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ISOLATION AND MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF *CRYPTOCOCCUS NEOFORMANS* FROM FECAL MATTER OF URBAN PIGEONS (*COLUMBA LIVIA* L) IN ELEMENTARY SCHOOLS IN MONTERREY, MEXICO.

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Abstract: The fecal matter of urban pigeons (*Columbia livia* L) is considered a potential source for the development of many yeasts of importance in Public Health that affect man, such as *Cryptococcus* spp, and are abundant in public areas. In the metropolitan area of Monterrey, state of Nuevo Leon, Mexico, there are reports of the presence of *C. neoforms* and *C. albidus*. However, there are no reports of this microorganism existing in elementary schools, so the objective of this work was to inspect fecal matter of pigeons in different schools from seven municipalities in the metropolitan area of Monterrey, Mexico to detect *Cryptococcus* spp by using specific culture media and PCR. **Results:** From the 88 fecal matter samples collected in the different educational centers from the seven municipalities corresponding to the metropolitan area of Monterrey, in 35 of them could be recovered *Cryptococcus neoformans*, which correspond to 39.7% of isolated strains and 60.3% of negative samples. **Conclusions**: The Cladogram analysis of sequences that amplify the *CAP59* gene from our isolations aligned with the sequences reported in Genbank, show that strains Cr2 (KX349217) and Cr4 (KX349218) are clustered with *C. neoformans* variety *grubii* with a 100% homology with the sequence GQ850225 reported in the Southeast of China and 99% with the sequence reported in Japan (Genbank AB066115). According to the results, it is important to establish control measures of these birds in these elementary educational centers and to prevent the fecal accumulation, avoiding the exposure of this yeast to the children of these schools.

Keywords: Urban pigeon (*Columbia livia* L), Cryptococcosis, *neoformans*, *grubii*, Mexico.

INTRODUCTION

Cryptococcus neoformans is an encapsulated saprophyte yeast, which is

present in natural form as much as in the host and the environment (Nayak *et al*., 2010). Generally, this microorganism shows a low virulence in immunocompromised individuals; however, it can significantly affect immunocompetent people (Lazera *et al*., 2000; Harris *et al*., 2021; Pyrgos *et al*., 2013), sometimes causing cryptococcosis meningitis (Galnares-Olalde *et al*., 2014). Therefore, it has been frequently associated with the exposure of the patient to bird droppings (Cafarchia *et al*., 2006b). According to immunological basic properties to capsular polysaccharide can be recognized serotypes A, B, C and D. Serotype A is *C. neoformans* variety *grubii,* D and AD correspond to *C. neoformans* variety *neoformans*. Serotypes B and C to *C. neoformans* variety *gattii* (Kwon-Chung *et al*., 2002, 2011; Simwami *et al*., 2011). *C. neoformans* variety *grubii* is characterized for a worldwide distribution, with optimal growth in bird droppings and detritus of different tree species (Casali *et al*., 2003; Meyer *et al*., 2003; Elhariri *et al*., 2015). *C. neoformans* variety *neoformans* (serotypes A and D) same as variety *grubii* have been associated with bird droppings (García-Hermoso *et al*. 1997; Soltani *et al*., 2013). Although *C. neoformans* variety *gattii* (serotypes B and C) presence has been restricted only to tropical and subtropical regions, those serotypes have been detected in Vancouver, Canada (Stephen *et al*., 2002).

Bird fecal matter, particularly from urban pigeons (*Columba livia* L), has been considered a potential source for the development of many yeasts of importance in Public Health that affect man, which are abundant in public places. Such is the case of *Cryptoccocus* spp and *Candida* spp. (Chee and Lee, 2005), those yeasts can spread in the environment and lately infect people through the inhalation of propagules (desiccated yeast cells or spores) suspended in the air, capable of infecting lungs and disseminate systematically causing pathologies to man (Ellis and Pfeiffer, 1990). Another important yeasts, as *Rhodotorula* and *Trychosporon,* have been isolated from pigeon droppings and from the sewer of *American coot* (*Fulica americana* L), which have been implicated in intravenous nosocomial infections by catheterization (Cafarchia *et al*., 2006a).

