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Outbreak of protothecal mastitis in a herd of dairy cows in Poland

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Abstract

The study was carried out in a herd of 280 dairy cows in the North-Eastern part of Poland in the summer of 2011. During the period of 5-6 months before the study, mastitis cases resistant to routine antibiotic therapy were observed in this herd. Bacteriological examination of 280 milk samples collected from 70 cows with clinical or subclinical forms of mastitis was performed. Diagnosis of mastitis was made on the basis of anamnesis, clinical examination of the udder, macroscopic evaluation of secretion, California mastitis test (CMT), and results of bacteriological examination of milk. Protothecal mastitis was detected in 34 cows (12.6% of all cows in the herd). Algae belonging to *Prototheca zopfii* were isolated from 27 milk samples in pure cultures; in the remaining seven samples, mixed infections were identified (*P. zopfii* and *Staphylococcus* sp.). The acute form accompanied by elevated body temperature (40°C), pain and hot oedema of the udder, loss of appetite, and reluctance to move were observed in two cows immediately after delivery. The similar symptoms were also noted in three cows with mixed infections. The chronic form of protothecal mastitis was characterised by pasty oedema in the udder of slight painfulness and hard tissue consistency, as well as markedly reduced milk secretion. The macro- and microscopic changes in the mammary tissue was indicative of chronic, fusing microgranulomatous interstitial mastitis protothecosa.

Key words: cows, mastitis, *Prototheca zopfii*.

Introduction

Algae, belonging to the genus *Prototheca* of the family *Chlorellaceae*, are ubiquitous in nature (13, 30, 31). Only three known *Prototheca* species, i.e. *P. zopfii*, *P. wickerhamii*, and *P. blaschkeae* are considered as pathogenic for humans and animals (13, 21-23, 28, 32, 33, 35, 36). In animals, protothecal infections are most commonly chronic and systemic; in dogs they affect various organs and skin whereas in cows - the mammary gland. In cows, *P. zopfii* is the most frequently isolated (34). The two remaining species, i.e. *P. wickerhamii* and *P. blaschkeae*, sporadically cause mastitis in cows (12).

Typically, *P. zopfii* induces chronic subclinical or clinical mastitis, whose course is mild. However, the disease process in the udder leads to a rapid reduction in milk production and permanently elevated somatic cell counts in milk (3, 14). The histological changes are typical of chronic granulomatous mastitis (16).

Despite an increasingly high incidence of *mastitis protothecosa* cases, they are still sporadic although endemic events have been also described (10, 14, 27). The first case of *mastitis protothecosa* was reported by Lerche in 1952 (17). Since that time, algae have been isolated in Denmark (1), Hungary (15), Spain (2), Portugal (18), Italy (6), Serbia and Montenegro (27), Brazil (5, 8, 10), China (12), and Egypt (4). In Poland, single cases of mammary protothecosis were first reported in 2002 (20).

The purpose of this report was to characterise the endemic form of *mastitis protothecosa* in a herd of dairy cows in Poland.

Material and Methods

The study was carried out in a herd of 280 dairy cows in the North-Eastern part of Poland in the summer of 2011. During the period of 5-6 months before the

study, mastitis cases resistant to routine antibiotic therapy were observed in the herd. The course of mastitis was most commonly chronic with slight clinical symptoms and markedly reduced milk yield. In four cows calved in July and at the beginning of August, mastitis was characterised by such general symptoms as elevated temperature, lack of appetite, reluctance to move as well as strongly pronounced manifestations including oedematous and painful two udder quarters, and almost complete inhibition of milk secretion several days later. Due to udder lesions and almost complete inhibition of milk production, the cows were culled.

Bacteriological examination of 280 milk samples collected from 70 cows with clinical or subclinical forms of mastitis was performed. Diagnosis of mastitis was made on the basis of anamnesis, clinical examination of the udder, macroscopic evaluation of secretion, California mastitis test (CMT), and results of bacteriological examination of milk. At the beginning of the examinations, the affected cows were at various lactation periods (mean milk yield was 9,000 L per year) and 65 cows were during the dry period. The lactating animals were kept in a detached cowshed with beds covered with a layer of sand and small amounts of straw. The dry cows stayed on the unpaved paddock. Cows had *ad libitum* access to total mixed rations (TMRs), i.e. bulky feed-concentrate mixtures supplemented with nutrients and minerals. This method of feeding provides suitable concentrations of energy, proteins, and minerals in 1 kg of dry mixture.

