RESEARCH ARTICLE

First Report of Molecular Identification of *Prototheca zopfii* Genotype 2 as Causative Agent of Bovine Mastitis in Navsari, South Gujarat, India

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Abstract

Prototheca species is emerged as an important causative agent for bovine mastitis worldwide, and is responsible for great economic losses for dairy industry. A 5-year-old HF cow was referred to the Veterinary Clinical Complex of the College, Navsari (India) with a complaint of watery milk with flakes, anorexia and drop in milk production. On physical examination, udder appeared warm and swollen, while milk consistency was thin, watery with flakes. Milk sample obtained aseptically from the cow was inoculated aerobically on Sabouraud dextrose agar and blood agar plates. *Prototheca* colonies grew in pure and luxuriant form from mastitic milk on both the plates after 48 h of incubation. *Prototheca zopfii* was identified on the basis of colony morphology, staining characters and biochemical reactions. Molecular confirmation of *Prototheca zopfii* genotype 2 was performed by employing polymerase chain reaction (PCR). This report presents first isolation and molecular identification of *Prototheca zopfii* genotype 2 from a case of bovine mastitis from Navsari region, South Gujarat, India.

Keywords: Algae, Blood agar, Mastitis, PCR, Sabouraud medium.

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Introduction

Mastitis is globally an important production disease of dairy cows (FAO, 2014) and one of the major causes of economic losses to dairy farmers. Economic losses due to mastitis include decrease in milk production, lower milk quality, premature culling and treatment costs. Globally loss of 5100.75 to 8111.03 Rs per cow due to clinical mastitis has been reported (Hogeveen *et al*., 2011). In India, annual economic losses due to subclinical and clinical mastitis have been estimated to be Rs. 4151.1 and Rs. 3014.4 crores, respectively, with a total of Rs. 7165.5 crores (Bansal and Gupta, 2009). A wide variety of microorganisms including bacteria, fungi, yeast, and mycoplasma are responsible for causing mastitis (Bhat *et al*., 2017) and the occurrence depends on variables related to the animal, pathogen and environment (Radostits *et al*., 2007; Pal *et al*., 2019).

Prototheca species is considered as unicellular, achlorophyllic yeast-like algae that are normally found as saprophytes. *Prototheca* organisms are ubiquitous in nature and more variable in size and shape (Pal, 2007; Pal *et al*., 2014). Protothecal bovine infection is gradually progressive and often subclinical, making it difficult to recognize at early stage. It leads to mild changes in milk, with an increase in somatic cell counts (SCC), reduced milk production and a thin watery milk secretion containing white flakes (Cremonesi *et al*., 2012). Majority of *Prototheca* isolates from bovine mammary protothecosis came from *P. zopfii* genotype 2 and considered as to be the major causative agent of mastitis in dairy cows (Möller *et al*., 2007; Osumi *et al*., 2008; Bozzo *et al*., 2014; Capra *et al*., 2014; Milanov *et al*., 2016). Bovine mammary protothecosis leads to subclinical and clinical mastitis. So far, *P. zopfii* has been isolated worldwide from milk of cows with clinical and subclinical mastitis (Costa *et al*., 1996; Malinowski *et al*., 2002). Treatment for protothecal infections remains controversial, various therapeutic regimens have been attempted but without consistency in the clinical responses

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(Buzzini *et al*., 2008). Pal and Lee (1997) are credited to record protothecal mastitis in a 7-year-old HF cow for the first time in India. The algae was isolated in pure and luxuriant growth on blood agar as well as Sabouraud dextrose agar medium and the detailed microscopic morphology of the isolate was studied in PHOL (Pal, Hasegawa, Ono, Lee) stain (Pal *et al.,* 1990). This communication delineates the first report of molecular identification of *Prototheca zopfii* genotype 2 as causative agent of bovine mastitis in Navsari, South Gujarat, India.