Growth conditions of *Cryptococcus* spp are variable. However, tropical and subtropical climates associated to pigeon's droppings and tree detritus are more favorable for its development (Lazera *et al*., 2005). Nevertheless, there are isolated reports of the yeast in Monterrey, Mexico, in high temperature (40°C) climates and dry humidity, which looks like an adaptation to extreme climate by this microorganism (Canónico-González *et al*., 2013). The importance of these yeasts has been increasing, due to cryptococcosis cases in six hospitals in Monterrey, Mexico (Casillas-Vega, 2012). Likewise, a case of a mixed infection of *Mycobacterium tuberculosis* and *C. neoformans* in a young patient (9 years old) with systemic lupus erythematosus (SLE) has been reported recently at the Hospital Universitario "Dr. Jose E. Gonzalez" in Monterrey, Nuevo Leon (Martínez-Longoria *et al*., 2015). The presence of the disease in minors has aroused the interest in examining pigeon fecal matter in educational centers, due to the abundant presence of these birds at schools and, therefore, a big amount of droppings. There are no studies in Monterrey of the presence of *Cryptococcus neoformans* in educational centers, so the objective of this work was to inspect pigeon droppings in different elementary schools in seven municipal zones surrounding the metropolitan area of Monterrey, Mexico, to detect *Cryptococcus neoformans* by the use of specific culturing agar and PCR.

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MATERIALS AND METHODS

LOCATION

For the making of this study, the samples were taken from elementary schools in seven municipal zones of Monterrey City and the surrounding metropolitan area (Monterrey, San Pedro Garza Garcia, Santa Catarina, Escobedo, San Nicolas de los Garza, Guadalupe and Apodaca) with a 3,293 Km^2 surface, equivalent to 5.12% of the surface of the state of Nuevo Leon, Mexico.

SAMPLING

88 fecal matter samples were collected randomly in the educational centers from the seven municipal zones (Monterrey 18, San Pedro Garza Garcia 12, Santa Catarina 13, Escobedo 12, San Nicolas de los Garza 11, Apodaca 11, Guadalupe 11) during July and August 2015, using sterile spatulas and putting the samples in plastic bags with hermetic lock. Samples were taken directly to the Microbiology lab at the Veterinary School (Facultad de Veterinaria y Zootecnia) of the Universidad Autonoma de Nuevo Leon to be processed immediately.

SAMPLE PROCESSING

1g of each sample was put into 20ml of saline solution sterile at 0.85% with chloramphenicol (0.2 g/L). The saline solution with the fecal matter was stirred for 15 minutes in a shaker at 300 rpm, and let rest for 30 minutes. From the supernatant were taken 100 µL to culture using a Drigalsky handle in agar Staib, otherwise called *Guizotia abyssinica*-creatinine agar or Niger seed agar (ASN) (Staib *et al*., 1973). The samples were incubated at 37°C for 5 to 7 days. Shiny or opaque colonies in brown color, which are characteristic of the species, allow us to differentiate from other contaminating microorganisms. Dark colonies were cultured in new dishes with ASN agar to obtain pure culture. Also, subcultures were done in Sabouraud glucose agar (SGA) for morphologic and biochemical identification; melanin production in agar ASN, growth at 37°C, Urease production in Christensen agar, capsule formation (Pérez et al., 2003; Kwon-Chung and Varma, 2006). Growth was done with esculine agar for melanin detection in *C. neoformans* according to MacFaddin (2000), and McFadden and Casadevall (2000).

DNA EXTRACTION

Colonies obtained in ASN agar were processed using the modified Fastprep-24 Technique (MP Biomedicals), putting the samples in microtubes of 1.5 mL to rupture the cellular wall and capsule. The cells were put in 500 µL lysis buffer and 40 µl/L of K proteinase, the tube contained sterile ground glass with 15 pulses for a 5 minutes period. DNA extraction was performed according to the method proposed by Ferrer *et al*. (2001). The cells were incubated for 1 h at 65°C in 500 mL of extraction buffer (50 mM Tris-HCl, 50 mM EDTA, 3% sodium dodecyl sulfate, and 1% 2-mercaptoethanol). The lysate was extracted with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol). Then 65 µL of 3M sodium acetate and 75 µL of 1M NaCl were added to 350 µL of the supernatant, and the resulting volume was incubated at 4°C for 30 min. DNA was recovered by isopropanol precipitation and washed with 70% (vol/vol) ethanol, dried under a vacuum, and suspended in TE buffer (10 mMTris-HCl, 1 mM EDTA [pH 8]). DNA was cleaned with the Gene clean kit II (BIO 101, Inc., La Jolla, Calif.) according to the manufacturer's instructions.

