

INCIDENCE OF *PROTOTHECA* SPP. IN MILK PRODUCED BY DAIRY HERDS IN ASSIUT CITY

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ABSTRACT

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Bovine mastitis associated with *Prototheca* is considered a rare pathology, but is increasing in prevalence all over the world and therefore becoming more relevant to the dairy industry. A total number of 150 random samples (100 of buffalo's milk and 50 of cow's milk) were collected from dairy farms and dairy shops in Assiut city as well as from some villages around the city. Physical examination of all samples revealed absence of any visible abnormalities. California Mastitis Test (CMT) was carried out on all samples to detect the subclinical cases. 59 % of buffaloes and 68 % of cow's milk samples were positive. The average count of total yeasts and mold counts was 3.29×10^2 and 3.59×10^2 in examined samples of buffaloes and cow milk, respectively. Samples were examined for the presence of *Prototheca* spp. 12 % of buffalo's milk samples were positive. On the basis of colony morphology and microscopic appearance, after incubation at 38°C for 48–72 hrs, *Prototheca* spp. were isolated, identified and sub-cultured for further identification. *P. zopfii* and *P. wickerhamii* were found in 7 % and 5 % of the positive samples-respectively. In addition, *Prototheca* spp. was not isolated from any of examined cow's milk samples. Antibiotics sensitivity test was carried out to detect antimicrobial susceptibility of isolated microorganisms.

Key Words: *Prototheca* spp., subclinical mastitis, dairyherds.

INTRODUCTION

Subclinical mastitis is the most serious type of diseases as the infected animal shows no obvious symptoms and secretes apparently normal milk for a long time, during which causative organisms spread infection in the herd, so it is an important feature of the epidemiology of many forms of bovine mastitis (Bakken and Gudding, 1982).

Mastitis caused by *P. zopfii* alge is a disease of high producing, machine-milked dairy cows. It occurs worldwide in tropical and temperate climatic areas, and mostly appears sporadically in a therapy-resistant forms. However, in poorly managed dairy herds it may be endemic, causing serious economic losses as a result of decreased milk quality and quantity and culling of infected animals (Ricchi *et al.*, 2010).

The genus *Prototheca* consists of unicellular, aerobic, achlorophyllous, saprophytic algae with a wide distribution in natural environments. They have been isolated from such diverse sites as slime flux of trees, soil, animal faces and various aqueous sources including streams, rivers, lakes, marine water, fish ponds, swimming-pools and even tap water. They are ubiquitous inhabitants of domestic and municipal sewage and are seldom detected in sewage treatment systems. The algae can also be found in many food products, e.g. beef, pork, clams, crabs and dairy milk.

Eventually, *Prototheca* have been isolated from both wild and domesticated animals, they have been shown to transiently colonize human skin, fingernails, respiratory tract and digestive system. Though there are a variety of other sources from which they can be isolated, the main reservoir remains sewage water and animal waste (Pore *et al.*, 1983; Huerre *et al.*, 1993 and Wirth *et al.*, 1999). *Prototheca* spp. is colorless unicellular algae that are opportunistic organisms, pathogenic for humans and animals (Acha and Szyfres, 2003).

The genus *Prototheca* was established and six species of *Prototheca* have been recognized: *Prototheca moriformis*; *Prototheca stagnora*; *Prototheca ulemaea*; *Prototheca wickerhamii*; *Prototheca zopfii*; *Prototheca blaschkeae*. In 1952, *P. zopfii* was first identified as a pathogen of bovine mastitis associated with reduced milk production characterized by thin watery secretion with white flakes (Möller *et al.*, 2007). Although sporadic cases of protothecal mastitis have been observed, endemic cases of this mastitis have been occurring in many countries around the world (Hodges *et al.*, 1985; Costa *et al.*, 1996; Aalbaek *et al.*, 1998; Janosi *et al.*, 2001). Microalgae of the genus *Prototheca* are closely related to the algal genus *Chlorella* but lack chlorophyll (Ueno *et al.*, 2003; Roesler *et al.*, 2006 and Ricchi *et al.*, 2010). Though three species (*P. zopfii*, *P. blaschkeae*, and *P. wickerhamii*) can cause

bovine mastitis, *P. zopfii* is the most common organism responsible for protothecal mastitis and the other two species could only cause sporadic cases (Marques *et al.*, 2006 and Marques *et al.*, 2008). Since *P. zopfii* was first identified as a pathogen of bovine mastitis in 1952, the incidence of mastitis due to this microalga is steadily increasing and gaining more and more economic and public health importance (Costa *et al.*, 1996; Janosi *et al.*, 2001 and Roesler and Hensel, 2003). *P. zopfii* strains are often associated with wet conditions and organic matters, and widely dispersed in dairy environments) (Costa *et al.*, 1997; Buzzini *et al.*, 2004 and Bueno *et al.*, 2006).

