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Microbiological and Histopathological Investigations on *Prototheca* Mastitis in Dairy Animals

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Abstract: A total number of 100 and 300 milk samples from buffaloes and cows as well as 60 and 20 specimens from mammary parenchyma was collected for microbiological and histopathological examinations of Protothecosis in both species, respectively. Examined milk samples showed high scores with California Mastitis Test (CMT). Six buffalo's subclinical milk samples with percentage of 6% and seven cow's milk samples (Clinical and subclinical) with percentage of 2.33% were positive for *Prototheca spp*. Somatic cell count (SCC) and milk composition was investigated in this study. *Prototheca spp.* was detected in samples of 4 swabs took from 8 milking machine lines. Moreover, *P. zopfii* were detected in 8 (13.33%) and 2 (10%) of the collected buffalo and cow's mammary parenchyma specimens, respectively. After aerobic incubation for 24-72 h at 25-37°C, the visible *Prototheca* colonies were identified based on macro-microscopical morphology. Characteristic microscopic morula appearance of *Prototheca spp*. was observed by using light and phase-contrast microscopy. *Prototheca spp.* was stained by methyl-blue and Gram-stains. Biochemical characteristics of *Prototheca* were monitored using assimilation patterns of different types of sugars and Urease activity. *P. zopfii* was the most isolated species from the collected samples. Microscopical examination of mammary tissues revealed focal or diffuse, chronic and necrotizing interstitial mastitis associated with intralesional algal structures. Pronounced infiltrations of lymphocytes and macrophages with few neutrophils and multinucleated giant cells were observed. *Prototheca* was spheroid, ovoid, or elliptical with a prominent thick wall, contains several thick-walled autospores and was predominantly seen in macrophages which were markedly enlarged and both sporangiospores and sporangia were found. The algae were positively stained with PAS and Alcian blue as well as by GMS stains.

Key words: Protothecosis · Bovine mastitis · Milk composition · SCC · Histopathology

INTRODUCTION

Mastitis is the most frequent and expensive disease of dairy animals. It negatively influences the economic effectiveness of farms and the hygienic quality of milk [1, 2].

Protothecosis is a severe form of mastitis in cattle that is caused by colorless algae of the genus *Prototheca* [3].

Prototheca spp. is widely distributed worldwide. Originally, they were recovered from slime on trees. Also, they have been isolated from a variety of environmental sources, including plants, soil, mud stagnant ponds and marine water. They can also be found in water tanks, manure, teat dip containers, milking machine liners and faeces of rats trapped on dairies, indicating that *Prototheca spp.* are widely dispersed in dairy environments [4, 5].

Members of the genus *Prototheca* are aerobic, unicellular, microscopical and achlorophyllic algae related to the green algae of the genus *Chlorella*, but without chlorophyll that cause infectious diseases in human and animals [1, 2, 6, 7].

Prototheca spp. is oval or spherical in shape. They differ from bacteria and fungi in size and shape. The cell wall of *Prototheca* consists of outer (thinner) and inner (thicker) envelopes, while all Chlorella species (except for *Ch. prototecoides*) are characterized by a three-layer cell wall [5,8].

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They reproduce asexually by internal septation to produce 2 - 20 and sometimes 50 sporangiospores within a hyaline sporangium. The sporangiospores are arranged in a characteristic morula configuration and upon rupture of the sporangium, are released to develop into additional endosporulating forms (autosporulation) [1, 2, 4, 7].

Prototheca algae are saprophytic, but some species may turn into unusual opportunists causing pathology when the host immunological defenses are impaired or when predisposing factors occur, such as, in case of dairy cows, poor animal care and poor milking hygiene [3, 5, 6, 9].

Infection by these algae causes acute to chronic granulomatous mastitis, leading to reduced milk production and atresia of the udder [2, 10].

The purpose of this survey was to describe the natural occurrence of bovine mastitis caused by *Prototheca species* in Egypt. Moreover, special interests were given to the milk microbiology and the histopathology of the mammary parenchyma of slaughtered dairy buffaloes and cows.