PCR AMPLIFICATION

The genomic DNA samples (200 ng) of the yeast were amplified by PCR in a mixed reaction of 25 µL with a tampon PCR 1 x 5 µl, 200 µM of each triphosphate deoxyribonucleoside (dATP, dGTP, dCTP, dTTP), 0,3 mM de MgCl2, 0,4 µM of each primer (*CAP59F*: 5'-CCTTGCCGAAGTTCGAAACG-'3 and *CAP59R*:5'-AATCGGTGGTTGGATTCAGTGT-'3) (Chang and Kwon-Chung, 1994). 1.25 U/µL *Taq* DNA polymerase (Invitrogen, San Diego, Calif).

The amplification of gene CAP59 was made using the following protocol: 1 minute at 94°C followed by 30 cycles of 1 minute at 94°C, 30 seconds at 65°C and 1 minute at 72°C. A final extension of 15 minutes at 72°C. PCR reaction was performed in a MiniCycler thermocycler (TM MJ Research INC, NY, USA). As a positive control, the strain of *C. neoformans* var, *neoformans* serotype D (ATCC 28957) was used as reference. In each experiment negative controls were used. The amplified fragments were visualized by electrophoresis in agarose gel at 1.5% in tampon TBE (0.09 M Tris, 0:09 M Boric acid and 2M of EDTA, pH 8.3) at 60 V, using GelRed™ Nuleic Acid Gel Stain, 10,000 in water, under UV irradiation in the transilluminator MultiDoc-Lt. Digital Imaging System (Upland CA, USA). PCR products were sequenced in ASBI Prism 3130 system (Applied Biosystems, Foster City CA). The positive strand sequences were analyzed with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare with the sequences available of *Cryptococcus* spp. The sequences obtained from two samples were submitted to Genbank.

The phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 6 (Tamura *et al.*, 2013).

RESULTS

Figure 1 shows the distribution of sampling in the municipal zones of the metropolitan area of Monterrey, Nuevo Leon, Mexico. From the samples cultured in ASN agar and esculine agar, the suspicious strains of *Cryptococcus* spp showed dark coloration. As indicated on Table 1, 35 strains were isolated in the municipalities of Monterrey, San Pedro Garza Garcia, Santa Catarina, Escobedo, San Nicolas de los Garza, Guadalupe and Apodaca.

Macroscopic observation in ASN and esculine agar showed shiny mucous colonies of creamy white color. Later, after 4 to 5 days, colonies changed their color to dark brown. The observation in the microscope, using a negative stain with china ink, showed ovoid and globose yeasts with a diameter from 3 to 7 µm, capsuled and budding with a single outbreak. According to the biochemical characteristics, the strains recovered were identified as *C. neoformans* compared to positive control *C. neoformans* variety *neoformans* serotype D (ATCC 28957). Based on following parameters: Inositol (+), urease (+), lactose (-), $KN0_3$ (-), maltose (+), saccharose (+), galactitol (+), negative to modification of canavanine-glycine-blue of bromothymol at 28°C for 5 days.

From 88 fecal matter samples collected in different educational centers in the 7 municipal zones which correspond to the metropolitan area of Monterrey, in 35 of them was found *Cryptococcus neoformans*, which is equal to 39.7% of isolated strains and 60.3% of negative samples (Fig. 2).