Protothecosis has been reported in humans (gastroenteritis, bursitis, etc.) and in many other animal species. Bovine mastitis represents the main form of occurrence of protothecosis in cattle. Milk as well as dairy products, when contaminated with *Prototheca spp.*, represent a potential means of transmission of this zoonosis disease. In humans these microorganisms have been associated with cutaneous lesions (Segal *et al.*, 1976 and Venezio *et al.*, 1982), gastroenteritis (Iacoviello *et al.*, 1992 and Costa *et al.*, 1995) and bursitis (Nosanchuk and Greenberg., 1973 and Abhel *et al.*, 1980). *Prototheca spp.* have also been isolated from many other sources: blood, peritoneal abscesses (Cox *et al.*, 1974), peritoneal fluid (Sands *et al.*, 1991), liver (Chan *et al.*, 1990), the blood of an immunocompromised child (Heney *et al.*, 1991), from patients with AIDS (Kaminski *et al.*, 1992 and Laeng *et al.*, 1994) and from a patient with cancer (Gómez-Hernando *et al.*, 1996).

The transmission of infection caused by the microalgae usually occurs by means of direct contact with contaminated sources. Studies on the in vitro susceptibility profile of the *Prototheca* genus have revealed great resistance to conventional antimicrobial and antifungal agents (Buzzini *et al.*, 2004 and Tortorano *et al.*, 2008). These microalgae do not respond to routine mastitis therapy, and the only control measure to date has been the elimination of the infected cows (Marques *et al.*, 2006). However, some previous studies found in vitro susceptibility of *P. zopfii* strains to amphotericin B and nystatin, which are commonly used in human protothecosis therapy (Segal *et al.*, 1976; Marques *et al.*, 2006 and Tortorano *et al.*, 2008). These findings led us to test the in vitro susceptibility of our *P. zopfii* strains to the two mentioned drugs. Meanwhile, some other conventional antimicrobial and antifungal agents were also chosen for the in vitro susceptibility test in the present study.

Considering the increasing importance that is being given to the *Prototheca* species, mainly due to the economic losses for which they are responsible (concerning basically dairy cattle breeding), as well

as for representing a potential risk to public health, the purpose of this study was to examine the milk produced from dairy farms in Assiut for presence of *Prototheca spp.*

MATERIALS and METHODS

A) Collection, preparation and serial dilutions of samples: A total of 150 random samples of buffaloes and cow's milk were collected from different dairy farms and dairy shops in Assiut city to be examined in laboratory (100 samples of buffalo's milk and 50 samples of cow's milk). All samples were tested by the CMT, and then kept at 4 °C to be examined microbiologically for presence of *Prototheca spp.* and total yeast and mold count. Eleven ml of each of the examined samples were mixed with 99 ml of sterile 0.1 % peptone water and thoroughly mixed to give a dilution of 1/10, and then ten fold serial dilutions were carried out (A.P.H.A., 1992).

B) California Mastitis test (CMT) according to Schalm *et al.* (1971): Each 3 ml of milk sample was drawn in shallow cups in the CMT paddle then approximately equal volume of 3 ml of the commercial available CMT reagents was added to each cup and mixed together through swirling the paddle in a circular motion for few seconds. According to the visible reaction of the CMT, the results were classified into four scores: 0 = negative or traces (no change in consistency), 1 = slightly positive (+), 2 = positive (++) and 3 = highly positive (+++). Scores 1, 2 and 3 depend on the degree of gellation that were indicated by gelatinous mass (Schuppel and Schwoppe, 1998).

C) Enumeration of total yeasts and molds according to Harrigan and MacCance (1976) by using malt extract agar (containing 500 mg each of chlortetracycline and HCL chloramphenicol).

D) Isolation of *Prototheca spp.*: Aliquots of 100 µl of milk were streaked on Sabouraud dextrose agar (SDA) plates. Streaked plates were incubated under aerobic conditions for 48 hrs. at 37 °C. Yeast-like colonies (grayish and white to cream colored small colonies of 1 mm or 1–2 mm size, respectively, with yeast like appearance and odor were detected) were subcultured on SDA for 48 h at 37 °C. Species identification of *Prototheca* was based mainly upon morphological characterization of pure cultures (fig.1), lactophenol cotton blue (LCB) stained smears and carbohydrate assimilation profiling. The microscopical observation of *Prototheca* cells stained with lactophenol cotton blue which showed a typical appearance with ovoid to globose sporangia with sporangiospores in several developmental stages (fig.2) suggesting that they could belong to a different *Prototheca* species. The morphological appearance observed for the *Prototheca spp.* was similar to

previous description of other authors (Pore, 1998; DiPersio, 2001; Roesler *et al.*, 2006).

