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RESEARCH ARTICLE

Isolation and Molecular Identification of *Histoplasma capsulatum* from Respiratory Samples of Horses in Iraq

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ABSTRACT

This research was carried out to isolate*Histoplasma capsulatum* from respiratory samples of horses in Iraq using genomic detection.A total numbers of one hundred (100) nasal swabs andone hundred (100) blood samples were collected from equestrian clubs in Baghdad, DhiQar, Addiwaniya and Najaf provinces.All samples cultured on sabouraud dextrose agar (SDA) and brain heart infusion agar (BHIA) with chloramphenicol and cycloheximide and incubatedat (25°C and 37°C) for (4 weeks). The samples wereexamined macroscopically depending on shape, texture and color of the fungalgrowth and microscopically by preparing a mount smear with lactophenol cotton blue and examined under (40X). The diagnosisconfirmed by molecular techniques for the detection of*Histoplasma capsulatum* by using conventional PCR.So, the isolation of*Histoplasma capsulatum*was(4%)in both conventional examination and molecular detection innasal swabs,but in case of blood samples,the *Histoplasma capsulatum* isolatedby conventional methods was 3%,whereas the molecular detection appeared as 2%.

Keywords: *Histoplasma capsulatum*,Horses,Conventional PCR, Sequences analysis

INTRODUCTION

Histoplasmosis was first described in (1906) by Darling among the workers of the Panama Canal (Darling, 1909). The *H. capsulatum* is thought to cause approximately 500000respiratory infections a year in the central river valleys in the Midwestern and south-central United States (Chang, 2007).*Histoplasma capsulatum* is a fungal pathogen that can result in a wide range of clinical presentations, from asymptomatic to fatal infection. It usually causes lung disease called Histoplamosis or Darling's disease, according to Samuel Darling who found the pathogenic fungi in histopathologic specimens about a century ago, (Chang, 2007).*Histoplasma capsulatum* is a biologically interesting inhabitant of soil and mammalian hosts, a clinically significant cause of respiratory and systemic infection, and an excellent fungal

model of dimorphic cell development and facultative intracellular pathogenesis(Heitman.*et al*, 2006). *Histoplasma capsulatum* is unique in its dimorphism.

This fungus also causes Epizootic lymphangitis in equids and most common form of this disease is an ulcerative, suppurative, spreading dermatitis and lymphangitis; however, other forms including pneumonia or ulcerative conjunctivitis also occurs. Epizootic lymphangitis spreads most readily where large numbers of animals are assembled, and it was a serious problem during the early twentieth century, when large numbers of horses were stabled together. This disease continues to be a significant concern in some countries, where the prevalence in carthorses is nearly (19%), and economic losses from this disease are high. (Ameni and Terefe 2004).

MATERIALS AND METHODS

The collected samples

A total ofone hundred(100) nasal swabs and one hundred (100) blood samples of the horses were collected fromequestrian clubs in Baghdad, Dhi Qar, Ad diwaniya and Najaf provinces, during the period from October 2017 to the end of March 2018. The samples were kept in a cold until and processed in the laboratory, the nasal swabs inoculated in transport media before processed in laboratory.

Laboratory tests

All samples from horses were inoculated in Sabouraud dextrose agar with Chloramphenicol; incubated in (25° C for 2-4 weeks) , as well as they were inoculated in Brain heart infusion agar with Chloramphenicol 0.05 mg/ml and Cycloheximide (1%) ; incubated in (37° C for 2-4 weeks).

Isolated strains examined macroscopically according to their shape, texture and color of the fungal colonies on SDA and BHIA and microscopically by preparing a mount of smear with lactophenol cotton blue to identify fungal elements. So, the suspected isolated strains wereinoculated in a new BHIA to proceed to a molecular detection by conventional PCR(Alexander and Street, 2001).

Molecular diagnosis

PCR techniques were performed on isolated strains of the horses for the four provinces to detect of the *Histoplasma capsulatum* based on 18SrRNA gene. The method was carried out according to a description obtained by Wang *etal.,*(2014).The DNA extraction was done from isolated suspected strains by using EZ-10 Spin Column Fungal Genomic DNA Mini-Preps Kit(DNA Extraction Kit), (Bioneer, Korea). PCR master mix preparation by using primer pair: forward (5–TTGTCTACCGGACCTGTTGC–3) and reverse (5-CCTGGTGTGAAAAGGGGGTT-3) (Maxime™ PCR PreMix Kit (*i*-Taq)), then placed in PCR Thermocycler (MyGene. Bioneer. Korea).