The cladogram analysis (Fig 4) from sequences that amplify gene *CAP59* from our isolations aligned with the sequences reported in Genbank, shows that strain Cr2 (KX349217) and Cr4 (KX349218) are grouped together with *C. neoformans* variety *grubii* with an homology of 100% with the sequence GQ850225 reported in the Southeast of China

Fig. 1. Location of the diffetent points of sampling, where darker dots indicate positive samples for *Cryptococcus neoformans.*

Municipal zones	Samples	Positive	Negative
Monterrey	18	10	8
San Pedro	12	5	7
Santa Catarina	13	3	10
Escobedo	12	6	6
San Nicolás	11	4	7
Guadalupe	11	4	7
Apodaca	11	3	8
Total	88	35	53

Table 1. Ratio of samples collected in each municipal zone from the Monterrey metropolitan area.

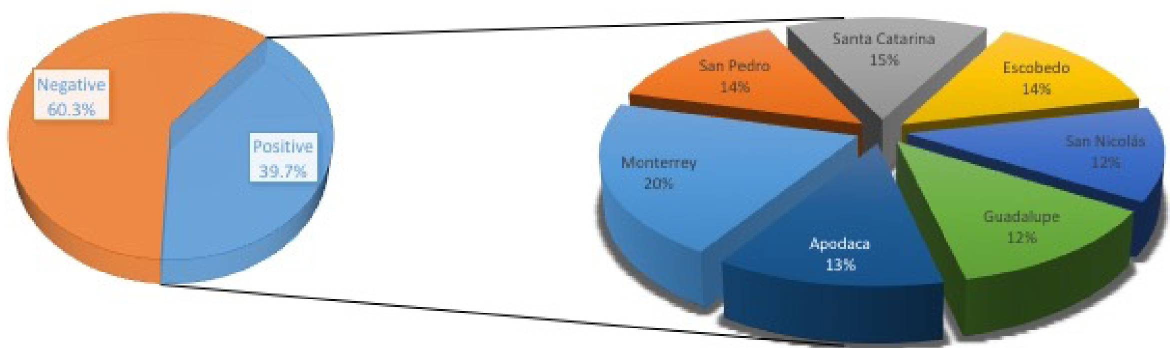

Fig. 2 Frecuency of isolation of *Cryptococcus neoformans* from fecal matter in the seven municipal zones.

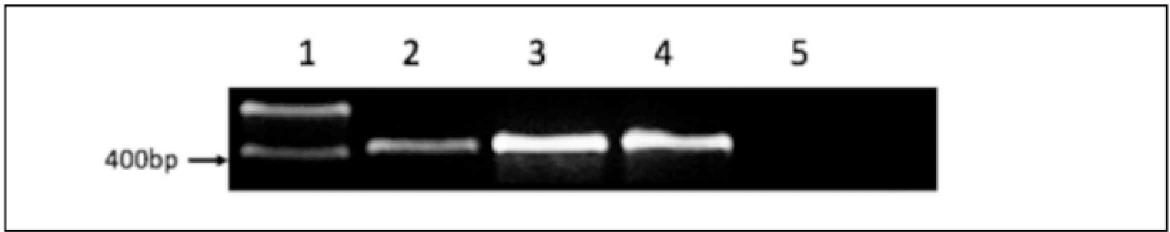

Figure 3 shows the amplified product of 400 pb in 5 of the 35 isolations studied with the reference strain and the primers which correspond to gene *CAP59*.

Fig. 3 Amplified products of gene *CAP59* in isolation of *C. neoformans*.

Lane 1: Molecular weight marker 400 bp; Lane2: control strain *C. neoformans* var. *neoformans* serotipe D (ATCC 28957); lanes 3 and 4 isolates and lane 5 negative control.

