Molecular Serotyping of *Cryptococcus neoformans* Isolated from Environmental Sources

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Abstract

In addition to study the genetic information of capsular polysaccharide gene by PCR assay .This study is also, aimed to determine the molecular serotyping among different environmental resources samples of *Cryptococcus neoformans*, by using phenotypic and molecular techniques.

So, this study is designed to investigate the molecular serotyping of *C. neoformans* at a genetic level in order to identify the serotypes belong to this yeast and to differentiate them from each other.

To achieve these goals, environmental resources samples were collected from different area of Najaf province during (2015). Culture tests are done for samples in The same aspect includes identification by molecular methods. The polymerase chain reaction method (PCR) is used to identify the *Cryptococcus* serotype genetically. Depending on amplification of genomic DNA, the entire isolates are diagnosed as Cryptococcus yeasts. However, the most recurring species are the *C. neoformans* that rated 80%, while the *C. gattii* that rated 20% is the less recurring one. The results of amplifing the genomic DNA, using specific primer, show bands with molecular weight that is (387 bp) approximately for 12 isolates. However, the other species showed band with molecular weight that was (359 bp).

The isolates are diagnosed genetically using the RFLP – PCR method as restriction enzyme with (Bsm F1, Hpa II) through which systematic important information appears for the genetic relations in the studied isolates that are related for two types.

PCR assay revealed that five different serotypic patterns are detected as: serotype A ,serotype B , serotype C , serotype D and serotype AD .It can be concluded that the PCR targeting the genomic DNA and the REFP – PCR are rapid and simple techniques for serotyping *C. neoformans* and are useful not only for discrimination of *C. neoformans* from its related species, but also for epidemiological study at the molecular level. This study provides the detection genetically serotyping of *C. neoformans* which is isolated from different environmental resources in Najaf province

Key words: *Cryptococcus neoformans*, *Cryptococcus gattii*, environmental sorces, Molecular serotyping, PCR, REA-PCR.

الخلاصة

بالإضافة الى معرفة لمورث (الكبسولة 59) باستعمال تقنية تفاعل سلسلة انزيم البلمرة في نماذج العزلات المأخوذة من مصادر بيئية مختلفة وكذلك هدفت الدراسة الحالية الى التنميط الجزيئي ضمن عزلات من مصادر بيئية مختلفة لخميرة المكورات الخبيئة ، باستخدام بعض التقنيات المظهرية والجزيئية.

تعتبر خميرة المكورات الخبيئة من الفطريات البازيدية المعزولة من مصادر بيئية مختلفة والتي قد تسبب مشاكل صحية خطيرة للانسان وبالاخص المرضى المثبطين مناعياً، وقد صممت هذه الدراسة للتحري عن التنميط الجزيئي لخمائر المكورات الخبيئة على المستوى الوراثي لتحديد الانماط المصلية العائدة لهذه الخميرة والتمييز بينها.

وللدراسة الجزيئية لجين الكابسولة (المحفظة) المتعددة السكريات باستخدام فحص تفاعل سلسلة انزيم البلمرة تضمن أيضاً التشخيص بالطرق الجزيئية، فقد تم استخدام طريقة تفاعل سلسلة انزيم البلمرة لتشخيص النمط المصلي للخميرة بصورة وراثية بالاعتماد على تضخيم الـ DNA. تم تشخيص العزلات كخميرة المكورات الخبيئة، فضلاً عن ذلك كان النوع الاكثر تردداً هو *neoformans* بلغ العدد الكلي بينما النوع *gattii* وكان الاقل تردداً. اظهرت نتائج تضخيم الـ DNA، باستخدام البادئ المتخصص، حزم بأوزان جزيئية (387 زوج قاعدة) لـ (12) عزلة, على اية حال النوع الثاني اظهر حزم بوزن جزيئي (359 زوج قاعدة). كما وتم تشخيص العزلات بصورة وراثية على مستوى نمط مصلي باستخدام طريقة RFLP مع الانزيمات القاطعة (Bsm F1) و (Hpa II) والتي من خلالها ظهرت معلومات مهمة للعزلات المدروسة التي هي متعلقة بالنوعين من الخميرة المدروسة.