E) Identification of *Prototheca Spp.*: Identification of *Prototheca Spp.* was carried out according to Dubravka *et al.* (2006) and Asfour and El-Metwally (2010). Streaked plates were incubated under aerobic conditions at 37°C for 48 h and monitored daily. From the growing colonies, wet microscopic smears, Gram and methylene blue stains were done. The preparations were examined using light and phase-contrast microscopy according to Cosmina *et al.*

(2009). Urease activity also applied to differentiate between *Prototheca spp.* and other yeasts.

F) Sensitivity test: Antimicrobial susceptibility of *Prototheca spp.* strains was tested by disk diffusion method on Müller Hinton's agar as described in CLSI, 2004 (formerly NCCLS) document. The plates were incubated for 48 to 72 hrs at 37 °C and the inhibition diameters were measured. The following antifungal and antibacterial antibiotics were tested: amphotericinB, nystatin, kanamycin, penicillin, gentamicin, neomycin, streptomycin, ampicillin, and amoxicillin.

RESULTS



Fig. 1: Sabouraud dextrose agar plate showing the organism at 28°C for 96 hrs.

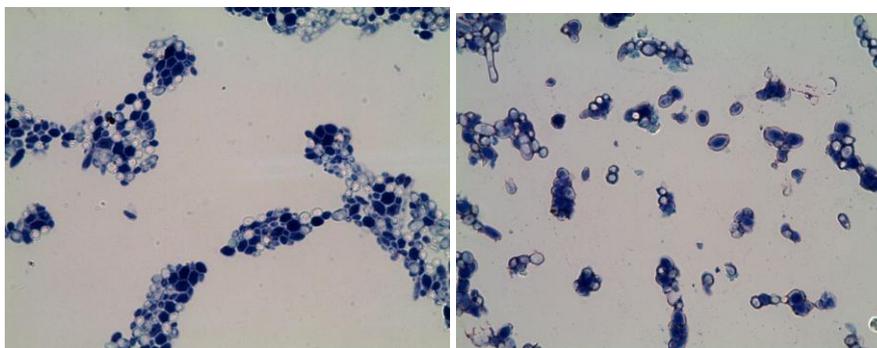


Fig 2: Micrographs of *Prototheca spp.* stained with methylene blue, 100 x magnification, grown on Sabouraud dextrose agar at 28°C for 96 hrs.

Table 1: Results of CMT on the examined samples.

Score	Cow's milk		Buffaloe's milk	
	No. of samples	%	No. of samples	%
+++ ve	6	12%	15	15%
++ ve	7	14%	21	21%
+ ve	21	42%	23	23%
-ve	16	32 %	41	41%
Total	50	100%	100	100%

Table 2: Statistical analytical results of total yeasts and molds count in the examined samples.

Type of sample	No. of examined samples	Positive samples		Count / g		
		No.	%	Average	Max.	Min.
Buffaloe's milk	100	80	80%	3.29x10 ²	1.27x10 ³	> 10
Cow's milk	50	38	76%	3.59x10 ²	2.4x10 ³	>10

Table 3: Frequency distribution of positive samples based on their total yeasts and molds count.

Count / g	Buffaloes milk		Cow milk	
	No. / 80	%	No. / 38	%
10 - < 10 ²	15	18.8%	6	15.8%
10 ² - < 10 ³	58	72.5%	28	73.7%
10 ³ - < 10 ⁴	7	8.7%	4	10.5%
Total	80	100%	38	100%

Table 4: Incidence of *Prototheca spp.* in the examined samples:

<i>Prototheca spp.</i>	Buffaloes milk		Cow milk	
	No./100	%	No./50	%
<i>Prototheca zopfii</i>	7	7%	0	0%
<i>Prototheca wickerhamii</i>	5	5%	0	0%
Total	12	12%	0	0%

DISCUSSION

Algae of the genus *Prototheca* are one of the few plant-like organisms that cause infections in humans and animals (Matsuda and Matsumoto, 1992; Möller *et al.*, 2007; Marques *et al.*, 2008). The pathogenesis of *Prototheca* organisms remains unclear, but they are believed to be of low virulence and mostly infect patients with various forms of immunosuppression (Jagielski and Lagneau, 2007).

