Bovine mammary protothecosis due to *Prototheca zopfi*i

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Mastitis due to *Prototheca zopfi*i was diagnosed in three of 28 cows in a dairy herd. As two cows continued to shed algae after 45 days they were slaughtered and organs were examined by cultivation, histology, immunohistochemistry and electron microscopy. Algae were restricted to the mammary glands and regional lymph nodes in which a granulomatous inflammation was seen. Algae were predominantly seen in macrophages but neutrophils also contained organisms. In macrophages both sporangiospores and sporangia were found, suggesting that intracellular proliferation may be responsible for the failure to overcome the infection. Serum samples from all cows were assayed for antibodies against *P. zopfi*i in an enzyme-linked immunosorbent assay (ELISA). Although the highest titre was found in an infected cow the difference between the mean values of the titre in infected and non-infected cows was not significant.

**Keywords** algae, bovine mastitis, *Prototheca zopfi*i, protothecosis

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**Introduction**

Protothecosis is caused by species of achlorophyllous algae belonging to the genus *Prototheca* [1]. Species of *Prototheca* have been isolated from a wide range of environmental sources, and sites characterized by wetness and the presence of organic matter [1,2]. During recent years some reclassifications within the genus have been made resulting in the three species *Prototheca stagnora*, *P. wickerhamii* and *P. zopfi*i [1]. Only the latter two have so far been reported to be pathogenic for humans and animals [1,3]. Although infrequently, cases of protothecosis have been diagnosed in humans [4–6], dogs [7,8], cows [9–12] and deer [13]. In cows, the udder is the target organ for infection and a long-lasting involvement can result from an ascending infection [9,11,14]. Based on experimental infections of cows [11,15] and different laboratory animals [4,12,15,16], *P. zopfi*i is regarded as a facultative pathogenic organism. In dairy herds, *P. zopfi*i may cause either sporadic [10] or widespread infections [11,14,17]. Information on the development of antibodies against *P. zopfi*i in serum of infected cows is sparse. In the electrophoretic assay, ultrasound-treated *P. zopfi*i cells are used as antigen [18,19]. By this assay, precipitating antibodies were found in only 40–42% of cows shedding *P. zopfi*i into the milk [18,19].

The two pathogenic forms of *P. zopfi*i are recognized *in vitro* and *in vivo*, i.e. sporangia containing multiple sporangiospores which are released following the rupture of the surrounding thecae [4]. In the bovine mammary gland, *P. zopfi*i causes chronic mastitis which is dominated by macrophages [9,11,14]; more rarely, epithelioid and giant cells may also be found [9,11]. Only scanty information concerning the pathogenesis and pathology of mammary protothecosis is available. From electron microscopic observations it has been found that algal cells, in different stages of degeneration, are contained by macrophages in the interstitium, sequestered between alveolar epithelial cells, and in the lumen of alveoli [14]. Because sporangia have not been observed in macrophages and algal elements have not been seen in neutrophils, it has been speculated that algae can not replicate within macrophages, and that neutrophils do not participate in the elimination of the microorganism, due to a prevention from uptake of algal organisms [14].

In the present work the serological response,
Table 1  Isolation and immunohistochemical in situ identification of P. zopfi in organs of two cows with protothecal mastitis

<table>
<thead>
<tr>
<th>Organs</th>
<th>Isolation AM</th>
<th>In situ localization</th>
<th>Isolation PM</th>
<th>In situ localization</th>
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<tr>
<td>Cow 1</td>
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<td>Cow 2</td>
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<td>Quarters</td>
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<td>Right front</td>
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<td>Left front</td>
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<td>Right rear</td>
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<td>Left rear</td>
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<tr>
<td>Lymph nodes</td>
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<tr>
<td>Right supramammary</td>
<td>−</td>
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<td>+</td>
<td>+</td>
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<td>Right profound inguinal</td>
<td>ND</td>
<td>ND</td>
<td>−</td>
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<tr>
<td>Left supramammary</td>
<td>+ +</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Left profound inguinal</td>
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<td>−</td>
<td>+</td>
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<tr>
<td>Other organs</td>
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<tr>
<td>Kidneys</td>
<td>−</td>
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<td>Lung</td>
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<td>Liver</td>
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</table>