اظهر اختبار تفاعل سلسلة انزيم البلمرة خمسة انماط مصلية مختلفة كانت محددة، النمط المصلي A ,النمط المصلي B النمط المصلي C النمط المصلي D والنمط المصلي AD وكانت الاختلافات معنوية قد ظهرت بين الانماط المصلية المختلفة. ومن هذا نستنتج بان اختبار تفاعل سلسلة انزيم البلمرة كان مهدفاً الـ DNA النووي وكان اختبار RFLP سريعاً وبسيطاً تقنياً لتنميط الخميرة جزيئياً ومقيداً ليس فقط لتفريق الخميرة عن الانواع ذات العلاقة بها ولكن مفيد ايضاً للدراسات الوبائية على المستوى الجزيئي. هذه الدراسة وفرت التنميط المصلي بصورة جزيئية لخميرة المكورات الخبيئة المعزولة من مصادر بيئية مختلفة في محافظة النجف.

نستنتج من هذه الدراسة بأن خميرة المكورات الخبيئة من الفطريات المرضية المنتهزة للفرص، ولها دور اساسي في احداث المرض في الكائنات (المضائف) المعتلة مناعياً – او المثبطة مناعياً – والذي تم تأكيد تشخيصه في هذه الدراسة باستعمال بادئات خاصة بهذه الخميرة.

الكلمات المفتاحية: المكورات الخبيئة, مصادر بيئية (PCR,PCR,REA–), تنميط مصلي وجزيئي.

Introduction

Cryotococcosis is potentially life-threatening invasive fungal infection and is often associated with disseminated disease. It mainly occurs in immunocompromised hosts such as acquired immunodeficiency syndrome patients and less frequently in immunocompetent patients. The most commonly affected sites in immunocompetent host are the lungs and central nervous system, while the lymph nodes usually remain unaffected.

The infection process begins with inhalation of infectious agents (spores of dessicated yeasts) resulting in a primary pulmonary infection, which can further disseminate to the central nervous system causing meningitis. Cryptococci are particularly difficult to treat with antifungal agents due to their ability to manipulate and exist within the host's immune response.

Species in the genus *Cryptococcus*, *C.neoformans* and *C.gattii*. *C.neoformens* (serotypes A, D, and AD) is found worldwide and causes cryptococcosis most frequently in AIDS patients *C. gattii* (serotypes B and C) is geographically restricted and is infrequently diagnosed in AIDS patients (Jianghan chen, *et al*; 2008).

Cryoptococcus neoformans and *Cryptococcus gattii* are the two etiologic agents of cryptococcosis. They belong to the phylum basidiomycota and can be readily distinguished from other pathogenic yeasts such as Candida by the presence of a polysaccharide capsule, formation of melanin, and urease activity, which all function as virulence determinants. infection proceeds via inhalation and subsequent dissemination to the central nervous system to cause meningoencephalitis. The most common risk for cryptococcosis caused by *C. neoformans* is AIDS, whereas infections caused by *C. gattii* are more often reported in immunocompetent patients with undefined risk than in the immunocompromised (Kyung *et al*., 2014)

A variety of characteristics have been implicated in *C. neoformans* success as a pathogen. The primary virulence factor of this pathogen, however, is the display of an elaborate polysaccharide capsule, which is required for virulence and is unique among the pathogenic fungi (Deepa *et al*., 2014). Polysaccharides impede the host immune response, and the size of the capsule affects phagocytosis of *C. neofarmans*; these in turn alter the balance of free and host cell-engulfed fungi, influencing key events, such as clearance, latency, and dissemination. Notably, capsule thickness changes dramatically in response to environmental conditions, mediated by signaling pathways that sense the host environment and detect nutrient limitation, increased temperature, and Co_2 levels to investigate etiological agent the fungal infections cryptococcosis and to identify *Cryptocossus* by isolating from environmental sources and determine

the serotypes and genotypes of these environmental isolates need using rapid methods which are sensitive and specific for diagnosis. (Anna Vecchiarelli, *et al*, 2013)

Conventional laboratory-based methods for diagnosis of fungal infection remain useful but are often slow and lack sensitivity. The newer rapid, sensitive and specific culture-independent methods for the diagnosis of Cryptococcal meningitis offer great promise for improved diagnosis and management of these serious infections (Michael A. p faller, 2015).

Molecular diagnosis techniques represent an important breakthrough in the diagnostic practice. In the present study used conventional PCR and restriction fragment length polymorphism analysis; two molecular methods, based on the sequence characteristics of a fragment of the CAP59 gone required for capsule biosynthesis both methods allow for the rapid and reliable identification of all *C. neoformans* serotypes.

