



Identification of *Prototheca zopfii* from Bovine Mastitis

F Zaini¹, A Kanani¹, *M Falahati², R Fateh¹, M Salimi-Asl³, N Saemi⁴, Sh Farahyar^{1,2},
A Kargar Kheirabad⁵, M Nazeri¹

1. Dept. of Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

2. Dept. of Parasitology and Mycology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

3. Dept. Of R&D, Behmedico Company, Tehran, Iran

4. Dept. of Nutrition, Allied Medical School, Tehran University of Medical Sciences, Tehran, Iran

5. Hormozgan Research Centre for Infectious and Tropical Disease, Hormozgan University of Medical Sciences, Hormozgan, Iran

(Received 21 Jan 2012; accepted 10 Jun 2012)

Abstract

Background: The aim of this study was identification of the epidemiology of *Prototheca zopfii* species from the milk samples of dairy cattle in Isfahan, central Iran.

Methods: Milk samples were obtained from 230 dairy cattle, 130 with and 100 without mastitis, in Isfahan. The samples were cultured in *Prototheca* Isolation Medium (PIM) and Sabouraud's dextrose agar. All *P. zopfii* isolates were identified by morphological and biochemical methods. Then, as a confirmatory test they were examined by genotype-specific PCR.

Results: Four *P. zopfii* strains (3.07%) were isolated from the 130 samples of dairy cattle with clinical mastitis and there was no isolation from totally 100 samples of healthy bovines without mastitis. Specific PCR product (about 946 bp) was detected in four isolates.

Conclusion: It seems that *P. zopfii* genotype II plays a key role in affecting bovine mastitis that confirmed other previous studies. Our study was the first, which identified the *Prototheca* species by traditional and molecular methods in Iran and Middle East as well.

Keywords: Bovine mastitis, *Prototheca zopfii*, Iran

Introduction

The algae of the *Prototheca* (*P.*) genus are in a close relation to green algae *Chlorella* but they lack of chlorophyll (1). There are five species of prototheca including *P. moriformis*, *P. stagnora*, *P. ulmea*, *P. wicherhamii*, and *P. zopfii* in the nature in saprophyte form and can be isolated from different environmental sources such as stool, soil, lakes, and mires. Protothecosis is a zoonotic infection as well as some species of *Prototheca* e.g., *P. zopfii*, and *P. wicherhamii* are etiological agents of human protothecosis (2). Krüger distinguished these species for the first time (1894) and in 1952 *P. zopfii* was identified as a bovine mastitis pathogen which affected and reduced milk production in dairy cat-

tle (3). There are many reports for endemic incidence as well as some case reports of the infection in many regions of the world (4). In last decade, the incidence of the bovine mastitis has been increased (5-9). Bovine mastitis due to *Prototheca* species has been demonstrated by some other investigations. In these studies, different aspects of the infection have been considered such as epidemiological aspects (4,7,10,11), clinical symptoms (5,6,9,12), anti-microbial sensitivities (13,14) and histopathologically (15,16). *P. zopfii* genotype II as a predominant pathogen for the mastitis (3,17,18). *Prototheca zopfii* has been differentiated to three biotypes (17). Studies using 18S rRNA gene

*Corresponding Authors: Tel: (+9821) 88622653, E-mail address: candidasp@gmail.com

Available at : <http://ijph.tums.ac.ir>

sequencing, obviously confirmed that the *P. zopfii* had three different biotypes. Recently biotypes I and II are considered as Genotypes I and II, respectively and biotype III as a *P. blaschkeae* (18). In the most cases of *Prototheca* infections that can lead to mastitis in cow, *P. zopfii* genotype II was identified as a predominant causative pathogen in bovine mastitis (3).

The aim of this study was identification of the epidemiology of the disease through biochemical and PCR methods in isolated *P. zopfii* species from the milk samples of dairy cattle with and without mastitis in Isfahan, Iran.

