*, found 38 mutations in five *C. neoformans* isolates between 13 transversion (refers to the substitution of a (two ring) purine for a (one ring) pyrimidine and 25 transition a point mutation that changes a purine nucleotide to another purine (A ↔ G) or a pyrimidine nucleotide to another pyrimidine (C ↔ T)) and 0 deletion nucleotide.

The first clinical *Cryptococcus neoformans* isolate showed 100% compatibility as shown, and score (857) and expect (0.0) with the wild type, While the clinical *Cryptococcus neoformans* isolates number (5 and 6) showed 99% compatibility as and score (845, 854) respectively and expect (0.0) with the wild type of of ITS gene from Gene Bank as shown in (Figures- 3 and 4). Clinical isolates of *Cryptococcus neoformans* isolates number (2 and 4) showed 98% compatibility as sho, and score (838, 814) respectively and expect (0.0) with the wild type of of *ITS* gene from Gene Bank as shown in (Table) and (Figure). The clinical isolates of *Cryptococcus neoformans* isolates number (3) showed 97% compatibility as shown in (table), and score (801) and expect (0.0) with the wild type of of *ITS* gene from Gene Bank as shown (Figure).

Cryptococcus neoformans isolate JKMMVBHU3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence ID: KJ175191.1Length: 538Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
838 bits(928)	0.0	481/491(98%)	0/491(0%)	Plus/Plus

Query 1
TGCTTAATTGCACCACATGTGTTTTTCTTTGAACAAATTTCTTTGGCGGTGGGCCCAGTC
60
Sbjct 48
TGCTTAATTGCACCACATGTGTTTTTTATTGAACAAATTTCTTTGGTGGCGGGAGCAATC
107
Query 61
CTACCGCCAGAGGTTATAACTAAACCAAACTTTTTATTTACAGTCACACCAGATTTATCA
120
Sbjct 108
CTACCGCCAGAGGTTATAACTAAACCAAACTTTTTATTTACAGTCAAACTTGATTTATCA
167
Query 121
TTACAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCA
180
Sbjct 168
TTACAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCA
227
Query 181
GCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTGAACGC
240

Sbjct 228
GCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTGAACGC
287
Query 241
ACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTTCTCCCTCAAA
300
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 288
ACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTTCTCCCTCAAA
347
Query 301
CCCCCGGGTTTGGTGTTGAGCAATACGCTAGGTTTGTTTGAAAGAATTTACGTGGAAACT
360
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 348
CCCCCGGGTTTGGTGTTGAGCAATACGCTAGGTTTGTTTGAAAGAATTTACGTGGAAACT
407
Query 361
TATTTTAAGCGACTTANGGTTTATCCAAAAACGCTTATTTTGCTAGTGGCCACCACAATT
420
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 408
TATTTTAAGCGACTTANGGTTTATCCAAAAACGCTTATTTTGCTAGTGGCCACCACAATT
467

Query 421
TATTTCATAACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAAT
480
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 468
TATTTCATAACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAAT
527
Query 481 AAGCGGAGGAA 491
|||||||||||
Sbjct 528 AAGCGGAGGAA 538

Cryptococcus neoformans isolate JKMMVBHU3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence ID: KJ175191.1Length: 538Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
801 bits(888)	0.0	466/479(97%)	0/479(0%)	Plus/Plus

Query 1
TGCTTAATTGCACCACATGTGTTTTTTATTGAACAAATTTCTTTGGTGGCGGGAGCAGTC
60
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 48
TGCTTAATTGCACCACATGTGTTTTTTATTGAACAAATTTCTTTGGTGGCGGGAGCAATC
107
Query 61
CTACCGCCAGAGGTTATAACTAAAACCAATTTTTTATTAACTGTCAAACTTGATTTATCA
120
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct 108
CTACCGCCAGAGGTTATAACTAAACCAAACTTTTTATTTACAGTCAAACTTGATTTATCA
167
Query 121
TTACAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCA
180
Sbjct 168
TTACAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCA
227
Query 181
GCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTGAACGC
240
Sbjct 228
GCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTGAACGC
287
Query 241
ACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTTGAGCGTCGTTTCTCTCTCAAA
300
Sbjct 288
ACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTTCTCCCTCAAA
347