The aims of the present study are:

- To assess the serotype for *Cryptococcus neoformans* isolates, to molecular techniqal uses, To determine the fungal isolate associated with the environmental sources in Najaf provence.
- To determine the molecular serotype in the fungal isolate.

Objective of the Study

and molecular technique

- Confirmed identification of the strains and serotypes by the use of PCR technique by using of the Cryptococcal polysaccharide gene.
- Molecular characterization for serotype by using the specific sequences for these serotypes by PCR technique, or restriction fragment length polymorphic DNA technique.

The Isolation and identification of the yeast:

The isolation was submitted to the PCR technique.

Fermentation test:

This test had been used to determine the ability of the isolates to ferment (glucose, maltose, lactose, inositol, raffinose and sucrose) by using the carbohydrate broth, this test had been done by putting a colony from the growing yeast in a stabbing method in the test tube of the carbohydrate broth, then incubated in 30˚c for 24 – 48 hrs. the changing in the color media from pink to yellow would give an indication of the positive results of the test.

Identification by PCR:

For Molecular Identification to the Cryptococcus PCR technique was used by utilizing primer pairs for fifteen samples. CAP59 gene amplification, two primers (CH-CAP 59 Forward (5′- CCTTGCCGAAGTTCGAAACG -3′), and CH-CAP59 Revers (5′-AATCGGTGGTTGGATTCAGTGT- 3′) were designed from the conserved regions of the CAP59 gene. DNA was extracted using a method below.

Preparing the primers:

The primers were prepared depending on manufacturer's instructions by dissolving the lyophilized primers with TE buffer to form stock solution with concentration of 100pmol/µl, after spinning down and stay overnight at 4C^{o}, primers working solution were prepared by diluted the stock solution with TE buffer, using the equation $C_1V_1=C_2V_2$ to get final working solution (20pmol/µl) for both primers.

Culture and Harvest the isolates:

Cyrptococcus isolation were growth in 3 ml Sabouraud Dextrose broth to the early stationary phase for 24hr at 37^{o}C with agitation in shaking incubator. The cultures were harvested and concentrated by centrifugation for 5minutes at 6000rpm, and discarded the supernatant . Yeast cells washed 3 times with normal saline, and repelleted in 1.5ml eppendorf tube at 12000 rpm for 5 minutes.

DNA Template Extraction and purification:

The Cryptococcus DNA extraction was performed according to [Promega technical manual] for Isolating Genomic DNA from Yeast, as follows:

- One ml of a Cryptococcus species [culture grown for 20 hours in Sabouraud Dextrose broth] was added to a 1,5ml micro centrifuge tube.
- The tubes were centrifuged at 14,000 r.p.m for 2 minutes to pellet the cells. The supernatant was removed.
- The cells were resuspended thoroughly in 293µl of [50 mM EDTA].
- Seven and a half µl of [20mg/ml lyticase] were added and gently mixed 4 times by pipette.
- The samples were incubated at 37°C for 60 minutes [to digest the cell wall]. Then cooled to room temperature.
- The samples were centrifuged at 14,000 r.p.m for 2 minutes and then the super natant was removed.
- Three hundred µl of [Nuclei Lysis solution] were added to the cell pellet and gently mixed by pipette.
- One hundred µl of [protein Precipitation solution] were added with vortex vigorously at high speed for 20 seconds.
- The samples were left [on ice] for 5 minutes.
- Centrifugation was done at 14,000 r.p.m for 3 minutes.
- The supernatant containing [the DNA] was transferred to a clean 1.5 ml microcentrifuge tube containing [300 µl of room temperature isopropanol].
- The tubes contents were gently mixed by [inversion] until the thread – like [strands of DNA] form a visible mass.
- Centrifugation was done at 14,000 r.p.m for 2 minutes.
- The supernatant was carefully decanted and the tubes were [drained on clean] absorbent paper. 300 µl of room temperature 70% ethanol were added and the tubes were gently inverted several times to wash [the DNA pellet].
- Centrifugation was done at 14,000 r.p.m for 25 minutes. All of the ethanol was carefully aspirated.
- The tubes were drained on [clean absorbent paper] and the pellet was allowed to [air dry] for 15 minutes.
- Fifty µl of [DNA Rehydration solution] were added.
- One point five µl of [RNase solution] were added to each purified DNA sample with vortex for 1 second. The tubes were centrifuged briefly for 5 seconds to collect the liquid and incubated at 37°C for 15 minutes.
- The DNA was [rehydrated] by incubating overnight at 4°C.
- The DNA stored at 2°-8°C.