Sequence analysis

The genetic analysis done by phylogenetic tree method between local *Histoplasma capsulatum* horse isolates and NCBI-Blast submission *Histoplasma capsulatum*. Then the identification species isolates were submitted into of NCBI-GenBank.The DNA sequencing analysis was conducted by using Molecular Evolutionary Genetics Analysis version 6.0 (Mega 6.0) and Multiple sequence alignment analysis of the partial small subunit rRNA gene based on ClustalW alignment analysis and the evolutionary distances were computed using the Maximum Composite Likelihood method by phylogenetic tree UPGMA method (Wang *et al.,* 2014).

RESULTS

Conventional macroscopic method recorded 4 (4%) positive out of 100 swabs, and 3 (3%) positive out of 100 blood samples in horses. Not all microscopy positive specimens were found positive by PCR only one had been negative as in Tab. (1).

Microscopic appearance

Two types of conidia were produced on the hyphae. The macroconidia, or tuberculate conidia, are (8 to 15 μm) in diameter and have distinctive projections on their surface; the microconidia are small (2 to 4 μm) and smooth walled. Fig. (2).

PCR

Genomic DNA samples obtained from suspected isolates were subjected to molecular analysis by PCR, using 18S rRNA gene specific primers in order to identify *Histoplasma capsulatum*.PCR of positive samples employed in the research exhibited distinct band of (476 bp) on agarose gel confirming the presence of *Histoplasma capsulatum* (Fig. 3).

Macroscopic appearance

After four weeks,the mold was grainy to cottony in appearance, and became increasingly brown in color from white over time. Yellow/yellow-orange reverse (Fig. 1).DNA sequence results for Histoplasma capsulatum (2-6) showeda close relate to NCBI blast *Histoplasma capsulatum*(AF129547)according to phylogenetic tree analysis between local Histoplasma capsulatum isolate and NCBI blast Histoplasma capsulatum isolate at homologue sequence identity (100%) and then local Histoplasma capsulatum isolate (2-6) were submitted to NCBI gen bank at accession number (MH745422, MH745423, MH745424, MH745425 and MH745426) (Table 2).

DISCUSSION

The results of macroscopic and microscopic appearance of *Histoplasma capsulatum* isolated from nasal swabs and blood samples of horses in four provinces in Iraq,which were Baghdad, Dhi Qar, Addiwaniya and Najaf, revealed (3.5%)with characteristic features of *histoplasma capsulatum*(4/100 from nasal swabs and 3/100 from blood samples) were the fungal colonies appeared with grainy cottony appearance and became increasingly brown in color from white over time,yellow/yellow-orange reversewithtwo types of conidia. The macroconidia, or tuberculate conidia with distinctive projections on their surface and microconidia which were small and smoothly walled,so it gives the characterstic appearance of *Histoplasma capsulatum* which is agrees withAl-Ani *et al.*(1998), Ameni and Siyoum (2002) andKauffman(2007).

Isolation percent of*Histoplasma capsulatum* in this research was (3.5%), that indicates presence of this dimorphic fungi in horses without any other clinical signs related with *Histoplasma capsulatum,* but it also revealed that there were animals (horses) which were carriers (asymptomatic) to such pathogenic systemic fungi and also have ability to induce the disease related to predisposing factors and this disagree with (Al-ani and Al-delami in 1986),who were isolated *Histoplasma capsulatum* in conducting with incidence of epizootic lymphangitis with(6.4%) in Iraq ,and similar to that wasMesafint *et al.* 2018) whom estimated prevalence of Epizootic lymphangitis (EL) in (23.2%)in carthorses in Ethiopia.

The confirm diagnosis in this research was done by using PCR conventional technique so in all six positive cases, sequencing of the (476-bp amplicons) demonstrated the presence of *Histoplasma capsulatum* DNA, and this was

further confirmed by sequencing of a large sample of clones. PCR methods targeting the 18S rRNA region have previously been used to identify *Histoplasma capsulatum* and have the additional advantage of enabling strain sequence variation to be explored,region sequences shown a high degree of conservation, which was agree with the minimal diversity that was reported among *Histoplasma* spp. by Raquel*et al.* (2013) and Scantlebury *et al*(2016).In this researchfungal cells were used as template for the PCR amplification procedure. Molecular approaches have been developed to provide more rapid and accurate identification of fungi compared with traditional microscopic methods. The 18S rRNA regionshave been usedextensively for PCR-based systems to detect and identify fungal pathogens. Molecular identification of *Histoplasma capsulatum* can be based on the sequences of the 18S rRNA followed by a similarity search in public databases even though this may be hampered by the poor quality of databases in terms of sequence quality, sequence length and taxonomic group or other updates. (Al-ani *et al.* 1998), recorded the appearance of *Histoplasma capsulatum var farciminosum* in twelve suspected cases of horses suffered from Epizootic lymphangitis, and diagnosed presence of *Histoplasma capsulatum var farciminosum,* in contrast to results in this research which confirmed detection of *Histoplasma capsulatum var capsulatum* from asymptomatic carriers of horses by molecular techniques.