AM: ante mortem. PM: post mortem. Number of algae isolated/detected: − = none; + = few; ++ = moderate; +++ = numerous.
*Abnormal appearing secretions. ND: not done.

measured by a highly sensitive enzyme-linked immunosorbent assay, in a herd of dairy cows with mammary protothecosis caused by P. zopfi is presented. Elucidation of the histopathological response as observed by means of light and electron microscopy is recorded.

Materials and methods

Animals

Owing to high leucocyte counts, bovine mammary secretions from 20 Holstein–Frisian cows in a dairy herd comprising 28 cows were collected for cultivation. Five days later a second sample of secretions was collected from three cows from which algae had been cultured. Four weeks after the first samples were taken, mammary secretions and sera were obtained from all cows in the herd. Because two cows with mastitis (nos 1 and 2) continued to shed algae they were slaughtered 2 weeks later (Table 1). Following slaughter, tissue from all quarters, the regional lymph nodes of the udder (i.e. ln. supramammarii and ln. inguinaliis profunde dexter and sinister), liver, lungs, kidneys and the spleen were collected for culture and histopathology.

Examination of secretions

All samples of secretions were analysed by the Californian Mastitis Test (CMT) [20], and cultured according to Aalbørk et al. [10]. Algae were identified by cell morphology and standard assimilation criteria [21].

Pathology

Following gross inspection tissue samples from all quarters, lymph nodes and visceral organs were fixed in 10% buffered formalin, dehydrated and embedded in paraffin. Sections for immunohistochemistry were mounted on StarFrost Plus adhesive slides (Axel Johnson Lab System, Denmark). Sections were stained with haematoxylin and eosin (H&E), periodic acid-Schiff (PAS), Grocott’s methenamine silver (GMS), Luna’s stain for eosinophils, and Van Gieson’s stain for connective tissue. The content of algae within tissues was graded.

Immunohistochemistry

Immunohistochemical staining of P. zopfi was performed by a peroxidase antiperoxidase (PAP) technique originally developed for the study of experimental murine protothecosis [16]. In the technique, a polyclonal rabbit antibody raised against P. zopfi was used as the first reagent. The rabbit PAP immunocomplex (DAKO, Denmark) was bridged to the primary reagent by a swine antirabbit antibody (DAKO, Denmark). The reaction was developed in 3-amino-9-ethylcarbazole...
(AEC) (Fizzing, Denmark) in a 20 mM citrate buffer, pH 5-1, giving a brown-red colour at locations where peroxide was present. Sections were counterstained in Harris haematoxylin before reading. Immunostaining of macrophages was performed by the demonstration of CD68 antigen by a slight modified technique developed by Ackermann et al. [22] for bovine macrophages. Briefly, a monoclonal murine antibody EBM11 (DAKO, Denmark) was applied on protease XXIV 0.5% Tween 20. Following an additional fixation in 2.5% glutaraldehyde and postfixation in 1% osmium tetroxide the samples were dehydrated in acetone and embedded in Vestopal W (Serva, Germany). Survey sections were stained in toluidine blue while sections for transmission electron microscopy were stained in uranyl acetate and lead citrate. Sections were examined by a JEM 100 B transmission electron microscope.

**Electron microscopy**

Fragments of paraffin-embedded mammary tissue corresponding to algal-containing areas observed in sections made from the paraffin block were removed and deparaffinized. Following an additional fixation in 2.5% glutaraldehyde and postfixation in 1% osmium tetroxide the samples were dehydrated in acetone and embedded in Vestopal W (Serva, Germany). Survey sections were stained in toluidine blue while sections for transmission electron microscopy were stained in uranyl acetate and lead citrate. Sections were examined by a JEM 100 B transmission electron microscope.

