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MOLECULAR IDENTIFICATION AND THE FIRST RECORD OF *PROTOTHECA* SPECIES FROM HUMAN PROTOTHECOSIS IN IRAQ

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ABSTRACT : The current study included a collection of 275 clinical samples from four different cases: burns with varying degrees of combustion ranging from (3% -80%), urinary tract infection (UTI), leukemia and dialysis samples. It has been distributed (82, 94, 46, 53), respectively. The samples were collected from Hillah General Teaching Hospital, lobby burns, Margan hospital and period in Iraq, from September 2018 to March 2019. The main objective of the present study was to investigate, isolate, and diagnose different types of *Prototheca* in human infections by using genetic technique polymerase chain reaction (PCR). The results also showed that, the diagnosis genetically to four types of *Prototheca* species responsible for human protothecosis infection in burns with varying degrees of combustion, urinary tract infection (UTI), leukemia and dialysis, for the first time in Iraq, including *Prototheca zopfii* genotype1, *Prototheca zopfii* genotype2, *Protoheca stagnora* and *Protoheca blaschkeae* by using genes *(Proto 18s P. zopfii* genotype 1, *Proto 18s P. zopfii* genotype 2, *Proto 18s P. stagnora* and *Proto 18s P. blaschkeae).* Finally, the current study revealed that, new recorded and the main causative agent responsible for Protothecosis in Iraq is *Prototheca zopfii* genotype 1(45.4%), followed by *Prototheca zopfii* genotype 2 (27.6%), followed by *Prototheca blaschkeae* (15.1%) and *Prototheca stagnora* (11.8).

Key words : Molecular identification, new record, *Prototheca* species, human protothecosis.

INTRODUCTION

The only plants (Microalgae) known to cause infections in humans and animals, the colorless, unicellular green algae genus *Prototheca* (Von Bergen *et al*, 2009; Satoh *et al*, 2010). *Prototheca* is part of Trebouxiophyceae Class (Nedelcu, 2001). Protothecosis is a possible zoonotic disease that can be transmitted to humans by infected milk bovine mastitis (Bozzo *et al*, 2014). The *Prototheca* Microalgae are closely associated with Chlorella greenalgae but lack chlorophyll (Jagielski and Lagneau, 2007).

Five species of *Prototheca* are present in nature and can be isolated from various environmental sources such as stool, soil, lakes and mires, including *P. moriformis*, *P. stagnora*, *P. ulmea*, *P. wicherhamii* or *P. zopfii* (Buzzini, *et al*, 2004). Recent studies suggest that there are six types of *Prototheca* included six species identified, namely: *Prototheca wickerhamii*, *P. zopfii*, *P. blaschkeae*, *P. stagnora*, *P. ulmea* and *P. cutis* (Satoh *et al*, 2010).

Protothecosis is a zoonotic infection, as well as certain *Prototheca* species, e.g. *P. zopfii* and *P. wicherhamii*, are human protothecosis etiological agents (Buzzini *et al*, 2004). These species were first distinguished in (1894) by Krüger, while in livestock with mastitis in 1952, the first case of *Prototheca* infection was reported (Lerche, 1952).

P. zopfii is consideredone of the major pathogens for a bovine mastitis that affected and decreased dairy livestock production (Osumi *et al*, 2008). Infections from *Prototheca* spp have been occurring since then reported in humans, domestic and wild animals. *Prototheca* spp is widespread in the environment, especially in organic substances with a high level of humidity (Scaccabarozzi *et al*, 2008).

Prototheca is a genus of a microscopic, single cell, a sexual auto sporulation based a chlorophyll microalgae with a variable number of Autospores (Malinowski *et al*, 2002). These algae are ubiquitous and saprophytic but, if host immunological defenses weakened orpoor livestock and poor milking hygiene occur, some species may become unusual pathological (Roesler and Hensel, 2003). At the local level, this disease (Protothecosis) and its causes (*Prototheca* spp) have not received the required attention

despite the great damage it causes to human and animals and a few studies, however, have been performed on this genus. Al-Dabbagh (2007) isolated *Prototheca* spp morphologically and biochemically from bovine Mastitis in Mousil city. *Prototheca zopfii* was isolated from the aborted placenta of ewes in AL-Najaf city (Faris *et al*, 2013). *Prototheca zopfii* was detected in wounds of animals (AL-Tameemi and Khalaf, 2013). On the other hand, our study was the first study isolated and identified *Prototheca* spp from human by using molecular methods in Iraq. The current study was aimed to isolation and identification of causative agent of Protothecosis in Iraq by using molecular methods.