Cryptococcus neoformans var. grubii (Filobasidiella neoformans serotype A) New York AF248136
Cryptococcus neoformans A/D, southeast China GQ850242
Cryptococcus neoformans var. grubii (Filobasidiella neoformans serotype A) New York AF248135
Cryptococcus neoformans var. grubii, USA CP003820
Cryptococcus neoformans var. grubii ((Filobasidiella neoformans serotype A) New York AF337639
Cryptococcus neoformans A/D, France EF392816
Cryptococcus neoformans A var. grubii France EF392812
GQ850246
Cryptococcus neoformans var. grubii (Filobasidiella neoformans serotype A) southeast China GQ850225
Cryptococcus neoformans var. grubii (Filobasidiella neoformans serotype A) southeast China AB066115
Cryptococcus neoformans var. grubii (Filobasidiella neoformans serotype A) Japan
Cryptococcus neoformans, México KX349218
Cryptococcus neoformans, México KX349217
Cryptococcus neoformans var. neoformans, var. gattii, Japan AB066136
Cryptococcus neoformans A/D, France EF392813
Cryptococcus neoformans var. neoformans, New York AF248143
Cryptococcus neoformans var. neoforman, USA, Chr1 AE017341
Cryptococcus neoformans, USA L26508
Cryptococcus neoformans var. neoformans, USA AF248144

0.15 0.10 0.05 0.00

Fig. 4. Phylogenetic relationship between 16 sequences obtained from Genbank, and 2 generated in this research.

and 99% with the sequence reported in Japan (Genbank AB066115).

DISCUSSION

Cryptococcosis is described as a systemic mycosis caused by propagules which transport a large amount of microorganisms, among them *C. neoformans,* which are present in the environment and that can be inhaled by humans, mainly immunocompetent patients causing respiratory and neurological diseases. This yeast, in addition to be found in fomite, can be located also in bird fecal matter, in saprophyte form in fruit, milk, wood, soil and root vegetables, especially potatoes and carrots (Curo *et al*, 2005). Isolation of *C. neoformans* in routine agar medium from places where there is no high contamination is relatively easy (Mitchell and Perfect, 1995). However, when we try to isolate from fecal matter, it is required to use mediums that help us to differentiate from other microorganisms in the lab. In the present study, Niger medium was used, which was optimum for isolation and differentiation of the yeast, recovering 35 strains in total. According to the results obtained in this research, with regard to the recovery of *C. neoformans* (39.7%) from fecal matter using specific mediums, were similar to what Caicedo *et al*. (1996) reported, with a 46% the same as Chavarria *et al*. (2011) with 36.5% positivity. However, there are reports with bigger recovery as 63.6% (Xavier *et al*., 2013), possibly due to the abundant quantity of trees and a high presence of pigeons in sample site. Likewise, this study surpasses what was reported by other researchers in Mexico and other countries, where they just reached a 20% recovery (Rivas *et al*., 1999; Castañón-Olvares *et al*., 2000; Quintero *et al*., 2005; Canónico-González *et al*., 2013). All strains of *C. neoformans* obtained in the study were recovered from dry fecal matter from pigeons that were present in backyards of the different elementary educational centers, avoiding to take fresh fecal matter due to previous reports that this yeast cannot be recovered from fresh fecal matter (Mishra *et al*., 1981).

The PCR from samples isolated from fecal matter were 100% positive to *C. neoformans* using primers that amplified gen CAP59 and obtaining an amplified product of an approximate size from 370 to 400bp, equal to the obtained by Siachoque *et al*. (2010), from a suspension with *C. neoformans* and *C. gattii*. Nakamura *et al*. (2000) obtained different results to the ones found in this study using a different amplification program, where they got a 600bp product of amplification.

Other technics could be used and different regions to identify these yeasts like the Hyperbranched Rolling Circle Amplification (HRCA) from Trilles *et al*. (2014). This technique can be used with great potential for the diagnosis of cryptococcosis and implement a fast treatment for a patient with this disease. The information gathered in the study helps a lot to locate pigeon presence in our elementary student population and to detect and notify the existence of yeasts that can be implicated in cryptococcosis. This is a result from what was reported by Martinez-Longoria *et al*. (2015) about a case of severe meningoencephalitis caused by a co-infecction of *C. neoformans* and *Mycobactrium tuberculosis* in Monterrey, Nuevo Leon, Mexico.