**Enzyme-linked immunosorbent assay (ELISA)**

An ELISA for the monitoring of antibodies against *P. zopfi* in bovine serum was developed and applied at its optimal conditions. Briefly, wells of polystyrene microtitre plates (Nunc-Immuno Plate, Maxisorp, Denmark) were coated with 100 µl of somatic antigen of *P. zopfi* [16] containing 1.9 µg ml⁻¹ protein in 50 mM carbonate buffer, pH 9.6, and incubated for 1 h at 37 °C and then overnight at 4 °C. To each well 100 µl of the bovine serum samples diluted 1:100 in PBS-T-TBSA (phosphate buffered saline (PBS) containing 0.05% Tween 20 and 1% bovine serum albumin (Sigma, USA) was added, and the plates were incubated for 1 h at 37 °C. Then, 100 µl of peroxidase-conjugated rabbit antibovine immunoglobulins (DAKO, Denmark) diluted 1:1000 in PBS-T-TBSA was added to each well. After 1 h of incubation at 37 °C, 100 µl of an ortho-phenylenediamine (OPD) solution (0.8 mg ml⁻¹ in 50 mM citrate buffer, pH 5-2, containing 10 µl 30% H₂O₂ per 10 ml) was added to each well. The OPD solution was obtained by dissolving four OPD-tablets (KEM-EN-TEK, Denmark) in 10 ml H₂O. The reaction was stopped after 5 min with 50 µl of sulphuric acid, 1N, and the optical density was read at 492 nm by an Easy Reader EAR 400 (SLT-Lab Instruments, Austria). Before each incubation the plates were washed five times with PBS containing 0.5% Tween 20.

Samples of serum were tested in triplicate and the interassay coefficient of variation was kept at 5-7%.

**Statistics**

The significance of serological results was evaluated by Student’s *t*-test with a significance *P*-value of 0.05.

**Results**

Abnormal-appearing mammary secretions having clots and flakes were only observed in cow 1, which continued to shed algae (Table 1). However, all secretions from which algae were grown (Table 1) had a positive CMTF value of 4 or 5. All isolated algae were identified as *P. zopfi* [21]. The organs from which it was cultured and demonstrated in situ appear in Table 1.

Affected quadrants (Table 1) had only a slight increased density, and apart from some enlargement and moistening of the submammary lymph nodes of cow 2, no macroscopic lesions were recorded in any extra-mammary sites.

Affected quadrants and lymph nodes of both cows revealed identical histomorphological patterns. Within affected quarters, a heterogeneous picture of algal proliferation and cellular reaction was observed. Within lobules, usually not all alveoli were affected. The contents of organisms and accompanying tissue response within and around infected alveoli, and to a limited extent also excretory ducts, varied from an acute to a chronic type. Especially in the early stages of infection of alveoli protothecal cells multiplied within the lumen and epithelial lining of the alveoli (Fig. 1). CD68 positive cells containing algae within the epithelial lining of the alveoli and excretory ducts were often vacuolated (Fig. 2). Within the lumen, multiplication occurred within the cytoplasm of sequestered macrophages in which different stages of development and degeneration of the algae could be seen (Fig. 3A and B). During early stages of infection, algal cells were observed within the cytoplasm of neutrophils which had been excreted to the lumen of alveoli (Fig. 3C). Many chronically infected alveoli were involated, and epithelial hyperplasia was often observed. The chronic inflammatory
of the excretory ducts, algal cells were contained by intra-epithelial macrophages. Many CD68 positive macrophages, filled with a homogeneous PAS-positive material which failed to react in the PAP-staining for *P. zopfii*, were often seen around alveoli. Within the regional lymph nodes (Table 1) accumulation of macrophages containing single and sporulating forms were localized in the paracortical and paratrabeicular sinuses (Fig. 4).

In all tissues the application of the PAP-technique for *P. zopfii* resulted in a strong reactivity (Fig. 2). Chloroplasts were not observed within algae when examined by electron microscopy (Fig. 3).