Materials and Methods

Milk samples obtained from 230 dairy cattle, 130 with and 100 without mastitis, in Isfahan. The samples first were cultured in *Prototheca* Isolation Medium (PIM), supplemented with chloramphenicol (100µg/ml) and Subouraud- dextrose- agar for 7 days at 27°C. Then the *P. zopfii* species were identified by morphological and biochemical methods. Genotypes of the species isolates from mastitis were analyzed by genotype-specific PCR. In order to perform PCR, certain primers for the genotypes were employed (19). In order to consider the three genotypes of *P. zopfii* and primer designing through the analysis of the 18S rRNA gene sequencing, acquired from GeneBank # [AY973040](#), [AY 940456](#), [AY973041](#) (specific for genotypes I, II, and III, respectively), was used Gene runner, MEGA 4 and oligo analyzer software were employed to achieve this goal (18).

Four isolated *P. zopfii* samples recultured in Subouraud's Dextrose agar medium at 27°C for 48 hours for DNA extraction by using Invisorb Spin Plant Kit (Invitex GmbH). Afterwards, 18S rRNA gene was amplified by PCR. Genotype-specific primers are listed in Table 1.

PCR amplification (25 µl/reaction) was carried out with the master mix (0.3 µl (5 U/µl) TAQ Polymerase, 3 mM MgCl₂, 1 µl (65 ng) genomic DNA, 1 µl (50 µM) each primer and 0.5 µl (10 mM) (dNTP), 2.5 µl buffer (10X) .The PCR was started with an initial denaturation step at 95 °C

for 4 min followed by 40 thermal cycles of denaturation at 95 °C for 1 min, annealing at 66 °C for 55 s, and DNA synthesis at 72 °C for 55 s, and finally at 72 °C for 8 min.

Table 1: Genotype-specific primers used in this study

Specific primer	Target	Sequences
PZGT 1-A/f PZGT 1-A/r	Genotype 1-specific PCR	5'- CGCGCAAATT ACCCAATCC- 3' 5'-GCCAAGGCC CCCCGAAG- 3' 5'-CGCGCAAATT
PZGT 2-A/f PZGT 2-A/r	Genotype 2-specific PCR	ACCCAATCC- 3' 5'-GTCGGCGGG GCAAAAAGC- 3' 5'-CAGGGTTCGA
PZGT 3-A/f PZGT 3-A/r	Genotype 3-specific PCR	TTCCGGAGAG- 3' 5'-GTTGGCCC GGCATCGCT- 3'

Results

The *P. zopfii* species cultured in *Prototheca* Isolation Medium (PIM), supplemented with chloramphenicol (100µg/ml) (Fig. 1) and Subouraud- dextrose- agar (Fig. 2).

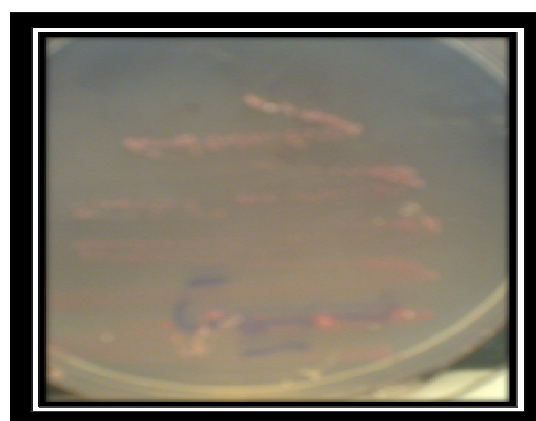


Fig.1: Characteristic colony morphology of *P.zopfii* genotype 2 on differential *Prototheca* Isolation Medium at 27°C after 7 days (Source: Authors)



Fig. 2: Colony morphology of *P.zopfii* genotype 2 on Subouraud Medium (Source: Authors)

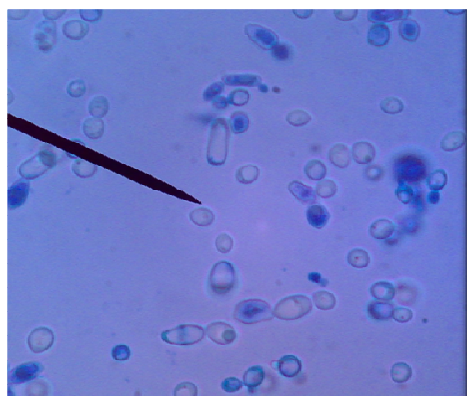


Fig.3: Methylene blue staining of *P. zopfii* genotype II isolated from milk samples obtained from diary cattle with mastitis

Then the *P. zopfii* species were identified by morphological (Fig. 3) and biochemical methods (Table 2).

