Identification of Prototheca zopfii from Bovine Mastitis

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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 cattle (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 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
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 caw, 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 (100 f.g/ml) and Subouraud- dextrose- agar for 7 days at 27ºC. 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 27ºC for 48 hours for DNA extraction by using Invisorb Spin Plant Kit (Invitek GmbH). Afterwards, 18S rRNA gene was amplified by PCR. Genotype-specific primers are listed in Table 1.

PCR amplification (25 f.l/reaction) was carried out with the master mix (0.3 f.l (5 U/f.l) TAQ Polymerase, 3 mM MgCl2, 1 f.l (65 ng) genomic DNA, 1 f.l (50 f.M) each primer and 0.5 f.l (10 mM) (dNTP), 2.5 f.l buffer (10X) .The PCR was started with an initial denaturation step at 95 ºC for 4 min followed by 40 thermal cycles of denaturation at 95 ºC for 1 min, annealing at 66 ºC for 55 s, and DNA synthesis at 72 ºC for 55 s, and finally at 72 ºC for 8 min.

Table 1: Genotype-specific primers used in this study

<table>
<thead>
<tr>
<th>Specific primer</th>
<th>Target</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>PZGT 1-A/f</td>
<td>Genotype 1-specific PCR</td>
<td>5’-CGCGC_AAATT_</td>
</tr>
<tr>
<td>PZGT 1-A/r</td>
<td>Genotype 1-specific PCR</td>
<td>5’-GCC_AGGCC_CCCGAAG-3’</td>
</tr>
<tr>
<td>PZGT 2-A/f</td>
<td>Genotype 2-specific PCR</td>
<td>5’-CCCG_AAATT_CGCGC_3’</td>
</tr>
<tr>
<td>PZGT 2-A/r</td>
<td>Genotype 2-specific PCR</td>
<td>5’-ACCC_AATCC_GCAAAGC_3’</td>
</tr>
<tr>
<td>PZGT 3-A/f</td>
<td>Genotype 3-specific PCR</td>
<td>5’-TTCC_GGAG_3’</td>
</tr>
<tr>
<td>PZGT 3-A/r</td>
<td>Genotype 3-specific PCR</td>
<td>5’-GTTGG_CCC_5’</td>
</tr>
</tbody>
</table>

Results

The P. zopfii species cultured in Prototheca Isolation Medium (PIM), supplemented with chloramphenicol (100 f.g/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 27ºC after 7 days (Source: Authors)
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.

**Table 2: Characteristics that discriminate *Prototheca zopfii***

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Absorption</th>
</tr>
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<tbody>
<tr>
<td>Glucose</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
</tr>
<tr>
<td>Trehalose</td>
<td>-</td>
</tr>
<tr>
<td>Propanol</td>
<td>+</td>
</tr>
<tr>
<td>Glycerol</td>
<td>+</td>
</tr>
</tbody>
</table>

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

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

**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-
A medium 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 veterinary 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.

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References