MATERIALS AND METHODS

Milk samples and specimens from mammary gland parenchyma were collected through a period of 18-months during 2008-2009in Egypt.

A-Milk Samples: Four hundreds milk samples (100 from buffaloes and 300 from cows) from different farms were collected from cases suffering from clinical and, subclinical mastitis according to California Mastitis Test, CMT (high score) and recurrent mastitic animals with record of resistance to antibiotic treatment. The proper method was used for collection of milk samples for microbiological examination according to Zurakowski [11].

A composite sample of all four teats was collected, cooled and maintained at approximately 4°C. SCC, milk composition analysis (Milk Scan Bently 150) and microbial culture were applied on the collected milk samples.

The samples were transported to the laboratory and preincubated at 37°C / 24h then 50 uL was streaked onto blood agar (with 8% sheep blood), MacConkey agar and Sabouraud dextrose agar plates containing inhibitors of normal microbial flora (0.05 mg/ml chloramphenicol). Streaked plates were incubated under aerobic conditions at 25- 37°C/ 24-72 h. The microbial growth was monitored daily. The presence of other pathogenic microorganisms (yeasts and bacteria) was also investigated by plating milk samples in Manitol salt agar and Edward's media. Of the colonies grown on Sabouraud dextrose agar after 48 hours of incubation, wet microscopic smears, Gram and / or methyl-blue stains were done. The preparations were examined using light and phase-contrast microscopy according to DiPersio [8].

Sensitivity test was done to detect antimicrobial susceptibility using different antibiotics as Penicillin (10µg), Streptomycin (10µg), Amoxicillin (10µg), Amoxicillin + Glaviolinic acid (30µg), Florphinicol (30µg), Gentamicin (10µg),Neomycin (30µg), Cloxacylin (5µg),Ampicilln (10µg), Oxytetracycline(30µg) and Tetradelta (Novobiocin (100 µg)+ Neomycin sulphate (105 µg)+ Procine penicillin (100 µg)+ Dihydro streptomycin (100 µg) + Predaziolone (10 µg)).

B-Milking Machine Inflation Swabs: Eight swabs were taken from milking lines, jars and cubs of positive *Prototheca* farms and managed as mentioned before.

C-Tissue Samples: eighty tissue samples (60 from buffaloes and 20 from cows) from mammary glands were collected from adult buffaloes and cows from different abattoirs. Each tissue sample was divided into two parts, one part was put in a small polyethylene bag in an ice box under aseptic conditions for microbiological examination (using the same media mentioned before in milk) and the second part was immersed in 10% neutral buffered formol saline for histopathological evaluation. These samples were collected randomly among the slaughtered animals and examined visually and through palpation.

The fixed specimens were trimmed, washed and dehydrated in ascending grades of alcohol, cleared in xylene, embedded in paraffin and sectioned at 4-6 u thickness. The samples were stained with Haematoxylin and Eosin stain (H and E) in addition to Periodic Acid Schiff stain (PAS), Alcian blue stain and Gomori Methenamine Silver stain (GMS) used as special stains [12]. The slides were examined using light microscope.

Identification of *Prototheca Spp.*: according to Dubravka *et al.* [10] and Padhye *et al.* [13].

Sugar assimilation was used for identification of *Prototheca spp.* using glucose, glycerol, fructose, mannose, trehalose, maltose, lactose. Urease activity also applied to differentiate between *Prototheca spp.* and other yeasts.

RESULTS

As a result of characteristics of the herd, bad hygienic measures and information given by farmers, mastitis due to *Prototheca spp.* was suspected and for that reason microbial and histopathological examinations were carried out.

Microbiological Investigation: Milk obtained from infected animals was of watery appearance with flakes, but without systemic symptoms of infection, the affected quarters were firm on palpation and painless with history of decreased milk yield.