Table 2: Characteristics that discriminate *Prototheca zopfii*

Characteristics	Absorption
Glucose	+
Galactose	-
Sucrose	-
Trehalose	-
Propanol	+
Glycerol	+

(+), absorption, (-), no absorption

Four *P. zopfii* strains (3.07%) were isolated from the 130 samples of dairy cattle with clinical mastitis. There was no isolation from totally 100 samples of healthy bovines without mastitis. PCR was performed by newly designed primers for genotype II of *P. zopfii* and specific PCR product (about 946 bp) was detected in four isolates. Four *P. zopfii* mastitis isolates were thus identified as *P. zopfii* genotype II (Fig. 4). The results of genotype-specific PCR also showed that the three genotypes obviously are different in 18S rRNA gene composition. Furthermore, 18S rRNA gene sequencing of genotype III was clearly different from genotypes I and II.

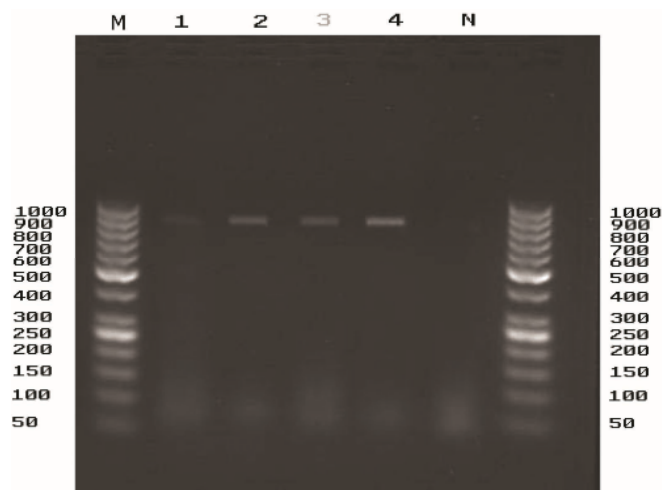


Fig. 4: The result of the PCR for *P. zopfii* genotype II. Agarose gel electrophoresis (2%) of amplified PCR products, performed by specific primers for genotype II. Lane M: DNA ladder, lane 1-4: the milk samples of the bovines with mastitis, lane N: negative control

Discussion

Based on the results of this study as well as other previous studies, it seems that *P. zopfii* genotype II plays a key role in causing bovine mastitis (2-5). This study was performed for the first time in Iran on the bovines with and without mastitis. In order to find *P. zopfii* in the milk samples, specific me-

dium was used for detection and isolation of *Prototheca* species (20).

In 1999, Melville et al. performed a study, which considered the sensitivity of *P. zopfii* to pasteurization. They showed different sensitivity of 40 strains of *P. zopfii* to different times and temperatures of pasteurization (21).

This study was also important from the point of view of veterinarian studies and humans' health since bovine mastitis can affect milk production by the cattle and reduce it. On the other hand, the *P. zopfii* is resistant to pasteurization and, therefore can easily transmit to its consumers and potentially cause health problems. Consequently, more studies should be carried out in this field and in other geographical parts of Iran, using more milk samples from bovines with mastitis in order to coordinate and confirm the results of recent study. The results showed *P. zopfii*, genotype II, is responsible for causing bovine mastitis in Iran as same as other geographical parts of the world (3, 17, 19).

Protothecosis is a zoonotic disease, which can be transmitted to the human by consuming milk and cause intestinal infections and enteritis because of its resistance to pasteurization (21). As a result, it is important and crucial to consider and identify these microorganisms in milk, because they can be potentially harmful to the human health. This study was the first study, which identified the *Prototheca* species as causative agents of bovine mastitis, by traditional and molecular methods in Iran and Middle East as well.

We hope that this study paves the way for further studies and investigations to be carried out in this field.

Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc) have been completely observed by the authors.

Acknowledgments

This study was part of MSc thesis supported by Tehran University of Medical Sciences (grand no: 704/P). The authors declare that there is no conflict of interests.