PCR of DNA samples:

PCR of DNA samples was carried out in a final reaction volume of 25µl in thermal cycler, quantity and concentration of various components used for PCR of DNA samples are shown in Table (1).

Table (1): Quantity and concentration of various components used in PCR

No.	Various components used in PCR	Concentration
1	Robust hot start ready mix contains 2mµ $MgCl_2$, at 1X	12.5µl
2	Forward primer (10µm) 10 pmole/µl	2µl
3	Reverse primer (10µm) 10 pmole/µl	2µl
4	Template DNA (62,5 ng)	3µl
5	Distilled water	5.5µl
6	Final reaction volume	25µl

Gene amplification: (Thermocycling conditions):

According to the Experimental Protocol of AccuPower® TLAPCR pre Mix tube, the PCR reaction mixture was prepared as follows:

- Three µl template DNA (62.5ng) and 4µl of each primer (10 pmole/µl, 2µl forward and 2µl reverse), were added to each [AccuPower® TLAPCR Pre Mix tube].
- Distilled water was added to [AccuPower® TLAPCR Pre Mix tubes] to the final volume of 25 µl.
- The tubes were mixed with vortex to dissolve the lyophilized blue pellet, and briefly spined down.
- Preforming PCR of samples (The PCR reaction conditions was listed in table7).

All sequences were amplified individually ,using [oligonucleotide primers], their sequence and size of the primers were listed in (table 8). As describe by [Lee, *et al*., (2004)]

Steps and conditions of thermal cycling in PCR were shown in table (2)

Table (2): PCR cycling conditions:

Step	Temperature	Time	No. of cycles
Initial denaturation	95°c	10 min.	1 cycle
Denaturation	95°c	30 s	35 cycles
Annealing	60°c	30 s	35 cycles
Elongation	72°c	45 s	35 cycles
Final elongation	72°c	7 min.	1 cycle

Specific sense primers for Cryptococcus neoformans designed to amplify DNA fragment by PCR shown in table (3)

Table (3): Primers used in this study

Organism	Primer	Sequence (5-3)	Length
C. neoformans	Cap 59 forward	CCTTGCCGAAGTTCGAAACG	42bp
	Cap 59 reverse	AATCGGTGGTTGGATTCAGTGT	

Electrophoresis and Visualization:

The gel electrophoresis method was done according to as follows:

1. Preparing the Gel:
   - A 1% agarose gel or the running buffer was made by mixing 1g agarose with 90ml distilled water and 10ml (10 X TBE buffer).
   - The mixture was heated in a microwave oven until all the agarose powder was melted and the solution started to boil in 250ml flask.
   - The gel solution was then left to cool (to approximately 65°c).
   - Five µl of ethidium bromide solution (10mg/ml) were added after the mixture was cooled, and gently mixed.
   - The gel was poured slowly into a gel rack, the comb was set at one side of the gel, and any bubbles in the solution removed. The gel was allowed to del (20 to 30 minutes).
   - After 20 minutes, when the gel had solidified, the comb was removed, [the gel, together with the rack], was soaked into a chamber with 1 X TBE gel running buffer. The gel was placed with the wells facing the electrode that provide the negative current (Cathode).
2. Loading and Running the Gel:
   - A DNA ladder was loaded into the first well. This was used to determine the absolute size of the separated DNA strand by comparing their migration with that of the ladder.
   - The samples were loaded into the wells (5 µl for each) without adding loading dye).
   - The lid of the electrophoresis champer was closed and the current was applied. The gel was ran at 70 volts for 1 hour.
   - The ethidium bromide stained gel was visualized under UV light and photographed.

Identification of *Cryptococcus neoformans*

Finally by the polymerase chain reaction (PCR) technique using specific primers.

Fermentation Test:

This test was done by using the carbohydrate broth to detect ability to ferment sugars, so that all the isolated of yeast do not appear any ability to ferment the sugars, and this appears when the color of the media do not change from the pink to the yellow color as Shawn in (Tab. 4).