A-Buffalo's Milk Samples: Among the total number of examined milk samples from buffaloes suffering from subclinical mastitis with high CMT scores (100), six milk samples were positive for *Prototheca spp.* with percentage 6%. The average SCC was over 2,000,000 cells /ml. The averaged milk composition of the positive *Prototheca* samples showed low fat (4.15%), lactose (2.15%), total solids (11.42 %) and protein (3.19%) contents.

B-Cow's Milk Samples: From the total cow's milk samples (300), seven samples were positive for *Prototheca spp.* with percentage 2.33%.

The Total Milk Samples Were Divided Into:

- **Samples Collected from Subclinical Mastitic Cases:** CMT showed high scores in 270 samples. *Prototheca spp.* were detected in 5 milk samples with percentage of 1.85% (from total subclinical samples) and SCC ranged between 3,988,000 – 175,000 cells /ml. The milk composition of the positive *Prototheca* samples showed low fat (1.91%), lactose (2.23%), total solids (9.76 %) and protein (2.67%) contents.
- **Samples Collected from Clinical Mastitic Cases:** A total number of 30 milk samples was collected from clinical mastitic cases. Two samples were *Prototheca* positive with percentage of 6.7% (from total clinical samples).

C-Tissue Samples: Among the total number of examined mammary tissue specimens obtained from buffaloes (60) and cows (20), eight (13.33%) and two samples (10%) were positive for *Prototheca zopfii,* respectively.

D-Cultural Features: After aerobic incubation for 24-72 h at 25-37°C, visible grown *Prototheca* colonies were mostly irregularly margined, with granular surface and a compact central protrusion. Sometimes smooth, larg, white or creamy consistency and yeast smell were seen.

Colonies grown on blood agar are mostly very small and pale gray. If the isolation medium does not contain growth inhibitors, *Prototheca* colonies may be overgrown by bacteria after prolonged incubation. Since it is slow-growing, the organism may easily be missed in routine practice if incubation is terminated after 24 h.

Microscopy, it is indispensable to distinguish *Prototheca spp.* from yeasts, since differentiation is not possible on the basis of their cultural features. Characteristic microscopic appearance of *Prototheca spp.* was quietly observed by examining wet preparations using light and phase-contrast microscopy, when formations described as "morula" or "mulberry" are visible (Fig. 1). *Prototheca spp.* is easily methyl-blue (Fig. 2a, b, c and d) and Gram-stained, but heat-fixation may induce morphological impairment. In Gram-stained preparations, positively stained spores and Gram-negative empty sporangia are visible.

Several environmental bacterial pathogens such as *Streptococcal spp., Ecoli, other coliform* and *Coagulase negative Staphylococci* bacteria were commonly isolated from milk and tissue specimens. *Staphylococcus aureus* and *Streptococcus agalactiae* were also recovered but less frequently than the environmental organisms. Sensitivity test using different antibiotic discs was done for detection of inhibition zones for these bacteria and it was noted that these bacteria were resistant to the used antibiotics.

Prototheca spp. was detected in 4 swab samples collected from 8 milking machine inflation (Fig 3a and b).

E-Identification of *Prototheca* Spp.:

**Assimilation patterns of *Prototheca Species* by using of different types of sugars were applied to differentiate between *P. zopfii* and *P. wikerhamii.*

Table (1) Sugar assimilation patterns:

Prototheca Species	No. of isolates (23)	Glucose	Glycerol	Fructose	Mannose	Trehalose	Maltose	Lactose
P.wickerhamii	3	+	+	+	+	+	–	–
P. zopfii	20	+	+	+	–	–	–	–

**Urease activity: *Prototheca* failed to hydrolyze urea within 7 days.

Histopathological Investigation: Histopathological examination of the collected mammary parenchyma tissue spicemens was done to confirm the microbiological isolation of *P. zopfii.*

A-Gross Pathology: The examined mammary glands had hard consistency and characterized by diffuse fibrosis, thickening and ectasia of mammary ducts.

B-Histopathological Appearance: Mammary gland tissues revealed focal or diffuse, chronic and necrotizing interstitial mastitis associated with intralesional algal structures (Fig 4).