The presence of microorganisms in milk, many of which are responsible for zoonoses, represents a factor that compromises its quality and safety. Therefore, bearing in mind the objective of reducing the microbial content of milk and also of eliminating microorganisms potentially harmful to humans, procedures for the thermal treatment of milk have been developed.

The present results showed that the average count of total yeasts and molds were 3.29x10² and 3.59x10² in the examined samples of both buffaloe's and cow's milk, respectively (Table 2 and 3) with minimum

count > 10 in both buffaloe's milk and cow's milk. Majority of positive samples occurs between 10² - < 10³ in a percent of 72.5% and 73.7% in the examined samples of buffalo's and cow milk, respectively. High scores of CMT could be recorded in both samples of buffaloes and cow milk where 59 and 68 % of examined samples where positive for CMT, respectively (Table 1).

Prototheca genus were isolated from subclinical mastitis buffaloes and shows a good development in aerobic conditions at 37 °C and the culture appears to be visible after 36 to 48 hours. The colonies are irregular ice crystal in shape (Fig., 1).

Table 4, revealed that *Prototheca spp.* are isolated in 12 % of the examined buffaloes milk samples. *P. zopfii* detected in 7 % while *P. wickerhamii* isolated from 5 % of examined milk of buffaloes. *Prototheca spp.* were not detected in cow milk.

The reported level of infection within dairy herds is generally under 10% of milking cows. The present recorded results in this assay are relatively similar to

that given by Buzzini *et al.* 2004 (4.7% of samples possessed cells of *Prototheca spp.*). Also, our recorded results were in parallel with Benites *et al.*, 1999; Corbellini *et al.*, 2001; Bexiga *et al.*, 2003; Kirk and Mellenberger 2011; who isolated *P. zopfii* from milk samples in percentages 9; 5; 2.2 and 5.4, respectively. While some reports have shown higher rates of isolated *P. zopfii* with percentages of 10.7 - 39% (Pore *et al.*, 1983; Costa *et al.*, 1996; Costa *et al.*, 1997 and Jensen *et al.*, 1998). Scaccabarozzi *et al.* (2008) could isolate *P. zopfii* from 11.9% of milk samples. Recently, Zaini *et al.* (2012) could identify four *P. zopfii* strains (3.07%) from the 130 samples of dairy cattle with clinical mastitis and there was no isolation from totally 100 samples of healthy bovines without mastitis. While, Gao *et al.* (2012) isolated *P. zopfii* strains from 17 of 23 quarters, which suffered CM in the outbreak, and 7 of 46 CM recovered quarters before the outbreak.

Comparing the present results by these obtained by Egyptian researcher, it is revealed that higher percentages of *P. zopfii* and *P. wickerhamii* (22.2% and 9.3%) were recorded by Horya, (2011) when 150 samples of raw milk were examined. On the other hand, Asfour and Metwally, (2010) documented that *P. zopfii* were isolated in 6% of examined raw buffaloes milk and in percentage 2.33 % of raw cow milk.

P. wickerhamii is generally associated with human pathology presenting essentially cutaneous or subcutaneous lesions and also more rarely generalized infections (Zaitz *et al.*, 2006; Hightower and Messina, 2007; Lass-Florl and Mayr, 2007; Narita *et al.*, 2008).

The common belief is that the transmission of an infection caused by *P. zopfii* occurs by means of direct contact of mammary glands with other contaminated sources on the dairy farm. Several studies have been devoted to the exploration and characterization of environmental sources of *P. zopfii* in dairy herds (Costa *et al.*, 1997; Scaccabarozzi *et al.*, 2008 and Osumi *et al.*, 2008). The wet weather may constitute an important risk factor in the multiplication and dissemination of the microalgae and result in the frequent reports of mastitis (Corbellini *et al.*, 2001). It has been reported that this agent can induce a persistent infection with intermittent shedding, due to its ability to infect and survive in macrophages and to invade the udder tissue (Roesler and Hensel, 2003). Thus, the infected udders may act as a reservoir of *P. zopfii* in the herd.

Results of antimicrobial and antifungal susceptibility tests in vitro indicated that these strains were resistant to majority of tested drugs, with the only exception of amphotericin B, nystatin, streptomycin, gentamicin, and amikacin.