The phylogenetic analysis of sequences from isolated strains in the present study and aligned against the sequences previously reported in the Genbank, show that sequence Cr2 (KX349217) and Cr4 (KX349218) get together in a group with *C. neoformans* variety *grubii* with a homology of 100% with the sequence Genbank GQ850225 reported in southeast China and 99% with the sequence Genbank AB066115 reported in Japan. This cladogram confirms microbiological identification done in our work. Those results agree on what was

found by Canónico-González *et al*. (2013) in studies done in Monterrey in 2 samples (Csl ID JQ794489 and Cs 9 ID JQ794497), in which *C. neoformans* were detected inside the group of *C. neoformans* variety *grubii*. However, in this research, gen 18S from rRNA was amplified, this made it different from the one we used in our work, in which we used primers that amplified capsular gene *CAP59*. Nevertheless, it is important to establish the presence of *Cryptococcus* from fecal matter and making deals with the authorities to establish an ecological control of the birds in the educational centers and to prevent fecal accumulation from pigeons, avoiding the exposure of this yeast to the children of these schools.

AUTHORS´CONTRIBUTION

JJHE: Conceived and designed the study, executed the experiment, made the sampling of the fecal matter and processed the samples, made the DNA extraction and PCR amplification.

ODGP: Made the DNA extraction and PCR amplification.

VRMM: Analyzed the phylogenetic and molecular data.

JAVC: Analyzed the phylogenetic and molecular data.

DRAG: Reviewed the wording and information of the article, the references, the tables and figures and the requested formats.

JPVV: Conceived and designed the study, executed the experiment, made the DNA extraction and PCR amplification.

All authors revised the manuscript and approved the final version.

REFERENCES

Cafarchia, C., A. Camarda, D. Romito, M. Campolo, N.C. Quaglia, D. Tullio and D. Otranto (2006a). Occurrence of yeasts in cloacae of migratory birds. Mycopathol. 161:229–34.

Cafarchia, C., D. Romito, R. Iatta, A. Camarda, M.T. Montagna and D. Otrato (2006b). Role of birds of prey as carriers and spreaders of *Cryptococcus neoformans* and other zoonotic yeasts. Med. Mycol. 44:485–492.

Caicedo, L.D., M.I. **Álvarez,** C.E. Llanos and D. Molina (1996). *Cryptococcus neoformans* en excretas de palomas del perímetro urbano de Cali. Colomb. Med. 27: 106-109.

Canónico-González, Y., J.M. Adame-Rodríguez, R. Mercado-Hernández and E.T. Aréchiga-Carvajal (2013). *Cryptococcus* spp isolation from excreta of pigeons (*Columbia livia*) in and around Monterrey, Mexico. SpringerPlus 2:632.

Casali, A.K., L. Goulart, L.K.R. Silva, A.M. Ribeiro, A.A. Amaral, S.H. Alves, A. Schrank, W. Meyer and M.H. Vainstein (2003). Molecular typing and environmental *Cryptococcus neoformans* isolates in the Brazilian state Rio Grande do Sul. FEMS Yeast Res. 3(4): 405-415.

Casillas-Vega, N.G. (2012). Fenotipificación y genotipificación del complejo *Cryptococcus neoformans* / *Cryptococcus gattii* en aislamientos clínicos del noreste de México. Tesis de Maestría en Microbiología Médica. Facultad de Medicina, Universidad Autónoma de Nuevo León. México.

Castañón-Olivares, L., R. Arreguin-Espinosa, G. Ruiz-Palacios and A. López-Martínez (2000). Frequency of *Cryptococcus* sp. and varieties in Mexico and their comparison with some Latin American Countries. Rev. Latinoam. Microbiol. 42: 35-40.

Chang, Y.C. and K.J. Kwon-Chung (1994). Complementation of a capsule- deficient mutation of *Cryptococcus neoformans* restores its virulence. Mol. Cell. Biol. 14: 4912-4919.

Chavarría, D.A., F.M.L. Henríquez and R.E.V. Recinos (2011). Aislamiento de *Cryptococcus neoformans* en muestras del ambiente contaminadas con excretas de palomas en diferentes zonas en el Salvador. Min. Rev. 2(1): 21-27.

Chee, H.Y. and K.B. Lee (2005). Isolation of *Cryptococcus neoformans* var. *grubii* (serotype A) from pigeon droppings in Seoul, Korea. J. Microbiol. 43:469–472.