MATERIALS AND METHODS

Samples collection

The present study was extended for the period between September 2018 to March 2019, included 275 patients with varying degrees of burns 82 patients, urinary tract infection 94 patients, Leukemia 46 patients, Dialysis 53 patients and the other 70 samples were collected from apparently healthy young people (control) group from Hillah General Teaching Hospital, lobby burns, Margan hospital. In this study, burns Patients included (30 males and 19 females with varying degrees of combustion ranging from (3%-80%)), Urinary tract infection patients (18 males and 35 females), Leukemia patients (14 males and 8 females), Dialysis patients (17 males and 11 females), with an age range (60±1.5 years).

Isolation of *Prototheca* species

Prototheca species were isolated by using Sabouraud's dextrose agar (SDA) and selective media it called Prototheca isolation medium (PIM) was prepared according to Pore (1973), from a purified agar base with the addition of selective agents that inhibit bacterial growth and cultivate *Prototheca*. Aliquots of different samples pre-incubated overnight and streaked on *Prototheca* isolation media (PIM). Streaked plates were incubated under aerobic conditions for 72 hr at 27ºC.

Purification of genomic DNA

Genomic DNA of *Prototheca* species was extracted by using Promega Genomic DNA Purification Kit, USA.

Primers

In this study, using four primers to detect diagnostic genes as shown in Table 1. The primers were supplied by Ligo, USA.

Polymerase chain reaction (PCR) protocols

The *Prototheca* genes were amplification by conventional polymerase chain reaction (PCR), the primer sets manufactured by Ligo, USA. The conditions for *Prototheca* genes are as following initial denaturation 95^0C for 4 minutes followed by Denaturation 95^0C for 1 minute; Annealing 61^0C for 55 second and Extension 72 for 55 seconds, which repeated for 40 cycles after that the final Extension 72C for 8 minutes. The PCR reaction mixture for gradient consisted of 5µl template DNA, 5µl master mix, 5µl of each forward and reverse primer in 20 µl of total reaction volume.

Ethical approval

A valid consist was realized from hospitals administration and from each patients and control before their inclusion in the study. For every patients or their followers, the procedure had been informed before the samples were collected, making completely sure that they understood the procedure that was to be carried out. The subjects were sentient that they had the right to reject to be included in the study without any detrimental effects.

RESULTS AND DISCUSSION

The study included the collection of 275 samples from different types of infections as follow; Burns 82 patients, UTI94 patients, Leukemia 46 patients, Dialysis 53 patients and the other 70 samples were collected from apparently healthy young people as control group (Table 2).

The study included samples from both gender distributed according to the Table 3.

The results demonstrated that, the highest rate of

Table 1 : Sequence of primers.

Gene		Primers	bp	Ref.
Proto 18s *P. zopfii* genotype 1	F	**GACATGGCGAGGATTGACAGA**	990	(Ahrholdt *et al*, 2012)
	R	**GCCAAGGCCCCCCGAAG**		
Proto 18s *P. zopfii* genotype 2	F	**GACATGGCGAGGATTGACAGA**	1050	(Ahrholdt *et al*, 2012)
	R	**GTCGGCGGGGCAAAAGC**		
Proto 18s *P. blaschkeae*	F	**CAGGGTTCGATTCCGGAGAG**	126	(Ahrholdt *et al*, 2012)
	R	**GTTGGCCCGGCATCGCT**		
Proto 18s *P. stagnora*	F	**CGCGCAAATTACCCAATCC**	660	(Abdel Hameed, 2016)
	R	**TCGGCGGGGCAAAAGC**		

infection for males was in burns injuries (19.7%) followed by UTI (11.8%), Dialysis (11.2%) and Leukemia (9.2%), while in female injuries was the highest rate of infection associated with urinary tract infection (23.0%) followed by burns (12.5%), Dialysis (7.2%) and Leukemia (5.3%) (Table 3), this may be due to the opportunistic of *Prototheca* nature.