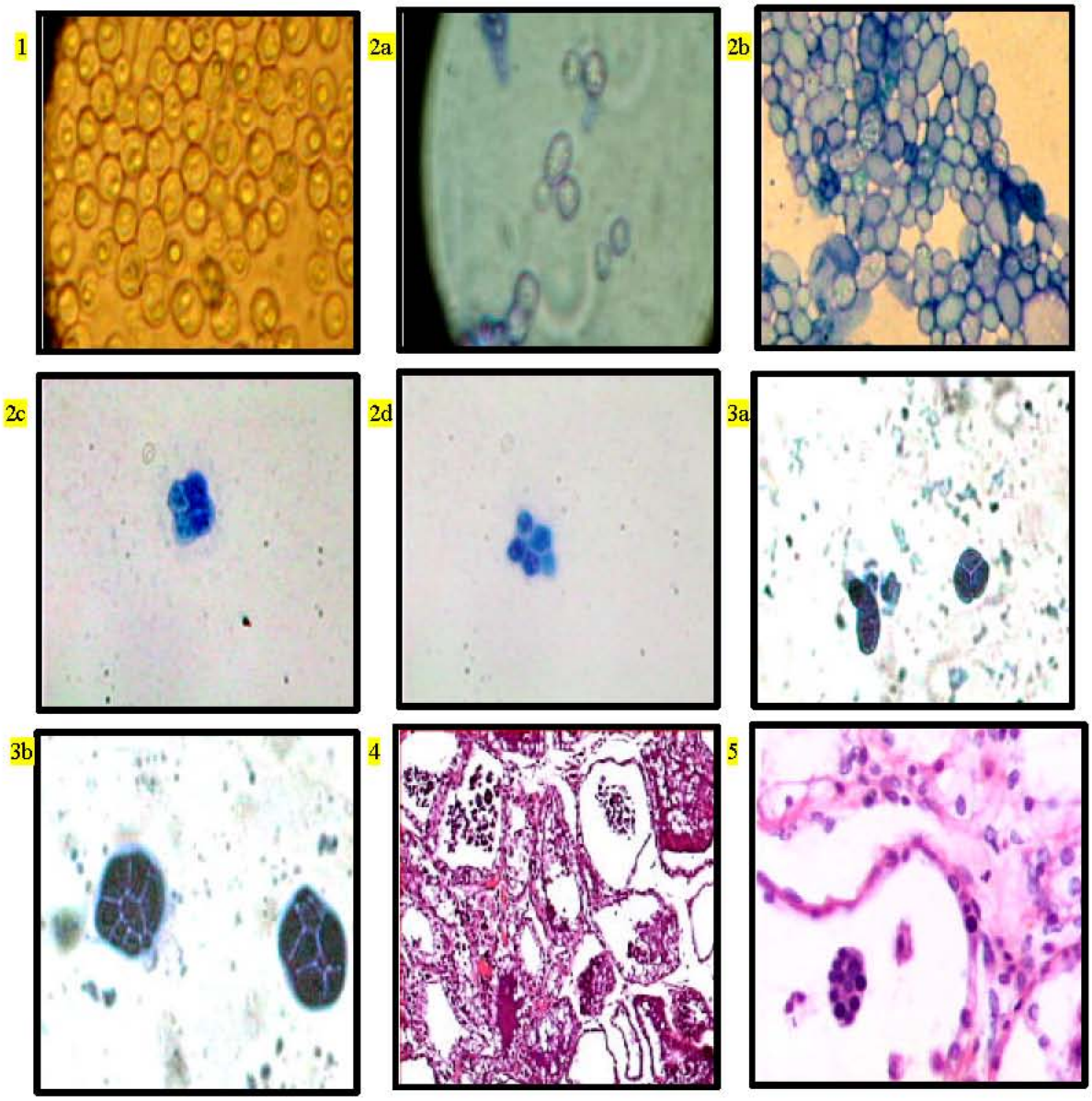

Fig. 1: Characteristic microscopic appearance of *Prototheca spp.* by examining wet preparations by using phase-contrast microscopy (Wet preparation, X100)

Fig. 2a: Characteristic appearance of *Prototheca spp.* using phase-contrast microscopy (Methyl-blue Stain, X100).

