ISOLATION OF PROTOTHECA ZOPFII FROM INFLAMED SECRETION OF UDDERS

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Twenty-eight Prototheca spp. strains were isolated from secretion of clinically and subclinically inflamed quarters of 25 cows. The strains showed hydrolytic activity of 6 enzymes, assimilated glucose and glycerol and were sensitive or intermediate sensitive to nystatin (85.7%), amphotericin B (42.8%), pimaricine (19.7%), gentamicin (62.5%), neomycin (41.7%) and streptomycin (29.2%). Based upon their morphologic features, assimilation patterns and antibiotic sensitivity, all isolates were identified as Prototheca zopfii.

Key words: cows, Prototheca zopfii, mastitis.

Mammary gland inflammation (mastitis) continues to be the most frequent and expensive disease of dairy cows (4, 14, 22). It negatively influences the economic effectiveness of farms and the hygienic quality of milk (10, 16, 22). About 150 species of microorganisms were found as etiological agents of mastitis. Apart of different species of bacteria (17, 22, 25), several other groups of microorganisms such as yeast, fungi and algae from Prototheca genus can cause an inflammatory process and alterations in the udder. The Prototheca genera constitute unicellular organisms without chlorophyll, with asexual reproduction by multiple splitting that form variable numbers of endospores (2, 6, 9, 23). Mastitis in cows is mostly caused by Prototheca zopfii and sometimes by Prototheca wickerhamii (9, 11). These algae are considered as the environmental pathogens of mastitis (7, 8). Protothecal mastitis occurs worldwide and appears sporadically in a therapy-resistant form (2, 5, 6, 11). Prototheca zopfii infection usually results in a chronic subclinical or mild clinical inflammatory process in the udder and is followed by a dramatic loss in milk production and a permanent increase in somatic cell count (11, 16). The histopathological findings could be characterized as a progressive interstitial mastitis associated with alveolar atrophy. Algae were seen in the alveolar lumen and interstitium, as well as in macrophages and neutrophils (5, 13).

Prototheca spp. was first described as a cause of mastitis by Lerch in 1952 (15). Since then Prototheca zopfii have been isolated from clinical and subclinical cases of mastitis in many countries (1, 12, 16, 23, 28). In Poland it was not described
up to date. The purpose of this report is to characterize the algal mastitis that was recognized in our laboratory.

**Material and Methods**

During two last years (2000 – 2001) bacteriological examinations of 11 224 milk samples taken from 4 850 cows with clinical and subclinical forms of mastitis were performed in the laboratory of Bydgoszcz Division of the National Veterinary Research Institute. Diagnosis of mastitis was made on the basis of anamnesis, clinical examination of the udder, macroscopic evaluation of secretion, California Mastitis Test (CMT), determination of somatic cell count (SCC) by Fossomatic 90 and bacteriological examination of milk. Quarter milk (inflamed secretion) samples were collected aseptically by research workers of the Department of Pathophysiology of Reproduction and Mammary Gland or (sometimes) by field veterinary surgeons. The teat ends were cleaned with alcohol swabs and allowed to dry. The first few streams were discarded and then 2 – 4 ml of secretion was collected in sterile tubes. Samples were cooled and immediately transported to the laboratory. Bacteriological examinations were performed according to commonly accepted rules (19).

Microorganisms that grew on Sabouraud’s agar were examined in light microscopy, tested for the carbohydrate assimilation, the hydrolytic activity and the antibiotic sensitivity. With the use of API 20 AUX test (BioMerieux) the ability to incorporate digested carbohydrates was examined. The activity of 19 hydrolytic enzymes was determined with the use of API ZYM test (BioMerieux), as described before (18).

Antimicrobial sensitivity was tested by disk diffusion method on YNB (Yeast Nitrogen Base - Difco) agar or on Mueller-Hinton’s agar. The following antifungal (Dom Handlowy Nauki, PAN Kraków) and antibacterial (Oxoid) antibiotics were tested: nystatin (100 µg), pimaricin (10 µg), amphotericin B (10 µg), clotrimazole (10 µg), miconazole (10 µg), ketoconazole (10 µg), thioconazole (10 µg), fluconazole (10 µg), itraconazole (10 µg), and fluocytosine (0.5 µg), as well as gentamicin (10 µg), neomycin (30 µg), streptomycin (10 µg), amoxicillin (30 µg), tetracycline (30 µg), penicillin (10 µg), lincomycin (10 µg), cefoperazone (30 µg) and novobiocin (30 µg).