Isolation and identification of *Prototheca*

The diagnosis of *Prototheca* depends mainly on the morphological characteristics of the organism on the affected areas or directly on the damaged tissue. The genus of *Prototheca* is difficult to isolate compared to the rest of the microorganisms, this is due to the rapid growth of bacteria and fungus. After collecting the various pathogens, this samples cultivate on a special cultures media that provides the special dietary requirements for the growth of the *Prototheca*, in addition to containing the growth inhibitors of bacteria and other microorganisms for the purpose of diagnosing *Prototheca*. One of the most important of these media is *Prototheca* isolation media (PIM). The incubation under aerobic conditions at period of 5-7 days at 25°C is sufficient for the growth of *Prototheca*, this is consistent with the study of Pore (1973) and the other essential medium necessary to isolate *Prototheca* is Sabouraud's dextrose agar (SDA), supplemented with chloramphenicol (0.05µg/ml) under aerobic conditions at period of 24 hours at 37°C. After the incubation period, the colonies can be distinguished as smooth, white, Creamy colonies, round to spherical shape (Fig. 1).

From the growing colonies, wet microscopic smears, methylene blue stains were done to increase the emphasis of the *Prototheca,* the preparations were examined using light microscopy to differentiate between *Prototheca* spp. and other yeasts according to Cosmina *et al* (2009) (Fig. 2).

Also, which showed a typical appearance of the characteristic ovoid to globose sporangia with sporangiospores in several developmental stages (the so-called morula form) (Pore, 1998; DiPersio, 2001; Roesler *et al*, 2006).

On the other hand, the results also demonstrated that the isolation of *Prototheca* distributed as follows 82 cases of burns only 49 (32.2%) positive, 94UTI 53 (34.9%), 46 Leukemia 22 (14.5%) and 53 Dialysis 28 (18.4%). On the other hand, the higher ratio was detected in UTI (34.9%) followed by Burns (32.2%), Dialysis (18.4%) and Leukemia (14.5%) (Table 4).

The results isolation and laboratory diagnosis (Fig. 3) showed that the most common isolates obtained were from Burns and urinary tract infection by 32.2% and 34.9%, respectively,when comparing the results; it was observed that the distribution of isolates and their types was different in the current study. This is due to several reasons, including the source of isolation and difference Geographical location of isolation, number of samples, and other influencing factors contributing significantly to their spreadadd to the degree of interest in hygiene and the type of sterilizers and disinfectants in the hospitals, as well as Neutrophils play an important role in host defense against different species of *Prototheca*, there are reports that patients with immunocompromised have Neutrophils Unable to kill *Prototheca* (Phair *et al*, 1981).

Table 2 : Types of cases including in this study and their percentage.

Cases	No.	%
UTI	94	34.2
Burns	82	29.8
Dialysis	53	19.3
Leukemia	46	16.7
Total	275	100.0

Table 3 : Association between *Prototheca* spp. isolated from each case with gender.

Cases	Gender				Total	
	Male		Female			
	No.	%	No.	%	No.	%
Burns	30	19.7	19	12.5	49	32.2
UTI	18	11.8	35	23.0	53	34.9
Leukemia	14	9.2	8	5.3	22	14.5
Dialysis	17	11.2	11	7.2	28	18.4
Total	79	52.0	73	48.0	152	100.0

Table 4 : Number and percentage of positive cases for *Prototheca* spp. growing on PIM and SDA with all cases including in this study.

Cases	No.	No. of Positive	%
Burns	82	49	32.2
UTI	94	53	34.9
Leukemia	46	22	14.5
Dialysis	53	28	18.4
Total	275	152.0	100.0

Table 5 : Relationship of *Prototheca stagnora* 18s gene with each case.