In total, milk was sampled for bacteriological tests from the lactating cows with lesions in the udder tissue or/and milk (positive CMT, macroscopic changes in milk) from 70 cows. This group included also cows that did not respond to routine antibiotic therapy. Milk samples were collected according to the accepted procedure. After cleaning, washing, and drying the udder skin, disinfecting the teat ends with 70% alcohol solution, milk was collected to sterile, properly labelled test tubes without preservatives, chilled to 4°C, and delivered to the Department and Clinic of Animal Reproduction in Lublin.

Bacteriological and mycological tests of milk were performed according to standard procedures (19): milk culture on the agar medium with 5% sheep blood added and on the Sabouraud medium with chloramphenicol, followed by 24-72-h incubation under oxygen conditions at 37°C; evaluation of morphology of isolated colonies and of Gram or methylene blue stained preparations. Identification was performed using API 20C AUX (bioMérieux, Poland) method (29). The test based on the susceptibility of each species of algae to clotrimazole according to Casal and Gutierrez (7) was also applied. This test was used to distinguish between *P. zopfii* and *P. wickerhamii*.

The mammary biopsy specimens from infected cows culled in the sanitary slaughterhouse were fixed in 10% buffered formalin, pH=7.2, and transferred

through series of alcohol, acetone, and xylene solutions prior embedding in paraffin blocks in the tissue processor (Leica TP-20). The 4 µm sections prepared with a sledge microtome (Leica SR-200) were stained with haematoxylin and eosin (HE), periodic acid-Schiff (PAS), and Grocott methenamine silver (GMS) analysed under a light microscope (Nikon Eclipse E – 600).

Results

The presence of various species of micro-organisms was found in 122 samples collected from clinically or subclinically affected udder quarters. Algae belonging to *Prototheca zopfii* were isolated from 27 milk samples in pure cultures; in the remaining seven samples, mixed infections were identified (*P. zopfii* and *Staphylococcus* sp.). In total, protothecal mastitis was detected in 34 cows (12.6% of all cows in the herd).

After 48 h of incubation on the Sabouraud agar, round, white colonies with a granular surface, 1-3 mm in diameter, resembling yeast-like fungal colonies, were observed. The microscopic preparations demonstrated oval-shaped sporangia containing endospores. In the API 20 C AUX, the strains assimilated only glucose and glycerol.

On the basis of the findings of clinical examinations, results of bacteriological tests, and somatic cell count (SCC), two forms of protothecal mastitis were found. The acute form accompanied by elevated body temperature (40°C), pain and hot oedema of the udder, loss of appetite, and reluctance to move were observed in two cows immediately after delivery, and in three cows with mixed infections; shortly, milk production substantially decreased or completely stopped. The chronic form of protothecal mastitis was characterised by pasty oedema in the udder of slight painfulness and firm tissue consistency as well as markedly reduced milk yield. The aqueous, grey inflammatory secretion contained flakes.

The macroscopic examination revealed the enlarged mammary gland of hard fibrotic consistency. Numerous, miliary, creamy-yellow papules, about 0.5-3 mm in diameter, were visible on section surfaces of individual udder quarters. The aqueous-mucous, opaque, yellow-white secretion containing fibre shreds was present in the lactiferous and teat sinuses (Fig. 1).

The microscopic examination of udder tissue biopsies demonstrated the presence of multifocal, often merging areas of chronic tissue inflammation with stromal fibrosis, which contained numerous forms of *Prototheca* sp. at various developmental stages. In HE staining, epithelial cells, necrotising, and separating from the basal membrane, were visible in the dilated mammary alveoli. The alveolar lumen contained numerous large tissue macrophages, less abundant lymphocytes and single neutrophils (Fig. 2). In the

macrophage cytoplasm and between damaged epithelial cells, various developmental forms of algae of *Prototheca* sp. were detected (Fig. 3). The majority of them were 2-15 μ m in diameter (single ones had 25 μ m), with the poorly stained cytoplasm and distinctly stained thin cell wall (Fig. 3).

Prototheca sp. cells were located predominantly in the cytoplasm of macrophages accumulated in the alveolar lumen and infiltrating the connective tissue stroma. Moreover, they were present between the separated epithelial cells and basal membrane and were scattered in the interstitial tissue and necrotic masses filling the outgoing efferent ducts (Fig. 4). Larger cells were identified as mother cells (sporangia) containing several to several dozen daughter cells (endospores) in the cytoplasm. The above-described developmental forms of *Prototheca* sp. were dark-brown in the methenamine Grocott staining (Fig. 5). In the lumen of dilated efferent ducts, high numbers of necrotic masses, normal and degenerative neutrophils, and developmental forms of algae were found (Fig. 6). The thickened interalveolar septa contained massive infiltration of mononuclear inflammatory cells, mainly lymphocytes and plasmacytes, focal infiltrations of macrophages, neutrophils and eosinophils, as well as fibroblast proliferation.