Fig. 2 b-d: Characteristic microscopic morula appearance of *Prototheca spp.* using light microscopy (Methyl-blue Stain, X100).

Fig. 3a-b: Prototheca spp. isolated from milking machine inflation swabs (Methyl-blue Stain, X100).

Fig. 4: Mammary gland tissues revealed focal or diffuse, chronic and necrotizing interstitial mastitis associated with intralesional algal structures (H&E Stain, X10).

Fig. 5: Dilated mammary acini associated with macrophages and lymphocytic infiltrations and destruction of their epithelium with *p. zopfii* found free in the mammary acini (H&E Stain, X40).

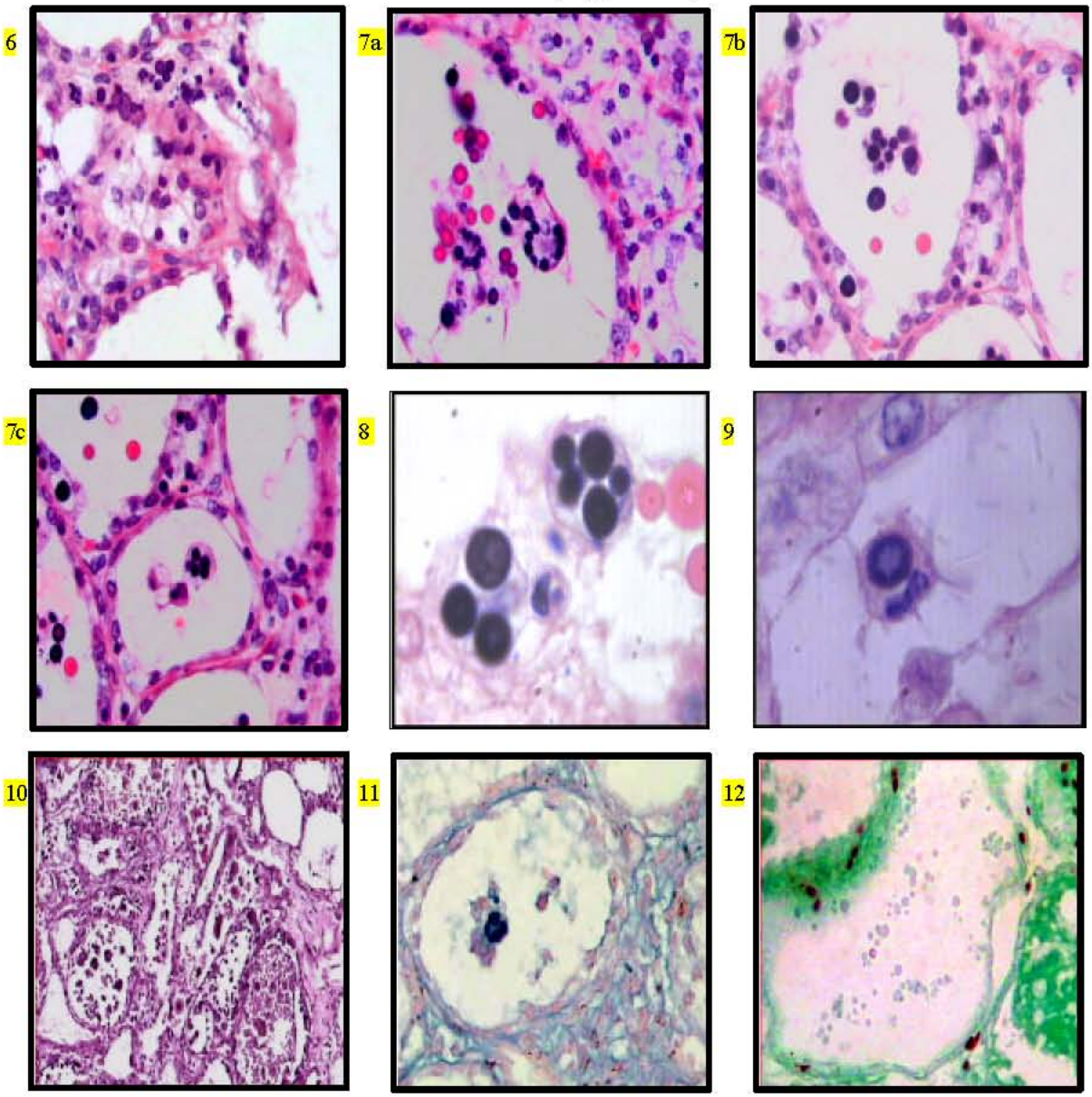

Fig. 6: Destruction of mammary acini associated with prominent inflammatory cells infiltration with many numbers of algae found free or between the epithelial cells and periacinar connective tissue (H&E Stain, X40).

Fig. 7a-c: Mammary acini showed infiltration of inflammatory cells with many numbers of *Prototheca* found free or in between the epithelial cells and in the lumen of destructed alveoli (H&E Stain, X40).

Fig. 8: *Prototheca* is a large spheroid nonbudding cell with a prominent thick wall, and contains several thick-walled autospores (H&E Stain, X100).

Fig. 9: Neutrophil was markedly enlarged and engulfed sporangiospore (H&E Stain, X100).

Fig. 10: The algae were positively stained with PAS stain and revealed variably abundant free or intracytoplasmic organisms (PAS Stain, X10).

Fig. 11: Blue rounded structure of *P.zopfii* could be seen against pink background (Alcian blue Stain, X40).

Fig. 12: The algae were positive reddish color (GMS Stain, X10).

There was no histopathological difference between several tissue specimens collected from the same animal. However, the samples from different animals showed irregularly lesions of varying degrees of severity. The inflammatory lesions were irregularly scattered among the mammary parenchyma.