Assimilation Test:

The *C. neoformans* showed their ability to assimilate Glucose, sucrose, Inositol and in weak manner, the Raffinose all members of the genus *Cryptococcus are* non – fermentative, assimilate Inositol and produce urease,

Table (4): The results of sugars fermentation and assimilation

Sugars	Fermentation	Assimilation
Glucose	**-**	**+**
Sucrose	**-**	**+**
Lactose	**-**	**-**
Maltose	**-**	**-**
Inositol	**-**	**+**
Raffinose	**-**	**+**

Furthermore, all the above tests were corresponding with a reference isolate of *C. neoformans* obtained from the Microbiology Department in the college of Veterinary Medicine in Baghdad University.

Molecular study:

Extraction of genomic DNA:

In the present study, the method used for extracting genomic DNA was previously described in details in chapter three. Fifteen samples of *Cryptococcus* spp. Were subjected to DNA extraction.

Briefly, the colonies obtained from cultures of different environmental sources were subject to DNA extraction utilizing (the genomic DNA mini kit/cultured).PCR product appeared as a single unique clear band on ethidiume bromide stain of gel electrophoresis.

The confirmation by PCR:

The use of the PCR to identify *C. neoformans* at variety, serotype and even molecular (genotype) levels become extended to improve diagnostic tests adversity of methods have been developed and this were agreement with.

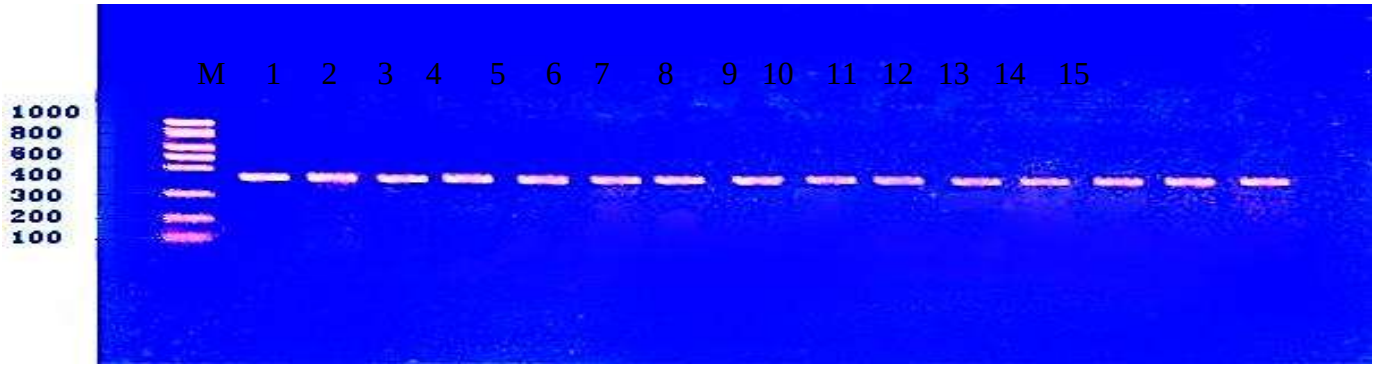
Molecular diagnosis by conventional PCR methods:

Molecular diagnosis had been made by conventional PCR for senotype determine for fifteen isolates, where complete genome was used, DNA template, by primer pairs, for isolated samples in this study, so all isolates were diagnosed as *Cryptococcus, C. neoformans* has been the most frequently.

The amplification result showed bands with molecular weight ~ 387bp for fifteen isolates of *Cryptococcus*, so these results were agreed with (Enache-Angaulvant *et al*, 2007).

Molecular diagnosis by PCR- RFLP methods:

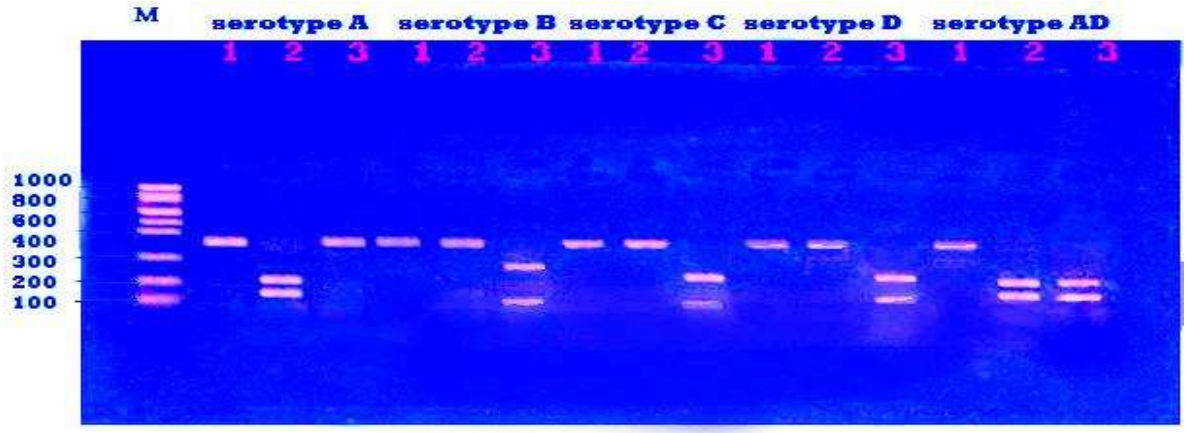
From this method, important and clear classified information were obtained from the relationship between *C. neoformans* spp., so in order to established clear molecular serotype for isolated yeast used in this study, amplification product had been obtained by the primer pairs specific with capsular polysaccharide gene (Cap 59), two restriction enzymes (HpaII) (BsmF1) had been used, so that results for digestion product with the two restriction enzymes, had been shown that there were variation in the studied serotype according to the band type in agarose gel electrophoresis.



(Fig.1): Electrophoresis amplification product on agarose gel for fifteen isolates of *C. neoformans* (387bp)

M: present the DNA marker (100bp). Lane: 1-15 indicate to fifteen isolates of C. *neoformans (agarose* gel 2%, 85 volt, 1.5hour).

From the resultes above that the band size was (387bp)and refers to the PCR product was *Cryptococcus neoformans.*



(Fig.2): Electrophoresis amplification product on agarose gel for fifteen isolates of *C. neoformans*

M: present the DNA marker (100bp). Lane:1-15 PCR product of C.*neoformans*/ C.*gattii complex*

(agarose *gel 2%, 85 volt, 1.5hour).*

All No.1 indicates to Amplicon(PCR product)

All No.2 Indicates to restriction enzyme $BsmF_I$ that was used in this study

All No.3 indicates to restriction enzyme HpaII that was used in this study

From the figure above the results show that the enzyme BsmFI was used for all isolated, but it works on serotype A,AD and did not work on another, serotype and this refers to the restriction site did not find in this fragment.

The definitive diagnosis of invasive fungal infections is usually based upon the isolation and identification of a specific etiologic agent in culture, and/or on the microscopic demonstration of the etiologic agent in histopathologic or other clinical specimens. The management of invasive fungal infections is, however, often hampered by difficulties in obtaining good specimens for histopathologic or microbiologic investigation, and by the fact that fungal cultures sometimes become positive only late in the infection. In this situation, other methods are essential to allow early diagnosis and treatment. New procedures, based on the detection of fungal

DNA in clinical material, are presently being developed (Chen, S.C., *et al*, 2002; Yeo, S.F., et al, 2002). These molecular tests offer great hope for the rapid detection and identification of difficult-to-culture organisms, and for rapid diagnosis directly from clinical samples. However, few DNA-based tests are currently available for routine clinical use.

The PCR assay was shown to be a valuable tool for detecting *Cryptococcus* organisms from different samples from infected organs content and that were consistent with (Al-Tamemy, 2009). Several advantages were reported for the PCR assay over the current conventional methods used to identify *Cryptococcus* species, the major advantage being the time taken compared to conventional methods which require several days to isolate and identify the organism, and that were agreement with. Another advantage is the need for minimal sample preparation because isolation of living organisms is not necessary. The assay is also not affected by contamination with other microbes that might be present in samples for isolation ,and that were constant with.

Amplification of DNA by PCR:

Amplification of DNA [extracted from colonies] was positive in all samples. According to the primer size, the amplicon runs at the level of 387bp on gel electrophoresis compared to a control DNA marker size.

Amplification of DNA reviled the presence of a single amplified product at the level 387bp in (15) samples of the environmental sources.