Query 301
CCCCCGGGTTTGGTGTTGAGCAATACGCTAGGTTTGTTTGAAAGAATTTACGTGGAAACT
360
Sbjct 348
CCCCCGGGTTTGGTGTTGAGCAATACGCTAGGTTTGTTTGAAAGAATTTACGTGGAAACT
407
Query 361
TATTTTAAGCGACTTANGGTTTATCCAAAAACGCTTATTTTGGTAGTGGCCACCACAATT
420
Sbjct 408
TATTTTAAGCGACTTANGGTTTATCCAAAAACGCTTATTTTGCTAGTGGCCACCACAATT
467
Query 421
TATTTCATAACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATTAA
479
Sbjct 468
TATTTCATAACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAA
526

Cryptococcus neoformans isolate JKMMVBHU3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence ID: KJ175191.1Length: 538Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
814 bits(902)	0.0	468/478(98%)	0/478(0%)	Plus/Plus

Query 1
TTGCTTAATTGCACCACATGTGTTTTTTATTGAACAAATTTCTTTGGTGGCGGGAGCAAT
60

Sbjct 47
TTGCTTAATTGCACCACATGTGTTTTTTATTGAACAAATTTCTTTGGTGGCGGGAGCAAT
106
Query 61
CCTACCGCCAGAGGTTATAACTAAACCAAACTTTTTATTTACAGTCAAACTTGATTTATC
120

Sbjct 107
CCTACCGCCAGAGGTTATAACTAAACCAAACTTTTTATTTACAGTCAAACTTGATTTATC
166
Query 121
ATTACAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGC
180

Sbjct 167
ATTACAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGC
226
Query 181
AGCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTGAACG
240

Sbjct 227
AGCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTGAACG
286

Query 241
CACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTTGAGCGTCGTTTCTCTCTCAA
300

Sbjct 287
CACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTTCTCCCTCAA
346
Query 301
ACCCCCGGGTTTGGTGTTGAGCAATACGCTAGGTTTGTTTGAAAGAATTTACGTGGAAAC
360

Sbjct 347
ACCCCCGGGTTTGGTGTTGAGCAATACGCTAGGTTTGTTTGAAAGAATTTACGTGGAAAC
406
Query 361
TTATTTTAAGCGACTTANGGTTTATCCAAAAACGCTTGCGGCGCTAGTGGCCACCACAAT
420

Sbjct 407
TTATTTTAAGCGACTTANGGTTTATCCAAAAACGCTTATTTTGCTAGTGGCCACCACAAT
466
Query 421
TTATTTCATAACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATC 478

Sbjct 467
TTATTTCATAACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATC 524

Cryptococcus neoformans isolate JKMMVBHU3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence ID: KJ175191.1Length: 538Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
845 bits(936)	0.0	475/478(99%)	0/478(0%)	Plus/Plus

Query 1
TTGCTTAATTGCACCACATGTGTTTTTTATTGAACAAATTTCTTTGGTGGCGGGAGCAAT
60
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 47
TTGCTTAATTGCACCACATGTGTTTTTTATTGAACAAATTTCTTTGGTGGCGGGAGCAAT
106
Query 61
CCTACCGCCAGAGGTTATAACTAAACCAAACTTTTTATTTACAGTCAAACTTGATTTATC
120
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 107
CCTACCGCCAGAGGTTATAACTAAACCAAACTTTTTATTTACAGTCAAACTTGATTTATC
166
Query 121
ATTACAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGC
180
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 167
ATTACAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGC
226
Query 181
AGCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTGAACG
240
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 227
AGCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTGAACG
286
Query 241
CACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTTCTCCCTCAA
300
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 287
CACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTTCTCCCTCAA
346
Query 301
ACCCCCGGGTTTGGTGTTGAGCAATACGTTGGGTTTGCTTGAAAGAATTTACGTGGAAAC
360
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 347
ACCCCCGGGTTTGGTGTTGAGCAATACGCTAGGTTTGTTTGAAAGAATTTACGTGGAAAC
406
Query 361
TTATTTTAAGCGACTTANGGTTTATCCAAAAACGCTTATTTTGCTAGTGGCCACCACAAT
420
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct 407
TTATTTTAAGCGACTTANGGTTTATCCAAAAACGCTTATTTTGCTAGTGGCCACCACAAT
466
Query 421
TTATTTCATAACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATC 478
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 467
TTATTTCATAACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATC 524
Cryptococcus neoformans isolate JKMMVBHU3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence ID: KJ175191.1Length: 538Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
854 bits(946)	0.0	478/480(99%)	0/480(0%)	Plus/Plus