Mammary acini were dilated and contained necrotic debris associated with macrophages and lymphocytes with numbers of algae found free or between the epithelial cells of the mammary acini and periacinar connective tissue. The algae were also found between the lining epithelium and basement membrane of the affected alveoli. The affected tissues showed severe destruction of their alveoli and some parts showed desquamation of the epithelium. Atrophy and vacuolar changes of acinar epithelium was evident (Fig 5 and 6) while other cases showed focal areas of alveolar epithelial necrosis.

Ducts were multifocal markedly dilated and contain abundant necrotic debris with inflammatory cells. Hyperplastic proliferation of the epithelial covering of the lactiferous ducts accompanied by focal or diffuse lymphocytic aggregation and infiltration at the subepithelial layer were noticed.

The interlobular septal interstitial of the mammary gland was moderately to severely expanded by fibrosis and prominent infiltration of lymphocytes, macrophages, plasma cells, few neutrophils with epithelioid cells and few multinucleated giant cells. Thickening of tunica media of blood vessels with focal necrosis of these tunica and vasculitis were predominant while some cases showed congestion.

In the lumen of the dilated alveoli and ducts, *Prototheca* which is a large nonbudding cell was seen. It is spheroid, ovoid, or elliptical with a prominent thick wall and contains several thick-walled autospores in different numbers and sizes (Fig 7a, b, c and 8). Algae were predominantly seen in macrophages and less frequently in neutrophils which were markedly enlarged and both sporangiospores and sporangia were found (Fig 9). Algae were degenerated and consisted of intact cell wall profiles which contained membrane fragments but lacked nuclei and cytoplasmic organelles.

The algae were positively stained with PAS stain and revealed variably abundant free or intracytoplasmic organisms (Fig 10). Also several blue rounded structures could be seen against pink background by Alcian blue stain (Fig 11) as well as by GMS, gave positive reddish color (Fig 12).