The serological response of the cows is shown in Fig. 5. Although the highest titre (0·41) of anti-*P. zopfii* antibodies was obtained in serum from an infected cow (no. 1), the difference between mean values of the titres in infected and non-infected animals was not significant ($t = 0·69 \sim P = 0·5$).

**Discussion**

Several bovine infections caused by the green algae *Chlorella* have been misdiagnosed as protothecosis [23–25]. To obtain an accurate diagnosis of protothecosis it is necessary to cultivate the organism, demonstrate an absence of chloroplasts [23] and/or specific immunohistochemical reactivity *in situ* [26,27].

In the present study, two documented cases of spontaneous bovine mammary gland infection with pure cultures of *P. zopfii* being recovered are described. The histopathological response in the mammary tissue of the cows is similar to that described previously [9,11,14]. However, that *P. zopfii* may proliferate within macrophages, or the proliferation stage may survive for some time following phagocytosis, was demonstrated by the observation of intact intracellular sporangia, a phenomenon which may help explain the chronic nature of the infection. In contrast to previous observations [14], it was found that neutrophils took up algae. Because they are able to kill *Prototheca* cells *in vitro* [28], it is tempting to speculate that they also, albeit to a lesser extent compared to macrophages, may participate in the *in vivo* control of mammary protothecosis.

The spread of algae to the regional lymph nodes tissue, dominated by infiltration by macrophages, lymphocytes, plasma cells and, occasionally, eosinophils. In such areas many of the macrophages contained algae. Macrophages with a content of degenerated algae, i.e. empty spherical cell wall profiles that were devoid of cytoplasmic organelles, were also frequently sequestered between epithelial cells (Fig. 3D), and sometimes into the lumen of alveoli. In the epithelial lining reaction was, apart from the proliferation of fibroblastic tissue, dominated by infiltration by macrophages, lymphocytes, plasma cells and, occasionally, eosinophils. However, the effect of this localization on local immune response remains to be determined. It is also only speculative that the organisms disseminate among quarters by lympho-haematogenic spread from one

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Bovine mammary protothecosis due to *Prototheca zopfi*

Fig. 3 Mammary protothecosis. (A) Algae in a desquamated necrotic macrophage in the lumen of an alveolus. One degenerated cell (D) is seen along with a single cell (S) and a dark sporangium containing four daughter cells. N: nucleus of macrophage. (B) An algal cell in a desquamated and necrotic macrophage. Note the non-solid appearance of the cell wall, the presence of lipid granules, and absence of chloroplasts within the cytoplasm. (C) A neutrophilic cell in the alveolar lumen containing a dense algal cell together with phagosomes containing milk protein granules. (D) Part of a macrophage in the alveolar epithelium. Within the cytoplasm of the macrophage several degenerated algal cells are seen. N: nucleus of macrophage. All sections were stained by uranyl acetate and lead citrate. Bars = 2 μm.

Quarter to another. Spread of algae to organs outside the mammary compartment has been reported in only a single cow, which had a concomitant necrotizing fungal mastitis [27].

In contrast to simple electrophoretic assays with lesser sensitivity [18,19], the present ELISA data demonstrate that both diseased and non-diseased animals possess antibodies against *P. zopfi* (Fig. 5). This situation parallels the serological response observed by other opportunistic pathogens of cows such as *Aspergillus fumigatus* [30]. It is likely that under normal farm conditions, cows are regularly exposed to *Prototheca* spp. through a non-infectious contact, e.g. through the gastrointestinal tract, which is supported by the
lesser extent by neutrophils. The occurrence of proliferating stages of algae within phagocytic cells may help to explain the chronicity of Prototheca mastitis. Although a strong serological response may be present in infected cows the titre in a single sample of serum is not valid for the diagnosis of bovine mammary protothecosis.

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References

Bovine mammary protothecosis due to *Prototheca* zopfi