References

1. Jagielski T, Lagneau P (2007). Protothecosis. A pseudofungal infection. *Med Mycol*, 17: 261-270.
2. Buzzini P, Turchetti B, Facelli R, Baadino R, Cavarero F, Mattaalia L, et al. (2004). First large-scale isolation of *Prototheca zopfii* from milk produced by dairy herds in Italy. *Mycopathologia*, 158: 427-30.
3. Osumi T, Kishimoto U, Kano R, et al. (2008). *Prototheca zopfii* genotypes isolated from cow barns and bovine mastitis in Japan. *Vet Microbiol*, 131: 419-423.
4. Hodges RT, Holland JTS, Neilson FJA, Wallace NM (1985). *Prototheca zopfii* mastitis in a herd of dairy cows. *New Zeal Vet J*, 33: 108-111.
5. Costa EO, Carciofi AC, Melville PA, Prada MA, Ribeiro AR, Watanabe ET (1996). Bovine mastitis due to algae of the genus *Prototheca* sp. *Mycopathologia*, 133: 85-88.
6. Costa EO, Ribeiro AR, Watanabe ET, Pardo RB, Silva JAB, Sanches RB (1996). An increase incidence of mastitis caused by *Prototheca* species and *Nocardia* species on a farm in Sao Paulo, Brazil. *Vet Res Commun*, 20: 237-241.
7. Costa EO, Carciofi AC, Melville PA, Prada MA, Schalch U (1996). *Prototheca* spp. outbreak of bovine mastitis. *J Vet Med*, 43: 321-324.
8. Aalbaek B, Stenderup J, Jensen HE, Valbak J, Nylin B, Huda A (1994). Mycotic and algal mastitis in Denmark. *Acta Pathol Microbiol Immunol Scand*, 102: 451-456.
9. Almeraya AP (1994). Aislamiento de *Prototheca* en un brote de mastitis bovina. *Vet Mex*, 25: 65-67.
10. Anderson KL, Walker RL (1998). Sources of *Prototheca* spp. in a dairyherd environment. *J Am Vet Med Assoc*, 193: 553-556.
11. Costa EO, Carciofi AC, Melville PA, Prada MS, Ribeiro AR, Watanabe ET (1994).

- Epidemiological studies on bovine protothecosis. In: World Buiatrics Congress, 18. Bologna, Italy: Proceedings, 853-855.
12. Kirk JH (1991). Diagnosis and treatment of difficult mastitis cases. *Agri-Practice*, 12:15-20.
 13. Segal E, Padhye AA, Ajello L (1976). Susceptibility of *Prototheca* species to antifungal agents. *Antimicrob Agents Chemother*, 10: 75-79.
 14. Camargo ZP (1978). Algas do g^o enero *Prototheca*. caracteristicas microbiologicas e imunologicas [Thesis]. Sao Paulo: Escola Paulista de Medicina.
 15. McDonald JS, Richard JL, Cheville NF (1984). Natural and experimental bovine intramammary infection with *Prototheca zopfii*. *Am J Vet Res*, 45: 592-595.
 16. Cheville NF, McDonald J, Richard J (1984). Ultrastructure of *Prototheca zopfii* in bovine granulomatous mastitis. *Vet Pathol*, 21: 341-348.
 17. Moller A, Truyen U, Roesler U (2007). *Prototheca zopfii* genotype2-the causative agent of bovine protothecal mastitis? *Vet Microbiol*, 120: 370-374.
 18. Roesler U, Moller A, Hensel A, Baumann D, Truyen U (2006). Diversity within the current algal species *Prototheca zopfii* a proposal for two *Prototheca zopfii* genotypes and description of a novel species, *Prototheca blaschkeae* spp. nov. *Int J Syst Evol Microbiol*, 56: 1419-1425.
 19. Aouay A, Coppee F, Cloet S, Cuvelier P, Belayew A, Lagneau P, et al. (2008). Molecular characterization of *Prototheca* strains isolated from bovine mastitis. *Med Mycol*, 157: 1-4.
 20. Pore RS (1973). Selective medium for the isolation of *Prototheca*. *Appl Microbiol*, 26: 648-9.
 21. Melville P, Watanabe E, Benites N, Ribeiro A, Silva J, Garino F, et al. (1999). Evaluation of the susceptibility of *Prototheca zopfii* to milk pasteurization. *Mycopathologia*, 146: 79-82.