Cases	No.	No. of Positive	%
Burns	82	5.0	27.8
UTI	94	8.0	44.4
Leukemia	46	1.0	5.6
Dialysis	53	4.0	22.2
Total	275	18.0	100.0

DNA extraction from *Prototheca* samples

Identification of *Prototheca stagnora* by 18s gene

The importance ofthe present molecular investigation focused on to isolate the different types of *Prototheca* species in clinical samples in Iraq. Molecular studies were used for differentiation between of pathogenic and non-pathogenic strains, which cause infection. In recent years, PCR tests have been developed based on 18S rDNA gene sequences to distinguish four genotypes of *Prototheca*. The novel step in this study was given a complete molecular characterization of the *Prototheca* strains segregation in clinical cases. The 18s gene is one of the most important and most widely used genes at the molecular level and a critical marker for the interaction of randomized polymerase (PCR) within the examination of natural biodiversity. In truth, the 18s sequence afterward given proves of the genus of *Prototheca*, which greatly supported a revolutionary change in diagnosis of *Prototheca* (Meyer *et al*, 2010). The results of electric transport on the gel revealed the distribution of positive samples according to the (Table 5).

In Table 5, which is revealed the Relation of *Prototheca stagnora* 18s gene with each cases from all cases 275 the distribution of *Prototheca stagnora* depending on 18s gene was as follow; 82 Burns casesonly 5 (27.8%) positive, 94UTI 8 (44.4%), 46 Leukemia 1 (5.6%) and 53 Dialysis 4 (22.2%). The higher ratio was detected in UTI (44.4%) followed by Burns (27.8%), Dialysis (22.2%) and Leukemia (5.6%). *Prototheca stagnora* was diagnosed by using *Proto 18s P. stagnora* gene (660bp) as appeared in the accompanying in Fig. 3.

Identification of *Prototheca zopfii* genotype1 and *Prototheca zopfii* genotype 2 gene

The results uncovered clearly that, the relationship of *Prototheca zopfii* genotype1 18s gene with each cases from all cases 275 the distribution of *Prototheca zopfii* genotype1 depending on 18s gene was as follow; 82 Burns cases only 22 (31.9.8%) positive, 94 UTI 19 (27.5%), 46 Leukemia 10 (14.5%) and 53 Dialysis 18 (26.1%). The higher ratio was detected in burns (31.9%) followed by UTI (27.5%), Dialysis (26.1%) and Leukemia (14.5%) (Table 6).

Table 7 explained the relationship of *Prototheca zopfii* genotype 2 18s gene with each cases from all cases 275 the distribution of *Prototheca zopfii* genotype 2 depending on 18s gene was as follow: 82 Burns cases only 13 (31.0 %) positive, 94 UTI 16 (38.1%), 46Leukemia 7 (16.7%) and 53Dialysis 6 (14.3%). The higher ratio was detected in UTI (38.1%) followed by burns (31.0%), Leukemia (16.7%) and Dialysis (14.3%).

Table 6 : Relationship of *Prototheca zopfii* genotype 1 18s gene with each cases.

Cases	No.	No. of Positive	%
Burns	82	22.0	31.9
UTI	94	19.0	27.5
Leukemia	46	10.0	14.5
Dialysis	53	18.0	26.1
Total	275	69	100.0

Table 7 : Relationship of *Prototheca zopfii* genotype 2 18s gene with each case.

Cases	No.	No. of Positive	%
Burns	82	13.0	31.0
UTI	94	16.0	38.1
Leukemia	46	7.0	16.7
Dialysis	53	6.0	14.3
Total	275	42	100.0

Table 8 : Relationship of *Prototheca blaschkeae* 18s gene with each case

Cases	No.	No. of Positive	%
Burns	82	9.0	39.1
UTI	94	10.0	43.5
Leukemia	46	4.0	17.4
Dialysis	53	0.0	0.0
Total	275	23	100.0