All examined mammary gland tissue samples were showed mixed infection of *prototheca* with *C. neoformans.*

DISCUSSION

Protothecosis is an infectious condition caused by achlorophyllic algae which are generally triggers a chronic inflammatory response restricted to the mammary gland. It is a wide-spread in the environment and can be found particularly in damp areas contaminated with manure or other organic matter which provides a source of nutrients [4, 5]. Malinowski *et al.* [1] discussed the cyclic and expanding event with *Prototheca* that is consumed through fecal contamination of feed and is able to pass unharmed through the gastrointestinal tract. The organism is excreted in feces, which is typically spread throughout the farm either mechanically or by the animals themselves. Upon gaining access to the mammary gland, through the teat orifice, *Prototheca* may be passed among herd mates through milking machines or to calves by feeding them with contaminated milk. Rodent and cat populations may act as a source of infection by consuming discarded milk and from defecating in feed areas.

In this study, it was noted that the affected udders produced watery milk with flakes in addition to marked reduction in the milk yield but without systemic symptoms of infection. This is in agreement with McDonald *et al.* [14] and Bueno *et al.* [15].

A percentage of 6% of buffalo's and 2.33% of cow's milk samples were positive for *Prototheca spp.* From the total collected mammary parenchyma samples 13.33 and 10% of buffaloes and cows were positive for *Prototheca spp.*, respectivly.

The reported level of infection within dairy herds is generally under 10% of milking cows. The present recorded results in this assay are coincided with those given by Buzzini *et al.* [4], Corbellini *et al.* [16], Benites *et al.* [17], Kirk and Mellenberger [18], Bexiga *et al.*[19] who isolated *P. zopfii* from milk samples in percentages 4.7, 9, 5, 2.2 and 5.4%, respectively. While some reports have shown higher rates of isolated *P. zopfii* with percentage of 10.7 – 39% [20 -24].

The affected buffaloes and cows with subclinically prototheca mastitis had a history of high somatic cell counts over 2,000,000 cells /ml or clinical mastitis that was not responsive to intramammary antibiotic treatment. These findings were concomitant with those given by Malinowski *et al.* [1], Milanov and Suvajdÿiã [5] and Hodges *et al.* [25]. The *Prototheca* subclinically mastitic animals showed low percentages of milk composition. These decreases in fat and lactose levels might be due to glycerol degrading and tissue damage which could be in accordance with Bueno *et al.* [15]. These changes in milk

composition explain the watery appearance observed in the infected animals and may be used as an accessory tool for sampling herds. It was observed that some animals showed low SCC, this result differs from that of Janosi *et al.* [26] who observed a permanent increase in SCC during lactation. Nevertheless a similar observation were recorded by Tenhagen *et al.* [27] who isolated *Prototheca spp.* from milk samples with SCC lower than 100,000 cells /ml. These findings alert us to the importance of not taking milk samples just from animals of high SCC that may cause miss identification of infected animals and failures eradication programs.

Of the five known *Prototheca* strains, *P. zopfii* and *P.wickerhamii* are considered pathogenic. Among animals protothecosis most commonly present in the form of bovine mastitis is *P.zopfii* as the primary pathogenic agent and only few cases have been reported with *P.wickerhamii* as the responsible agent. This is recommended in this study and supported by Pore *et al.* [20].

From the previous results and literatures it is known that algae can cause chronic mastitis that is difficult to be diagnosed and treated as cases of *prototheca* mastitis. Moreover, it may be misidentified as yeast mastitis because *Prototheca* resembles yeasts in their colonies and smell. The organism differs from *C. neoformans* and other yeast by the size, internal structure and lack of budding and *C. neoformans* rapid hydrolyzed urea after 6h while, *prototheca* failed to hydrolyze urea. Careful microscopic examination of *Prototheca* colonies will reveal sporangia either empty or filled with endospores. So far, no suitable serological test for the identification of infected animals is available for routine diagnosis [1, 3, 5, 28]. The failure of isolation of *Prototheca spp.* may be explained by the fact that they are readily over-grown by bacteria and fungi when culture is attemped from contaminated sources. Moreover, if media inhibitory to normal flora are not used, slower-growing colonies of *prototheca* may be overgrown by bacteria. Therefore, the most abundant growth was observed on Sabouraud dextrose agar [8, 29].