From the results show in the figure (1) the current study have diagnosed the *Cryptococcus* as shown in table (5):

Lane1:	serotype A	variety *grubii*	392bp
lane2:	serotype A	variety *grubii*	392bp
Lane3:	serotype A	variety *grubii*	392bp
Lane4:	serotype B	C. *gattii*	387bp
Lane5:	serotype B	C. *gattii*	387bp
Lane6:	serotype B	C. *gattii*	387bp
Lane7:	serotype C	C. *gattii*	359bp
Lane8:	serotype C	C. *gattii*	359bp
Lane9:	Serotype C	C. *gattii*	359bp
Lane10:	Serotype D	variety *neoformans*	375bp
Lane11:	Serotype D	variety *neoformans*	375bp
Lane12:	Serotype D	variety *neoformans*	375bp
Lane13:	Serotype AD	*C. neoformans*	387bp
Lane14:	Serotype AD	*C. neoformans*	387bp
Lane15:	Serotype AD	*C. neoformans*	387bp

From the results mentioned above, the present study showed that the PCR products that runs on agarose gel electrophoresis, treated with two restriction enzymes (BsmF1, HpaII), have different molecular weight, ranging from (359bp – 392bp), so the DNA band from serotype A have the highest molecular weight of 392bp, serotype B have the molecular weight of 387bp, serotype C have the lowest molecular weight of 359bp, serotype D have the molecular weight of 375bp, and the serotype AD have the molecular weight (as the serotype B) of 387bp.

From these results, explanation for the presence of multiple band in lines (2,6, 9,12,14,15) is due to the action of restriction enzymes, these multiple bands belong to *Cryptococcus neoformans* serotype AD (hybrid) lane 13,14,15 multiple bands belong

to *Cryptococcus gattii* serotype B, lane 4,5,6, multiple bands belong to *C. neoformans* serotype A lone 1,2,3, multiple bands belong to *C. gattii* serotype C lone 7,8,9, and so on.

The results obtained by (Enache- Angoulvant A. *et al*., 2007) confirmed these data, showing that two molecular methods (PCR – restriction enzyme analysis and length polymorphism analysis) for *C. neoforinans* serotype identification. Both are based on the sequence characteristics of a fragment of the CAP 59 gene required for capsule biosynthesis.

PCR-REA

Enzymatic restriction with BsmFI and HpaII gave three different patterns for serotypes A, D, and (B and C). Serotype AD strains exhibited a mixed A and D restriction profile. All strains were tested, and no profile variation among strains of a given serotype was observed. Serotype D and serotypes B and C, while having similar profiles (unique cutting site for HpaII and no cutting site for BsmFI), can be easily differentiated based on the size difference of the digested fragments. Serotypes B and C can be further differentiated by [AgeI digestion not used in this study] and gel electrophoresis. Two fragments corresponding to serotype B strains (one cutting site) were detected, while serotype C amplicons remained unchanged (no cutting site).

The current study based on molecular analysis of a fragment of the CAP59 gene appeared to be sensitive and specific, providing interpretable results for all C. *neoformans* sample tested (Guilhem, j., *et al*, 2003).

PCR-REA is an easy-to-use approach and requires only common molecular materials. Its value for distinguishing among fungal species has already been emphasized that were agreement with (Guilhem, j., *et al*, 2003). serotype identification by combining separate digestions with BsmFI and HpaII, followed by subsequent digestion with [AgeI] that was not used in this study when the pattern suggests a B or C serotype, can therefore be proposed (Table 6).

Table (6): Expected sizes (bp) of CAP59 gene fragments from different serotypes before and after restriction with BsmFI, HpaII enzymes

Assay or fragment	A	B	C	D	AD
Native amplicon	392	387	359	375	387
REA with BsmFI	201, 191	NRS	NRS	NRS	210,177
REA with HpaII	NRS	270, 117	256, 103	211, 162	

NRS, no restriction site.

More recently, serotyping has played a major role in the investigation of the endemic infection on Vancouver Island (Kidd, S.E., *et al*, 2004). Serotyping has no diagnostic value, but different treatments and outcomes corresponding to serotypes have been reported that use agrees with (Chen, Y.C., *et al*, 2000). Thus, considering that [the Crypto check kit (latron, Japan)] is no longer available, and in view of the relative complexity of obtaining monoclonal antibodies, new techniques need to be developed for the typing of C. *neoformans* strains.

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