This is the first research depend upon PCR-based detection of *Histoplasma capsulatum* from nasal swabs and blood samples of Iraqi equine. Our data suggests the identification of *Histoplasma capsulatum var. capsulatum* in horses, although further work is required to determine the timing of the development of histoplasmosis and investigate the potential for the early detection of *H. capsulatum* from nasal swabs and blood samples.

Conclusions:

According to results appeared in this research, *Histoplasma capsulatum*was isolated from apparentlynormal, healthy animals (horses) without clinical signs,so it was suggested carrier animals may appeared infection with any risk factors animals may suffered from them, so continuous observation and periodic inspection must be done to animals by using classical and modified conventional laboratorytests to confirm the devoid of animals from any infection in latent phase.

REFERENCES

1. Al-Ani, F. K., A. H. Ali, H. B. Banna(1998): *Histoplasma farciminosum* infection of horses in Iraq. Vet. arhiv 68, 101-107.
2. Al-Ani, F. K., A. K. Al-Delaimi (1986): Epizootic lymphangitis in horses: clinical, epidemiological and hematological studies. Pakistan Vet. J. 6, 96-99.
3. Alexander S. and Street D. (2001): In microbiology aphotographic atlas for the laboratory. Benjamincummings, an imprint of Addison Wesly longman, pp.51-52.
4. Ameni G and Terefe W.(2004):A cross-sectional study of epizootic lymphangitis in cart-mules in western Ethiopia. Prev Vet Med.66(1-4):93-9.
5. Ameni, G. and Siyoum, F., 2002: Study on histoplasmosis (epizootic lymphangitis) in carthorses in Ethiopia. Journal of Veterinary Science, 3, p.135-139.
6. Chang. Ryan. (2007):Histoplasmosis.http://www.emedicine.com/MED/topic1021.htm.
7. Collier; Balows L., A. and Sussman M.(1998): Topley & Wilson's Microbiology and Microbial Infections, 9th ed, vol. 4. Arnold, London, Sydney, Auckland, New York.
8. Darling(1909): The morphology of the parasite (Histoplasma capsulatum) and the lesions of histoplasmosis, a fatal disease of tropical America.Journal of Experimental Medicine, vol. 11, no. 4, pp. 515–531, 1909.
9. Dodge. H.J. (1965) : The association of bird-roosting site with infection of school children by Histioplama capsulatum. Am J Publ. Health 55:1203-1211.
10. Heitman; Joseph; Scott G. Filler; Aaron P. Mitchell and John E. Edwards.(2006):Molecular Principles of Fungal Pathogenesis. 1st ed. New York: ASM Press. (Heitman et al. 321-331).

11. Petrie A. and Watson P. (2006): Statistics for Veterinary and Animal Science, Second Edition. Ames: Blackwell Publishing, Pp: 24-112.
12. Wang Y.; Bo Pan, Jianhua Wu, Xinling Bi, Wanqing Liao, Weihua Pan, and Jun Gu (2014): Short Report: Detection and Phylogenetic Characterization of a Case of Histoplasma capsulatum Infection in Mainland China. Am. J. Trop. Med. Hyg., 90(6), pp. 1180–1183.
13. Mesafint M, Ashenafi A, Askale A, Gashaw E. (2018): Prevalence, associated risk factors and socioeconomic impact of Epizootic lymphangitis (EL) in carthorses in and around Gondar town. J Am Sci;14(2):77-83]. ISSN 1545-1003 (print); ISSN 2375-7264 (online). http://www.jofamericanscience.org. 10. doi:10.7537/marsjas140218.10.
14. Kauffman C.A.(2007): Histoplasmosis: a clinical and laboratory update. Clin Microbiol Rev. 20(1):115–32. doi: 10.1128/CMR.00027-06.
15. Scantlebury, C.E., Pinchbeck, G.L., Loughnane, P., Aklilu, N., Ashine, T., Stringer, A.P., Gordon, L., Marshall, M., Christley, R.M., McCarthy, A. (2016):Development and evaluation of a molecular diagnostic method to rapidly detect *Histoplasma capsulatum var. farciminosum*, the causative agent of epizootic lymphangitis, in equine clinical samples.J Clin Microbiol.54(12): 2990–2999.
16. Raquel C. T.; Christina M. S.; Mary E. B.; Takao K.; Eduardo B. (2013):PRP8 intein in cryptic species of Histoplasma capsulatum: Evolution and phylogeny

Table 1.Number of total and Positive.