The pathogenesis of *Prototheca* are still vague inhuman injury, where they are thought to be low virulence and often infect patients with various forms of immunosuppression (Jagielski and Lagneau, 2007). *P. zopfii* is often associated with chronic bovine mastitis in dairy herds and is responsible for massive economic losses in dairy herds (Bozzo *et al*, 2014). This is therefore important from a public health point of view because *P. zopfii* is transmitted to humans through contaminated milk and causes intestinal infection such as intestinal inflammation (Melville *et al*, 1999). *P. zopfii* adhesion to host cells, which is the most important step in the infection process, followed by colony colonization. This is consistent with Melchior *et al* (2006) and Akers *et al* (2015), which explores the adhesion and formation of biofilms of *P. zopfii* associated with mastitis. In this study, we have thoroughly studied, for the first time in Iraq, the involvement of *P. zopfii* genotype 1and 2 with human infections (Fig. 4).

The study showed a high incidence of *P. zopfii* infection, especially in burns, this is due to direct human contact with cows, in an environment where cattle breeding is frequent (McDonald *et al*, 1984).

On the other hand, *P. zopfii* is characterized as resistant to pasteurization (Zaini *et al*, 2012) and thus

Fig. 1 : Colony morphology of *Prototheca* spp. grown on Sabouraud's dextrose agar at 37°C for 24 hr.

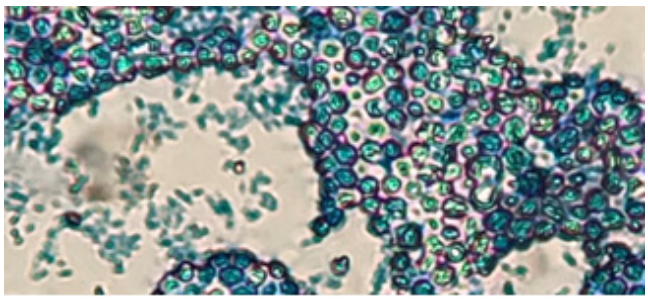

Fig. 2 : Micrographs of *Prototheca* spp. stained with methylene blue, 40x magnifications grown on Sabouraud's dextrose agar at 37°C for 24 hr.

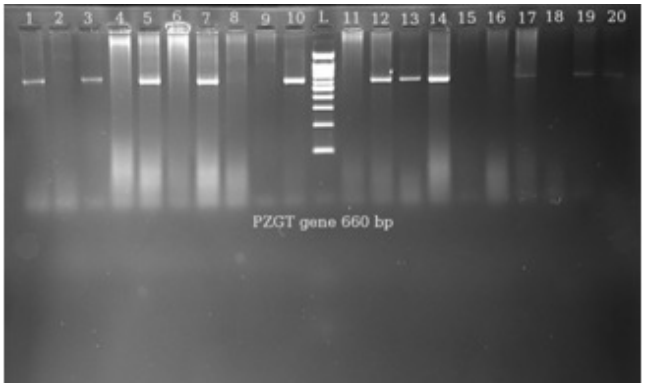

Fig. 3 : Electrophoresis of PZGT gene products. L lane contain the 100 bp DNA Ladder, 5% NuSieve® 3:1 agarose gel in 1X TBE buffer containing 5µl red safe. (1, 3, 5, 7, 10, 12, 13, 14, 17, 19, 20) Lanes positive results with 660 bp (2, 4, 6, 8, 9, 11, 15, 16, 18) negative results.

can be easily transported to its consumers and potentially cause health problems, since Protothecosis is an animal disease, can it is easily transmitted to humans through the consumption of milk contaminated with these organisms and caused infection because resistance to pasteurization (Melville *et al*, 1999).