Records on cows with chronic mastitis reported on a previous long-term antibiotic treatment; act as an important risk factor for the onset of protothecal mastitis [30].

In this study *Prototheca spp.* were isolated from milking machine inflation swabs following the milking of all cows even those not positive for *Prototheca* mastitis. These results are in agreement with Zurakowski [11] and Costa *et al.* [21] who reported that the majority of *prototheca* positive samples were recorded in milk and milking inflation swabs.

Hodges *et al.* [25] and Benites *et al.* [31] suggested that infection may have occurred as a result of teat sores as with all mastitis pathogens caused by trauma from a milking machine and the tendency for cows to lay down on a race, the surface of which was sometimes flooded by drain water in which *Prototheca* were present.

Histopathological changes of mammary tissue samples revealed that only 8 samples (13.33%) and 2 (10%) from buffaloes and cows respectively showed pathological lesions restricted to *P. zopfii.*

Mammary gland tissues revealed severe, focal or diffuse, chronic and necrotizing interstitial mastitis associated with intralesional algal structures. These were consistent with the given reports [1,2,10,14-17,21,25,26].

Zurakowski [11] and Cheville *et al.* [32] believed that the primary destruction of the organism by neutrophils is to be prevented by either the antiphagocytic effects of the algal cell wall material or by being sequestered within macrophages away from physical contact with neutrophils. Phagocytic resistance was claimed to the presence of sporopollenin which is a complex of oxidizing polymers of carotinoids and a component of the cell wall. The intracellular *P. zopfii* degenerates by progressive lysis of internal organelles with persistence of cell wall glycans as a defective response by host macrophages so that the pathogen causes inability to overcome the infection. Infected cows shedding large numbers of organisms in their milk and no firm evidences for spontaneous recovery, these may be contagious to herd via teat liners and poorly responsive to therapy.

As algae were predominantly seen in macrophages and less frequently in neutrophils which were markedly enlarged and both sporangiospores and sporangia were found. Janosi *et al.* [26], Milanov and Suvajdÿiã [5] and Lass-Florl and Mayr [28] described the presence of both sporangiospores and sporangia in macrophages, to be due to their ability to survive and replicate in these cells. In this respect, Cunha *et al.* [33] observed that *P. zopfii* promotes a high increase of H_2O_2 production by neutrophils from bovine milk during algae exposition accompanied by increase of antioxidant enzyme activities; however, this process did not affect *P. zopfii* death.

In the present work the collected mammary gland tissues were stained by different pathological stains. For the *Prototheca* visualization staining with (PAS) [15] and Alcian blue stain [34] as well as GMS [8, 10] is recommended.

Prototheca organisms may appear as green algae and light microscopy revealed not only similarities in size, shape and mode of reproduction but also a striking difference between the *Prototheca* organisms and green

algae. Unlike *Prototheca* species, the green algae contained abundant cytoplasmic starch granules that were PAS negative following diastase digestion. So PAS is particularly useful for differentiating the green algae from *Prototheca* cells in tissue [28, 35].

In this study, the histopathological examinations of the affected mammary glands indicated that, this process is a chronic mastitis with severe destruction of the mammary alveoli. Such mammary gland changes results in a progressive drop in milk production and a persistent infection poorly responsive to therapy [5, 22, 30].

In conclusion, 1-The program for mastitis control must include the *Prototheca* algae due to the increasing numbers of *Prototheca* isolation which indicates the need of a detailed evaluation of this problem as Prototheca infections affect on milk quality and quantity due to its destruction of mammary gland tissues. 2-Confirmatory stain with PAS on mammary tissue suspected *prototheca* infection is highly recommended for differential diagnosis from other green algae.

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