Query 1
TGCTTAATTGCACCACATGTGTTTTTCTTTGAACAAATTTCTTTGGTGGCGGGAGCAATC
60
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 48
TGCTTAATTGCACCACATGTGTTTTTTATTGAACAAATTTCTTTGGTGGCGGGAGCAATC
107
Query 61
CTACCGCCAGAGGTTATAACTAAACCAAACTTTTTATTTACAGTCAAACTTGATTTATCA
120
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 108
CTACCGCCAGAGGTTATAACTAAACCAAACTTTTTATTTACAGTCAAACTTGATTTATCA
167
Query 121
TTACAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCA
180
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 168
TTACAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCA
227
Query 181
GCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTGAACGC
240
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 228
GCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTGAACGC
287
Query 241
ACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTTCTCCCTCAAA
300
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 288
ACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTTCTCCCTCAAA
347
Query 301
CCCCCGGGTTTGGTGTTGAGCAATACGCTAGGTTTGTTTGAAAGAATTTACGTGGAAACT
360
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct 348
CCCCCGGGTTTGGTGTTGAGCAATACGCTAGGTTTGTTTGAAAGAATTTACGTGGAAACT
407
Query 361
TATTTTAAGCGACTTANGGTTTATCCAAAAACGCTTATTTTGCTAGTGGCCACCACAATT
420
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 408
TATTTTAAGCGACTTANGGTTTATCCAAAAACGCTTATTTTGCTAGTGGCCACCACAATT
467
Query 421
TATTTCATAACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAAT
480
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 468
TATTTCATAACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAAT
527

Cryptococcus neoformans isolate JKMMVBHU3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence ID: KJ175191.1Length: 538Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
857 bits(950)	0.0	477/477(100%)	0/477(0%)	Plus/Plus

Query 1
TTGCTTAATTGCACCACATGTGTTTTTTATTGAACAAATTTCTTTGGTGGCGGGAGCAAT
60
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 47
TTGCTTAATTGCACCACATGTGTTTTTTATTGAACAAATTTCTTTGGTGGCGGGAGCAAT
106
Query 61
CCTACCGCCAGAGGTTATAACTAAACCAAACTTTTTATTTACAGTCAAACTTGATTTATC
120
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 107
CCTACCGCCAGAGGTTATAACTAAACCAAACTTTTTATTTACAGTCAAACTTGATTTATC
166
Query 121
ATTACAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGC
180
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 167
ATTACAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGC
226
Query 181
AGCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTGAACG
240
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 227
AGCGAAATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTGAACG
286

Query 241
CACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTTCTCCCTCAA
300
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 287
CACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTTCTCCCTCAA
346

Query 301
ACCCCCGGGTTTGGTGTTGAGCAATACGCTAGGTTTGTTTGAAAGAATTTACGTGGAAAC
360
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 347
ACCCCCGGGTTTGGTGTTGAGCAATACGCTAGGTTTGTTTGAAAGAATTTACGTGGAAAC
406
Query 361
TTATTTTAAGCGACTTANGGTTTATCCAAAAACGCTTATTTTGCTAGTGGCCACCACAAT
420
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 407
TTATTTTAAGCGACTTANGGTTTATCCAAAAACGCTTATTTTGCTAGTGGCCACCACAAT
466
Query 421
TTATTTCATAACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATAT 477
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 467
TTATTTCATAACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATAT 523

Figure 4-Sequencing of sense flanking the partial *gliP* gene in *Cryptococcus neoformas* compared with standard *gliP*, obtained from Gene Bank. Query represents of sample; Subject represent of database of National Center Biotechnology Information (NCBI).

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