Curo, M., M. Salinas and J.C. Casquero (2005). *Cryptococcus neoformans* en excretas de palomas, suelo y aire de los palomares del perímetro urbano de ICA. Rev. Perú. Med. Exp. Sal. Pub. 22 (4): 262-264.

Elhariri, M., D. Hamza, R. Elhelw and M. Refai (2015). Eucalytus tree: A potential source of *Cryptococcus neoformans* in Egyptian environment. Int. J. Microbiol.
http://dx.doi.org/10.1155/2016/4080725

Ellis, D. and T. Pfeiffer (1990). Natural Habitat of *Cryptococcus neoformans* var. *gattii*. J. Clin. Microbiol. 28(7):1642-1644.

Ferrer, C., F. Colom, S. Frasés, E. Mulet, J.L. Abad, J.L. Alió (2001). Detection and identification of fungal pathogens by PCR and ITS2 and 5.8S ribosomal DNA typing in ocular infections. J. Clin. Microbiol. 39(8): 2873-2879.

Galnares-Olalde, J.A., S. Loza-Jalil, F. Gómez-Peña, O. Muñoz-Abraham, V. Pavia-Aubry and D. Luna-Gallardo (2014). Criptococosis meníngea en un paciente inmunocompetente: reporte de un caso y revisión de la literatura. Rev. Med. Hosp. Gen. Méx. 77(83): 109-113.

Garcia–Hermoso, D., S. Mathoulin-Pelisser, B. Cuprie, O. Ronin, B. Dupont and F. Dromer (1997). DNA typing suggest pigeon dropping as a source of pathogenic *Cryptococcus neoformans* serotype D. J. Clin. Microbiol. 35: 2683-85.

Harris, J., S. Lockhart and T. Chiller (2012). *Cryptococcus gattii*: where do we go from here? Med. Mycol. 50(2): 113–129

Kwon-Chung, K.J., T. Boekhout, B.L. Eickes and J. Fell (2011). Systemic of the genus *Cryptococcus* and its type species of *C. neoformans*. In: Heitman, J., T.R., Krozel, K.J. Kwon-Chung, J.R. Perfect and A. Casadevall (eds). *Cryptococcus* from human pathogen to model yeast. Washington DC: ASM Press. pp 3-15.

Kwon-Chung, K.J., T. Boekhout, J. Fell and M. Diaz (2002). Proposal to conserve the name *Cryptococcus gattii* against *C. hondurianus* and *C. bacillisporus* (Basidiomycota, Hymennomicetes, Tremellomycetidae). Taxon 51: 804-806.

Kwon-Chung, K.J. and A. Varma (2006). Do mayor species concepts support one, two or more species with *Cryptococcus neoformans*? FEMS Yeasts Res. 6:574-587.

Lazera, M.S., M.C. Gutierrez-Galhardo, M.A.S. Cavalcanti and B. Wanke (2005). Criptococose. In Coura, J.R. (org), Dinâmica das doenças infecciosas e parasitarias. Guanabara Koogan. Rio de Janeiro. p 1223-1235.

Lazera, M.S., M.A.S. Calvancanti, A.T. Londero, L. Trilles, M.M. Nishikawa and B. Wanke (2000). Possible primary ecological niche of *Cryptococcus neoformans*. Med. Mycol. 38(5): 379-383.

MacFaddin, J.F. (2000). Biochemical test for identification of medical bacteria. 3th edition. Lippincot Williams & Wilkins, Inc. USA. Chap. 2. Tests and esculin hydrolysis bile. p: 8-18.

Martinez-Longoria, C.A., N.E. Rubio-Perez, J.E. Rios-Solis and F. Garcia-Rodriguez (2015). Severe meningoencephalitis co-infection due to *Cryptococcus neoformans* and *Mycobacterium tuberculosis* in a child with systemic lupus erythematosus. Int. J. Inf. Dis. 33: 106-108.

McFadden, D.C. and A. Casadevall (2001). Capsule and melanin synthesis in *Cryptococcus neoformans.* Med. Mycol. 39(1): 19-30.

Meyer, W., A. Castañeda, S. Jackson, M. Huynh and E. Castañeda (2003). Molecular typing of Iberoamerican *Cryptococcus neoformans* isolates. Emerg. Infect. Dis. 9(2): 189-195.