Fig. 1. The cut surface area of the cow's udder with visible military, confluent nodules and muco-serous secretions with strands of fibrin in the lactiferous sinus

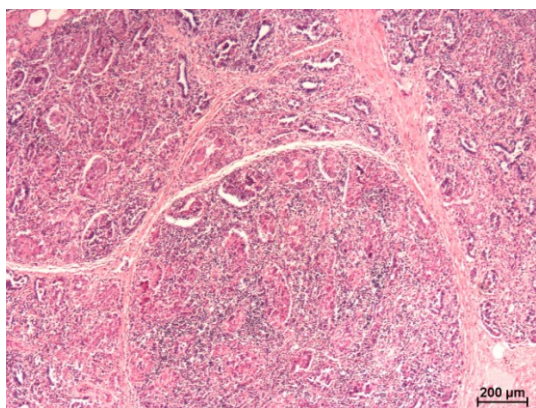


Fig. 2. The section of the glandular tissue of the cow's udder. Periacinar connective tissue infiltrated with lymphocytes and plasma cells and dilated acini containing numerous macrophages, inflammatory cells and desquamated epithelium. HE stain, 40x

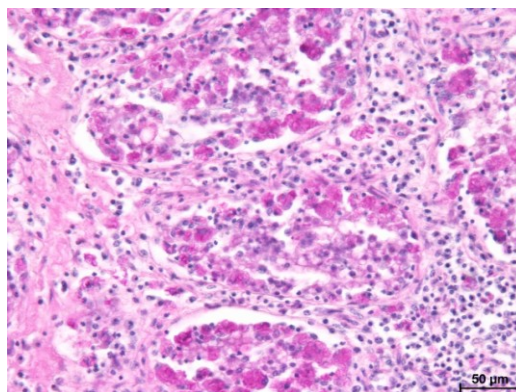


Fig. 3. Foamy macrophages containing in the cytoplasm *Prototheca* sp. organisms in the lumen of lactiferous acini. P.A.S. stain, 2000x

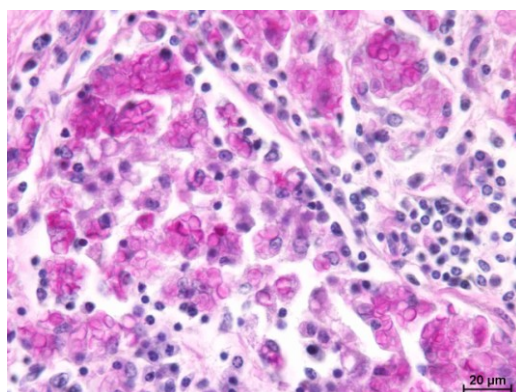


Fig. 4. Achlorophyllous algal organisms of the genus *Prototheca* sp. in different stages of development in the mammary gland tissue. P.A.S. stain, 400x

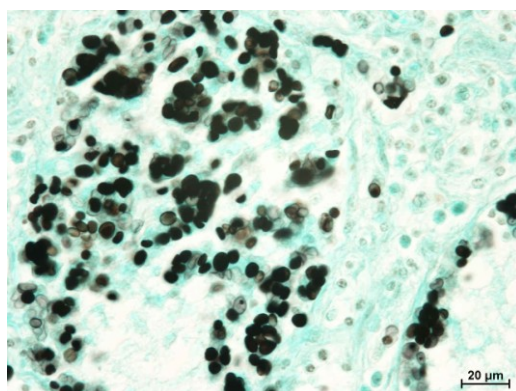


Fig. 5. Oval and spherical *Prototheca* sp. cells stained with Grocott's methenamine silver, 400x

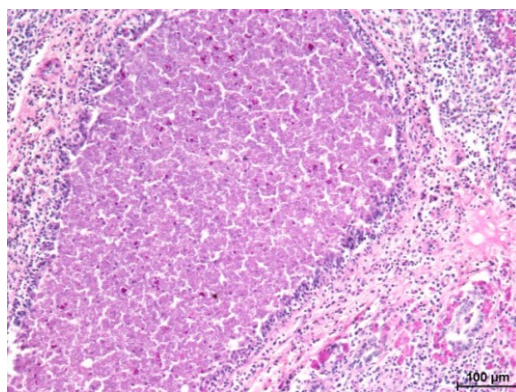


Fig. 6. The ectatic mammary duct filled with cellular debris and intensely pink stained spheroid cells of *Prototheca* sp. The connective tissue stroma infiltrated with mononuclear inflammatory cells. P.A.S. stain, 100x

The macro- and microscopic changes in the mammary tissue mentioned earlier were indicative of microgranulomatous, fusing chronic *mastitis protothecosa*.