Materials and Methods

A 5-year-old HF cow was referred to the Veterinary Clinical Complex, Veterinary College, Navsari (India) with a complaint of watery milk with flakes. The history revealed that animal was calved a month before and then after thin watery milk with flakes were observed along with anorexia and drop in milk production. The antibacterial and anti-inflammatory treatment was given repeatedly at field level that was found unresponsive. On physical examination, warm and swollen udder and thin watery milk with flakes was noticed. Aseptically collected milk sample was taken for cultural isolation and identification of the organisms. Primarily milk sample was inoculated on blood agar and incubated aerobically at 37° C for 24 h to rule out bacterial organisms. Also the sample was inoculated on Sabouraud dextrose agar (SDA) and aerobically incubated at 37°C. Colonies were observed on both the agar plates after 48 h of incubation. Microscopically, colonies were identified on the basis of wet mount preparation with lactophenol cotton blue (LPCB) and smear stained with methylene blue and Gram's stain. Biochemical tests and colony polymerase chain reaction (PCR) were carried out to confirm an unequivocal diagnosis. Two sets of primers were used to detect a fragment of *Prototheca* spp. (216 bp) and *Prototheca zopfii* genotype 2 (508 bp) as previously described (Capra *et al*., 2014). For identification of *Prototheca* spp., a set of forward primer TCGGAGTTAGCTGGTTCTCC and reverse primer ATTTTGGGGCCTTAACTGGT, while for *Prototheca zopfii* genotype 2 forward primer TGTAATAGATATTAGAAACGCAAC AAA and reverse primer GCAGCAGTAGGGAATTTTGG (Capra *et al*., 2014) were used. For colony PCR, colony was diluted in 20 µL nuclease free water and used as a template (DNA) for PCR. To this, mastermix (Thermoscientific) 12.5 µL primers (1 forward and 1 reverse) and NFW was mixed to prepare 25 µL of reaction mixture. DNA amplification was carried out using thermal cycler (ProFlex, Applied Biosystem) at the temperature profiles, *viz.,* initial denaturation at 94°C for 15 min, DNA denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min for 30 cycles and final extension at 72°C for 7 min. The amplified PCR products were separated electrophoretically on 1.5 % agarose gels and visualized by using UV (SynGene, Gene Genius Bio Imaging System, UK) and 50 bp DNA ladder (Genetix).

Results and Discussion

After 48 h of incubation, many soft, shiny, creamy white, circular colonies with slight margins were observed on the plates of both blood agar and SDA medium (Fig. 1, 2). Microscopically, characteristic morulas of *Prototheca* were observed by wet mount preparation with lactophenol cotton blue stain (LPCB). These revealed spherical to oval sporangia with endosporulating sporangiospores of varying sizes (Fig. 3). Colony stained by Gram's method revealed large yeast like cells with irregular size and shape (Fig. 4). Biochemically *Prototheca* spp. was confirmed by Galactose –ve, Glucose +ve and Trehalose –ve reactions (Fig. 5).

Prototheca spp. as well as *Prototheca zopfii* genotype 2 were successfully amplified and confirmed with N476-F and N476-R (amplicon size 216 bp) and N2-F and N2-R (amplicon size 508 bp), respectively (Fig. 6).

Bovine protothecal mastitis is characterized by deteriorating milk quality and quantity, thus imparting huge economic losses to dairy industry (Shahid *et al*., 2017). Protothecosis, mostly caused by *Prototheca zopfii* appears in the clinical and subclinical form of mastitis in dairy cattle (Ahrholdt *et al*., 2012). Acute clinical form of protothecal mastitis is generally characterized by high temperature (up to 40 °C), pain and hot oedema of the udder, anorexia and reluctance to move. In chronic form, slight pain, hard tissue consistency with pasty oedema in the udder, along with marked decrease in milk production with elevated somatic cell count, especially macrophages, is observed, which may

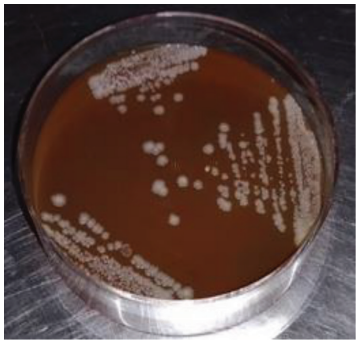

Fig. 1: *Prototheca* colonies on blood agar after 48 h of incubation

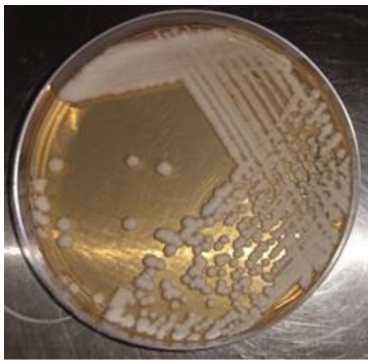

Fig. 2: Several colonies of *Prototheca* grew on Sabouraud dextrose agar after 48 h of incubation

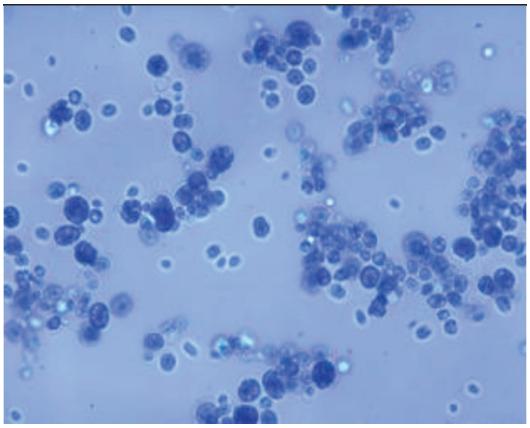

Fig. 3: Wet mount preparation showing *Prototheca zopfii* with endosporulating sporangiospores (LPCB stain)