As a result, it is important and crucial to identify these microorganisms as they possess a significant risk. *Prototheca zopfii* genotype 1 and *Prototheca zopfii*

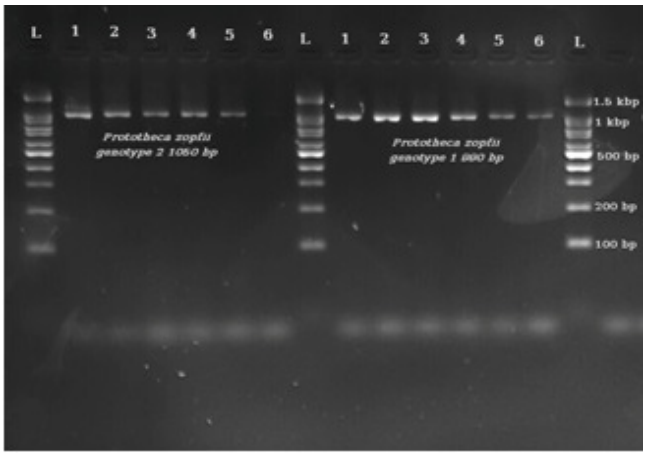

Fig. 4 : Electrophoresis of Proto 18s *P. zopfii* genotype 1 and Proto 18s P. zopfii genotype 2 gene products. L lane contain the 100 bp DNA Ladder, 5% NuSieve® 3:1 agarose gel in 1X TBE buffer containing 5µl redsafe. (From right 1-6) Lanes positive results 990 bp (from left 1-5) Lanes positive results 1050 bp (6 from left) negative results.

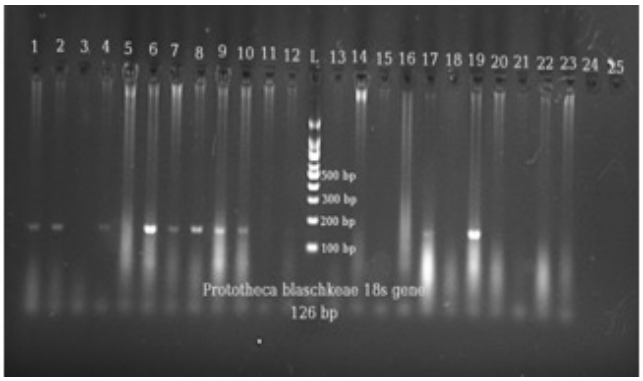

Fig. 5 : Electrophoresis of Proto 18s P. Blaschkeae gene products. L lane contain the 100 bp DNA Ladder, 5% NuSieve® 3:1 agarose gel in 1X TBE buffer containing 5µl redsafe. (1,2,4,6,7,8,9,10,17,19,) Lanes positive results with 126 bp5,16,18,20,21,22,23) negative results (24 and 25) Lane was control negative of PCR product.

genotype 2 was diagnosed by gene 18s (990 bp, 1050 bp) and our recorded results were consistent with the Ricchi *et al* (2010) (Fig. 5).

Identification of*Protothecablaschkeae* by 18s gene

The results elucidate that, the relationship of *Prototheca blaschkeae* 18s gene with all cases 275, the distribution of *Prototheca blaschkeae* depending on 18s gene was as follow: 82 Burns cases only 9 (39.1 %) positive, 94 UTI 10 (43.5%), 46Leukemia 4(17.4%) and 53Dialysis 0 (0.0%). The higher ratio was detected in UTI (43.5%) followed by burns (39.1 %), Leukemia (17.4%) and Dialysis (0.0%) (Table 8).

Previous researches indicates that *P. blaschkeae* was classified as the third biotype of *P. zopfii* where it was reported to be the non-pathogenic biotype, while the biotype II was associated with bovine mastitis (Roesler

and Hensel, 2003). The development of genetic studies and evolutionary studies of the 18S rDNA sequence of genes has been the Great credit in determining the specific molecular properties of *P. blaschkeae* and reclassifying the biotypes of *P. zopfii* to two genotypes. The first time the *P. blaschkeae* was isolated was from human onychomycosis (Roesler *et al*, 2006).

There is no previous researches indicating the contribution of *P. blaschkeae* in various human infections (e.g. burn injuries, UTI and leukemia infections) *Prototheca blaschkeae* was diagnosed by using Proto 18s*P. Blaschkeae* (126bp) (Ahrholdt *et al*, 2012) as appeared in the accompanying figure (Fig. 5).

CONCLUSION

Our study was the first timeRecord which detected genetically *Prototheca* species such as (*P. zopfii* genotype 1, *P. zopfii* genotype 2, *P. stagnora* and *P. blaschkeae*) in Iraq.

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