Types of samples	Total numbers	Positive by conventional methods	Positive by molecular detecting
Nasal swabs	100	4	4
Blood samples	100	3	2

Fig. 1: Macroscopic appearance of suspected isolates, *Histoplasma capsulatum*.

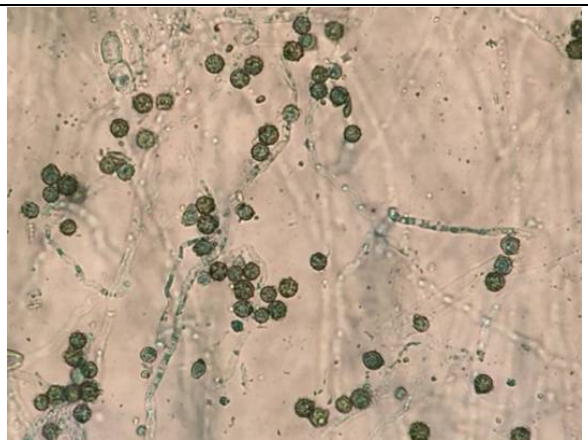

Fig. 2: microscopic appearance of suspected isolates *Histoplasma capsulatum*

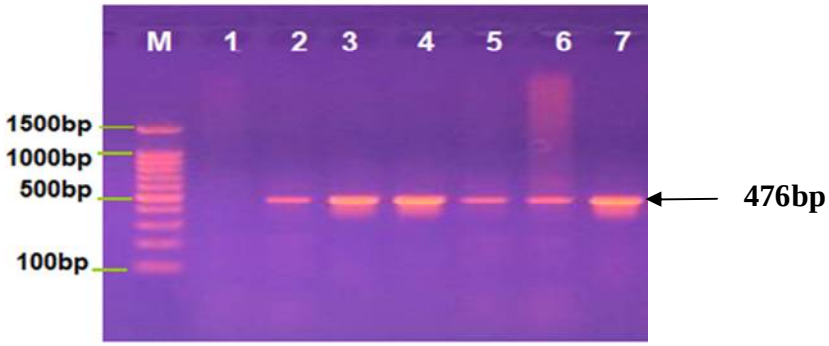

Fig. 3: Agarose gel electrophoresis image that shows the PCR product analysis of 18S ribosomal RNA gene in *H. capsulatum* positive isolates. Where Marker Ladder (1500-100bp), lane (1) negative isolates, and lane (2-7) *H. capsulatum* isolates at PCR product size 476bp.Sequence analysis

Table 2:NCBI-BLAST Homology sequence identity (%) between local *Histoplasma capsulatum* horse isolates and NCBI BLAST *Histoplasma capsulatum* isolates.

Local horse *H. capsulatum* isolatesNo.	Gen-Bank accession No.	NCBI BLAST Homology sequence identity	
		Genbank isolates	Identity (%)
2	MH745422	AF129547	100%
3	MH745423	AF129547	100%
4	MH745424	AF129547	100%
5	MH745425	AF129547	100%
6	MH745426	AF129547	99%