Mishra. S.K., F. Staib, U. Folkens and R.A. Fromtling (1981). Serotypes of *Cryptococcus neoformans* strains isolated in Germany. J. Clin. Microbiol. 14:106-107.

Mitchell, T.G. and J.R. Perfect (1995). Cryptococcosis in the era of AIDA-100 years after the discovery of *Cryptococcus neoformans*. Clin. Microbiol. Rev. 8(4): 515-548.

Nakamura, Y., R. Kano, S. Watanabe and A. Hasegawa (2000). Molecular analysis of *CAP59* gene sequences from five serotypes of *Cryptococcus neoformans*. J. Clin. Microbiol. 38 (3): 992-995.

Nayak, J.B., M.N. Brahmbahtt, C.V. Savalia, M. Pal and B.B. Bhanderi (2010). Cryptococcosis: A Garded Mycosis Threat. Res. J. Vet. Sci. 3: 101-112.

Pérez, C., S. Mata, C. Hartung, A. Roselló, M.T. Colella, C. Olaizola and M. Landaeta (2003). Preservation of *Cryptococcus* sp. with the Castellani method. Rev. Soc. Ven. Microbiol. 23:153-157.

Pyrgos, V., A. Seitz, C. Steiner, D.R. Prevost and P.R. Williamson (2013). Epidemiology of cryptococcal meningitis in the US: 1997-2009. Plos One 8: 1-6

Quintero, E., E. Castañeda and A. Ruiz (2005). Environmental distribution of *Cryptococcus neoformans* in the Departament of Cundinamarca Colombia. Rev. Iberoam. Micol. 22: 93-98.

Rivas, F., M.C. De Martín and V. Rojas (1999). *Cryptococcus neoformans* isolation from the soil in Panama. Rev. Med. Panama 24(1):4-6.

Siachoque, N., V.M. Jewtuchowicz, C. Iovannitti and M.T. Mujica (2010). Amplificación del gen *CAP59* en *Cryptococcus neoformans* y *Cryptococcus gattii* directamente a partir de una suspensión de levaduras. Rev. Argent. Microbiol. 42: 91-94.

Simwami, S.P., K. Khayhan, D.A. Henk, D.M. Aanensen, T. Boekhout, F. Hagen, A.E. Brower, T.S. Harrison, C.A. Bonnelly and M.C. Fisher (2011). Low diversity *Cryptococcus neoformans* variety *grubii* multilocus sequences types from Thailand are consistent with an ancestral Africa origin. Plos Pathog. 7, e1001343.

Soltani, M., M. Bayant, S.J. Hashemi and N. Pastechian (2013). Isolation of *Cryptococcus neoformans* and other opportunistic fungi from pigeon droppings. J. Res. Med. Sci. 18(1): 56-60.

Staib, F., M. Senska and D. Braunfarbeffekt (1973). Bei *Cryptococcus neoformans* auf *Guizotia abyssinica*-Kreatinin-Agar in Abhangigkeit vom Ausgangs pH Wert. Zentralbl. Bakteriol. Paraitendkd. Infekstionskr. Hyg. Abt. 1 Orig. Reihe A 225: 113-114.

Stephen, C., S. Lester, W. Black, M. Fyfe and S. Raverty (2002). Multispecies outbreak of cryptococcosis on southern Vancouver Island, British Columbia. Can. Vet. J. 43: 972-974.

Tamura, K., G. Stecher, D. Peterson, A. Filipski and S. Kumar (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30: 2725-2729.

Trilles, L., B. Wang, C. Firacative, M.S. Lazéra and B. Wanake (2014). Identification of the mayor molecular types of *Cryptococcus neoformans* and *C. gattii* by Hyperbanched rolling circle amplification. Plos One 9(4) e94648.

Xavier, T.F., A. Auxilia, M. Kannan, R. Freeda and S.R.S. Kumar (2013). Isolation and identification of *Cryptococcus neoformans* from pigeon dropping in Truchirappali district of Tamil Nadu, South India. Int. J. Curr. Microbiol. App. Sci. 2(11): 404-409.