**Results**

The presence of various species of microorganisms was found in 5 098 samples taken from clinically and subclinically affected udder quarters. Apart of staphylococci, streptococci, coliforms and other bacteria, yeasts and yeast-like organisms were isolated from 200 (0.4%) samples. These organisms grew on blood agar and on Sabouraud’s agar, but in 28 cases they differed from yeasts. Colonies on the blood agar were creamy-white or white-greyish, small, pasty, like CNS staphylococci or yeast after 48 h incubation. On Sabouraud’s agar they were dry with granular surface. Microscopic examination of fixed smears stained with methylene blue or Gram stain show large spherical or oval cells (sporangia) with or without daughter cells inside (endospores).
These microorganisms assimilated glucose and glycerol but not maltose, lactose, galactose, cellobiose, xylose, raffinose, trehalose, saccharose, sorbitol and arabinose.

Investigated strains were characterized by the activity of 6 enzymes. The highest activity of acid phosphatase, naphthol-AS-BI-phosphohydrolase, and then alkaline phosphatase, leucine arylamidase, esterase and lipase esterase (lowest) were noted. None of the strains exhibited activity of trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, cystine arylamidase, β-galactosidase, α-glucosidase, and β-glucosidase, α-fucosidase, and N-acetyl-β-glucosaminidase.

The organisms showed high levels of resistance to antifungal and antimicrobial drugs. All the strains were in vitro resistant to itraconazole, fluconazole, ketoconazole, clotrimazole, miconazole and thioconazole, and to lincomycin, tetracycline, amoxicillin, novobiocin, penicillin and cefoperazon. The most in vitro active antibiotics were: amphotericine B (35.7% sensitive strains and 7.1% intermediate sensitive), nystatin (21.4% and 64.3%), pimaricine (17.9% sensitive strains), gentamicin (58.3% sensitive strains and 4.2% intermediate sensitive), neomycin (12.5% and 29.2%) and streptomycin (4.2% and 25.0%, respectively).

Milk (inflamed secretion) from infected quarters appeared as normal or it was watery and sometimes a few flakes or clots were observed in the foremilk. The SCC oscillated from 6 816 000 to 23 495 000 in 1 ml of secretion from clinical mastitis and from 591 000 to 3 072 000 in milk from subclinically affected quarters. Local signs of acute mastitis such as edema and pain were noted in 10 cows. Other 7 cows had signs of chronic clinical mastitis (hardness), and 7 animals showed no clinical signs of mastitis. Eight sick cows belonged to one bigger farm (500 heads) and 16 animals belonged to 8 smaller farms. Most of the animals were earlier treated during lactation with intramammary products which contained various antibiotics.

**Discussion**

Based upon morphologic features, ability to assimilate carbohydrates and antibiotic sensitivity, all isolates were identified as *P. zopfii*. The examined strains differed in enzymatic the activity from yeasts that have shown activity of 10 – 12 hydrolytic enzymes (18). The resistance to antimycotic and antibacterial therapeutics of the strains was in close similarity to those stated by McDonald *et al.* (21). All their strains were resistant to clotrimazole, ketoconazole and miconazole and resistant to mafenixin and nystatin and 45.8% of strains were susceptible to amphotericin B.

Protothecal inflammation of the udder is often mild when compared to the bacterial one; however, it is invasive and results in a chronic granulomatous mastitis with a marked decrease in milk production (12, 20). The histological lesions are characterized by interstitial infiltrations of macrophages, plasma cells and lymphocytes (5). Infected quarters are refractory to treatment with standard antimicrobial therapy (5, 6). Diagnosis can be difficult, and infected animals often go unrecognized, allowing dissemination of the microorganism throughout the herd (3, 6). In some herds, *Prototheca* is 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 (8). Natural exposure of the udder to these environmental sources is the likely
route of infection (27). *Prototheca* may be a transient flora in the gastrointestinal tract. Thus, *Prototheca* in contaminated feed or water may pass through the gut, replicate and be unharmed to re-contaminate the environment (6). Environmental conditions, such as wetness, mud, and presence of organic material are conducive to the growth of *Prototheca* spp. (5, 6). These microorganisms were isolated from samples from dairy farms with or without a history of protothecal mastitis (2, 3).

From literature it is known that algae can cause chronic mastitis, difficult to diagnose and treatment. It seems, that udder inflammations caused by *Prototheca zopfii* occurred in the Bydgoszcz region earlier but they could be inadequately diagnosed as fungal mastitis or missed, because the colony morphology of *Prototheca* species is indistinguishable from that of yeasts (23, 26). So far, no suitable serological test for the identification of infected animals is available for routine diagnosis (24).

References