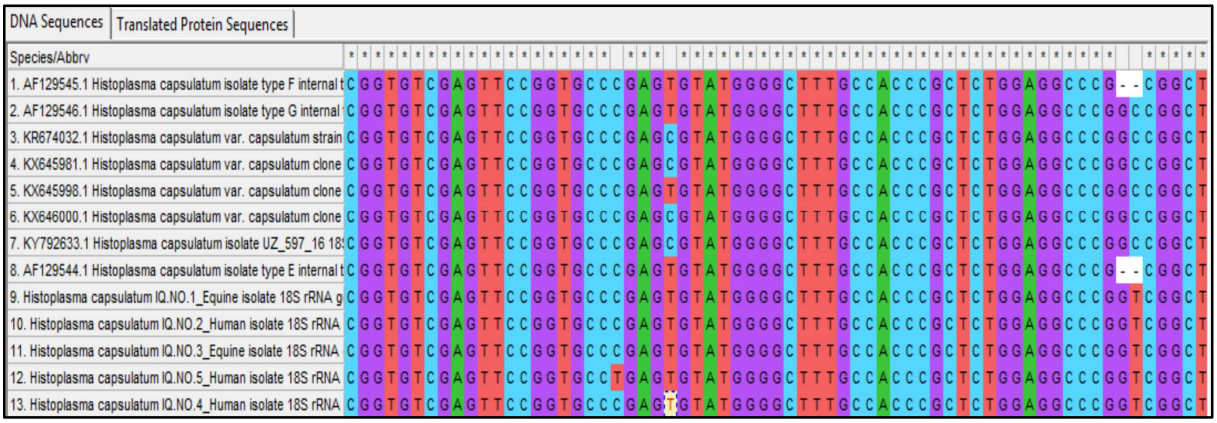

Fig.4: Multiple sequence alignment analysis of the partial 18S ribosomal rRNA gene sequence in local *Histoplasma capsulatum*isolates and NCBI-Genbank *Histoplasma capsulatum* isolates based ClustalW alignment analysis by using (MEGA 6.0, multiple alignment analysis tool). The multiple alignment analysis similarity was shown at (*) in nucleated sequence of 18 ribosomal gene.

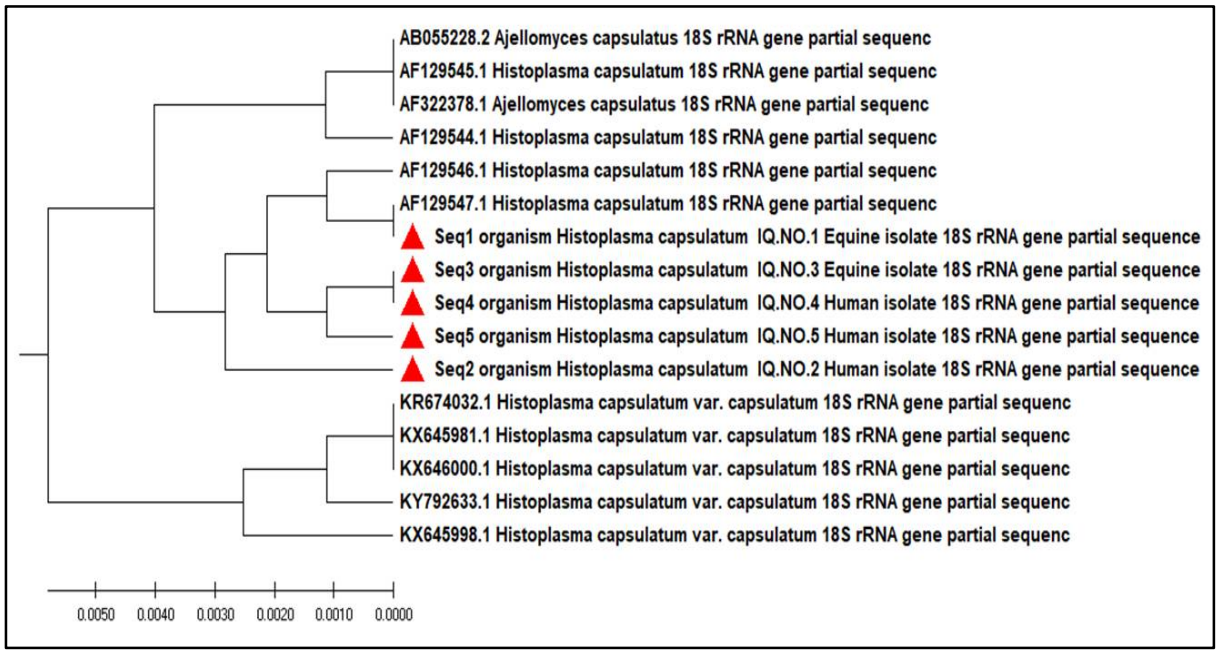

Figure (5): Phylogenetic tree analysis based on the partial sequence of 18S ribosomal rRNA gene in local *Histoplasma capsulatum*isolates that used for genetic confirmative detection analysis. The evolutionary distances were computed using the Maximum Composite Likelihood method by UPGMA phylogenetic tree (MEGA 6.0 version). The local Histoplasma capsulatum No.1-No.5 isolates were shown closed related to NCBI-Blast *Histoplasma capsulatum* (AF129547) at total genetic change (0.0010-0.0050).