Studies on the in vitro and in vivo susceptibility profile of the *Prototheca* genus have revealed great resistance to conventional antimicrobial, antifungal and antiseptic drugs (Costa *et al.*, 1996; Buzzini *et al.*, 2004; Bueno *et al.*, 2006; Marques *et al.*, 2006). *Prototheca spp.* isolates were classified on the basis of current taxonomic guidelines and identified as *P. zopfii*. Susceptibility tests carried out in vitro by using 25 antibiotic compounds revealed that the strains of *P. zopfii* were susceptible only to nystatin and amphotericin B (58 and 33% of total strains, respectively). Treatment for mastitis caused by *P. zopfii* is generally unsuccessful because the strains show in vitro resistance to most antibiotics commonly used in veterinary practice (Buzzini *et al.*, 2004). Our results were in agreement with some previous reports, which showed in vitro susceptibility of *P. zopfii* strains to these agents (Marques *et al.*, 2006). Since there are still no drugs with proven clinical efficacy against protothecal mastitis, although our results indicated sensitivity to some drugs in vitro, this does not guarantee that the drugs can be used for therapy against infections with this microalga in cows. However, a proposal about tentative treatment for CM cases using these drugs has been offered to veterinarians of the dairy farm.

P. zopfii has become an important cause of bovine mastitis in many countries. In the present study, to better understand the occurrence of one clinical mastitis (CM) outbreak due to *P. zopfii* (Gao *et al.*, 2012). *P. zopfii* is the most crucial organism that can cause protothecal CM associated with typical signs, including reduced milk production, increased density of the infected quarters, and thin watery secretion with white flakes (Ja'nosi *et al.*, 2001 and Moller *et al.*, 2007).

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 Egypt, using more milk samples from bovines with mastitis in order to coordinate and confirm the results of this study. The results showed *P. zopfii*, is responsible for causing bovine mastitis in Iran as same as other geographical parts of the world (Möller *et al.*, 2007; Aouay *et al.*, 2008 and Osumi *et al.*, 2008). 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 (Melville *et al.*, 1999). 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.

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

CONCLUSION

Milk as well as dairy products, when contaminated with *Prototheca* spp., represent a potential means of transmission of this zoonosis. These data suggest that *P. zopfii* may represent a serious risk in the studied herd, and this microalga could be an important potential pathogen causing mastitis in dairy herds in Egypt. It is indefinite that the infection source of this outbreak came from environment or previously infected quarters. Therefore, more samples should be collected from other environment sites and lactating quarters to define sources of this microalga. *P. zopfii*, due to its wide antibiotic resistance, could represent a serious risk in the studied herd. Providing clean and dry surroundings, improving milk procedures and culling chronically infected cows may contribute to prevent mastitis caused by this microalga.

Since the teat canal is the portal of entry for *P. zopfii*, cleaning and disinfecting the teats, milking machines, and animal housing facilities with a proper germicidal agent are among the most important preventive measures against protothecal mastitis.

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مدي تواجد ميكروب البروتوسيكيا في اللبن المنتج من القطعان الحلابة في مدينة أسيوط

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التهاب الضرع تحت الاكلينيكي احد أهم المشاكل الصحية التي تصيب الحيوانات الحلابة على مستوى العالم والتي تسبب خسائر فادحة للمربيين وتقلل من إنتاج الألبان وجودتها. يعد ميكروب البروتوسيكيا الذي يجمع بين خصائص الخمائر والطحالب احد مسببات الالتهاب تحت الاكلينيكي للضرع في الحيوانات الحلابة. في هذه الدراسة تم جمع 150 عينة من ألبان الأبقار والجاموس بواقع 50 عينة للبن البقري و100 عينة للبن الجاموسي من المزارع ومحلات الألبان المختلفة في مدينة أسيوط، وذلك لدراسة مدي تواجد أجناس البروتوسيكيا في هذه العينات. بداية تم فحص العينات وكانت كلها سليمة ظاهريا، تم اجراء اختبار كاليفورنيا لالتهاب الضرع على جميع العينات وذلك لتحديد عينات البان الحيوانات المصابة بالتهاب الضرع تحت الاكلينيكي. اظهرت نتائج الاختبار ان 59 % و 68 % من العينات كانت ايجابية في البان الجاموس والابقار علي التوالي. كان متوسط العدد الكلي للخمائر والفطريات في عينات ألبان الجاموسى والبقرى هي 3.29×10^2 و 3.59×10^2 علي التوالي. كما أوضحت النتائج ايضا ان 7 % من عينات الألبان الجاموسي و5 % منها كانت تحتوى على ميكروب البروتوسيكيا زوبفي (*P. zopfi*) وميكروب البروتوسيكيا وپكرهاميي (*P. wickerhamii*) على الترتيب بينما كانت عينات البان الأبقار خالية تماما من اى من هذه الميكروبات. هذا بالإضافة إلي انه تم عمل اختبار الحساسية للميكروبات المعزولة لبعض المضادات الحيوية للفطريات والبكتيريا.