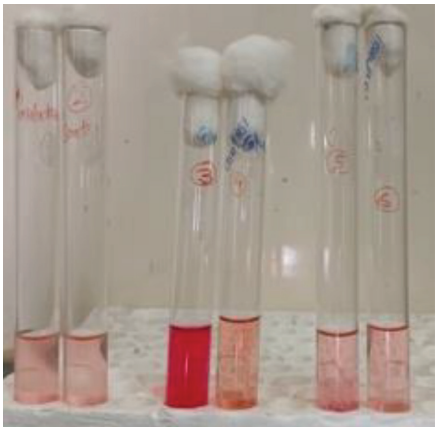

Fig. 5: Biochemical characteristics showing Galactose –ve, Glucose +ve, Trehalose –ve reaction

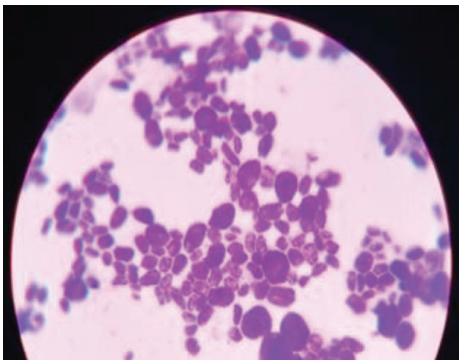

Fig. 4: Sporangia of *Prototheca zopfii* of varying size (Gram's staining)

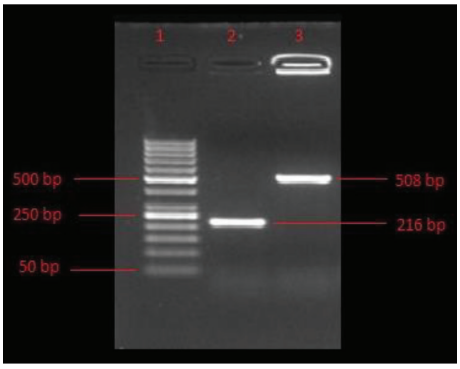

Fig. 6: PCR for *P. zopfii* genotype 2, Lane 1: DNA ladder (50bp), lane 1-*Prototheca* spp. (216 bp) and lane 2 *Prototheca zopfii* genotype 2 (508 bp)

even lead to culling of cow and ultimately result in high economic losses (Wawron *et al*., 2013). In the dry period, especially immediately before and after parturition, the mammary gland is highly susceptible to infections caused by environmental microorganisms (Cengiz and Bastan, 2015). It is important to mention that an open teat sphincter as well as some minute teat lesions caused by milking equipment constitutes a potential portal of entry of the infection (Milanov *et al*., 2016).

Diagnosis of protothecal mastitis depends on isolation and identification of the agent, but in many microbiology laboratories isolation and identification of *P. zopfii* from cow milk is not followed as routine practice. Protothecosis has traditionally been diagnosed by microscopic observation and physiological/biochemical tests of the isolated organism or pathological examination of the affected tissue (Pal and Lee, 1997; Pal, 2007). Recently, introduction of PCR and nucleotide sequencing of the ribosomal RNA gene (rDNA) has facilitated species identification of *Prototheca* (Hirose *et al*., 2018).

Prototheca zopfii grows readily on conventional laboratory media, such as blood agar, MacConkey and Sabouraud dextrose agar (Pal, 2007). The growth of *P. zopfii* on Sabouraud dextrose agar can be observed after 24-48 h at incubation temperatures between 25 ^{o}C and 37^{o}C (Milanov *et al*., 2016). Protothecosis is also important from public health point of view, as *P. zopfii* could be transmitted to humans through consumption of contaminated milk and cause intestinal infection and enteritis (Melville *et al*., 1999) because of its resistance to pasteurization (Zaini *et al*., 2012).

Conclusions

Mastitis is one of the most important economic diseases of dairy animals, so accurate and timely diagnosis of causative organisms has great significance. Colony PCR is a quick, easy and economic technique of great advantage for *Prototheca* species identification from field isolates. As case reports as well as isolation studies of *Prototheca* species were recorded globally and importance of this pathogen in bovine mastitis cases, clinicians should be aware of this remarkable organism.

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