Discussion

In the study, *mastitis protothecosa* occurred in the herd at the turn of July and August, *i.e.* the period of numerous rainfalls and high temperatures. The infection site was most likely the muddy earthen yard. Moreover, beds for cows in the detached sand-floored cowshed were in ideal conditions for the development of algae and mammary gland infections *via* the galactogenic route. Similar environmental conditions favouring the development of protothecal mastitis were described by other authors (8, 11, 12, 27). However, some authors (5, 9) did not isolate *Prototheca* sp. in the samples collected from the environment of infected cows, which can suggest another, unknown source of infection. On the other hand, the presence of algae in the environment does not determine the occurrence of udder infections, as *Prototheca* sp. was also isolated from the environment of healthy cows (5).

According to the literature data, *mastitis protothecosa* is usually subclinical, clinical with poorly pronounced symptoms, or chronic (5, 10, 15, 27). In most cases, infections affect single animals in a herd. In general, the percentage of affected cows is lower than 10 (4, 6, 8). However, cases of endemic protothecal mastitis were reported, in which the isolated *P. zopfii* ranged from 10% to 39% (10, 12, 14, 16, 27). Our findings confirm that despite low virulence of the algae, they can cause serious problems in the herd of dairy cows and induce endemic infections.

In the study, only milk changes and udder oedema were observed in most cases, which is consistent with the results published by other authors (15, 27). According to Milanov (27), clinical forms of mastitis were characterised by substantial enlargement, painlessness, and hard consistency of the infected udder quarter. In the majority of cases, clinical forms of protothecal mastitis were noted at the beginning of lactation. Similar cases were reported by other authors (14), which were also confirmed by our findings. *Prototheca* sp. most commonly affected cows immediately after delivery. Besides perinatal energetic imbalance, other factors predisposing to such infections were implicated, including *e.g.* poor phagocytic action against *Prototheca* sp., confirmed by the presence of macrophages capable of replication of sporangia and their descendants (4, 15, 27).

The acute form of *mastitis protothecosa* diagnosed in four cows in the study herd with distinctly pronounced general symptoms, such as high fever, lack of appetite, reluctance to move, and severe mammary gland symptoms can indicate that algae are also likely to induce this form of mastitis in dairy cows. These

four cows were culled in the sanitary slaughterhouse due to persistent oedema and lesions in the udder, lack of milk production, and reluctance to move despite the antifungal therapy. Thus, the observations of other authors were confirmed (15), demonstrating that the disease could cause financial losses due to reduced milk production, high SCC (3), and necessary culling of infected cows.

On the other hand, it was demonstrated that increased SCC was not characteristic for protothecal mastitis. In the milk of some cows infected with algae, low SCCs were detected as well as markedly decreased levels of other milk components, such as lactose and fat. The changes in milk composition are likely to be caused by mammary tissue damage, often irreversible (4, 5). In the study, SCC in cows infected with *P. zopfii* ranged from 200,000 to 15,000,000 cells/mL of milk. According to Malinowski (20), SCC fluctuated around 591,000-3,072,000/mL of milk in the subclinical and 6,000,000-23,000,000/mL of milk in the clinical form of *mastitis protothecosa*.

Our findings and results presented by other authors (15, 20, 27) revealed that mammary infections caused by *Prototheca* sp. result in irreversible changes in the mammary gland tissue. The histological lesions are characterised by interstitial infiltrations of macrophages, plasma cells, and lymphocytes (8, 16). *Prototheca* sp. induces chronic granulomatous interstitial mastitis with a marked decrease in milk production (15, 20, 24, 27).

The diagnosis of protothecal mastitis is difficult as the morphology of *Prototheca* sp. colonies cultured on the Sabouraud agar is not characteristic. These microorganisms can be interpreted as yeast-like fungi. Furthermore, mammary infections are often asymptomatic, thus the infected cows become the source of infection for healthy animals (9). According to the literature data, the bovine mammary gland can be a reservoir of *Prototheca* sp. due to their ability to survive in the udder during the dry period and infections caused by other microorganisms, since the administration of antifungal antibiotics is ineffective (12). Moreover, no cases of spontaneous recovery were reported (27).

Since the commonly used antifungal and antibacterial drugs are found ineffective, even when the *in vitro* susceptibility has been confirmed (unpublished data), the mastitis control programme should be followed, mainly to detect early subclinical asymptomatic inflammations. Furthermore, improved sanitary conditions regarding the environment and hygiene of milk collection are essential. Moreover, bacteriological and mycological tests of milk from mastitic cows are also crucial. Our results (unpublished data) and data presented by other authors (18, 25-27) disclose that currently there is no effective method of treatment of intramammary protothecosis. Therefore, cows with *mastitis protothecosa* should be eliminated from herds to avoid the spread of the disease.

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