Entry

Prototheca spp. in Bovine Infections

Simona Nardoni * and Francesca Mancianti ©

Department of Veterinary Sciences, University of Pisa, 56124 Pisa, Italy; francesca.mancianti@unipi.it
* Correspondence: simona.nardoni@unipi.it

Definition: Prototheca microalgae, although still considered uncommon etiologic agents, represent an insidious intruder, threatening cattle herd health and determining productive losses. Increasing numbers of clinical cases globally identified would indicate these microalgae as emerging pathogens. They can be isolated from a wide variety of environmental and non-environmental sources, due also to their ability to produce biofilm. This ability to spread and contaminate a huge variety of substrates, as well as the high resistance to elevated temperatures, renders Prototheca prevention a very hard task. In addition, early infection signs are subtle and difficult to detect. The poor response to conventional antimycotic drugs represents an additional challenge when facing this infection. Although it would seem unrealistic to completely eradicate the exposure risk of cows to these microalgae, the adoption of proper on-farm protocols and management, with the highest attention to hygiene measures, would be beneficial in reducing the magnitude of this problem. Keeping the attention focused on early diagnosis, together with the development of new, alternative, and effective agents and formulations, would be strongly advised to prevent, treat, and control Prototheca infections.

Keywords: microalgae; Prototheca bovis; Prototheca blaschkeae; Prototheca ciferrii; bovine protothecosis; mastitis; cattle

1. Introduction

Prototheca spp. (family Chlorellaceae, order Chlorellales, class Trebouxiophyceae) are achlorophyllous microalgae, widely distributed in the environment and repeatedly reported as responsible for human and animal disease. Bovine mastitis represents the most important form of Prototheca infection in animals and consists of clinical or subclinical forms. Dairy-cattle-associated Prototheca species are Prototheca bovis, Prototheca blaschkeae, and Prototheca ciferrii [1]. The ecology of Prototheca is not fully elucidated yet. These organisms can be recovered from animal waste, sludge, sewage, rivers, and fountains, preferring moist areas with high organic contents. Bovine protothecosis is reported to occur worldwide, in the presence of large dairy herds, mostly in tropical and temperate areas [2]. Predisposing factors to protothecosis are reported to be unclean or repeated intramammary infusions, and antibiotic drug treatments in the udder, where Prototheca would act as an opportunistic pathogen favored by antibiotic-induced suppression of the local flora [3–8]. Prototheca spp. can survive a wide range of environmental conditions as well as disinfectants [9,10]. Prototheca spp. can produce biofilm [11,12]. Bovine mammary gland can be infected by P. bovis following teat trauma by mechanical milking, and subsequent contamination of the teat orifice by environmental organic matter [13,14]. Infection of the mammary gland is often subclinical, without any visual sign, and can be revealed by raised somatic cell count only, although the high result is not continuous [6,15].

An environmental control approach would include action in stables, aisles, as well in milking parlor. The main strategy is devoted to controlling algal amounts in the environment enhancing hygiene measures by using conventional and natural disinfectants, as well as physical tools [16]. To date, no treatment protocol has been proven fully effective in controlling Prototheca spp. infection in dairy cows [2].
2. Taxonomy

Prototheca spp. (family Chlorellaceae, order Chlorellales, class Trebouxiophyceae) are achlorophyllous microalgae, strictly related to green algae belonging to the genera Helicosporidium (parasites of arthropods) and Chlorella (a rare human pathogen). The genus, recently revised [1], encompasses several species, namely Prototheca blaschkeae, Prototheca bovis (formerly Prototheca zopfii gen. 2), and Prototheca ciferrii (formerly Prototheca zopfii gen. 1), reported from bovine farms. Other pathogenic species such Prototheca cutis, Prototheca miyajii, and Prototheca wickerhamii are responsible for human and animal disease. Environmental species are Prototheca stagnora, Prototheca moriformis (formerly Prototheca ulmea), Prototheca tumulicola, and Prototheca portoricensis. However, further novel species Prototheca cerasi, Prototheca cookei, Prototheca pringsheinii, Prototheca xanthoriae, the re-defined P. zopfii, and Prototheca paracutis based on the evaluation of the mitochondrial cytochrome B (cytB) have been proposed [1,2,17,18]. Moreover, three new environmental species, namely Prototheca fontanaea, Prototheca lentecrescens, and Prototheca vistulensis, have been recently described [19].

The study on protothecoses, along with diseases caused by other zoopathogenic algae (Chlorella spp. and Helicosporidium spp.) deals with medical phycology after the symposium on 28 May 2009, entitled “Medical phycology: An emerging realm of microbiology”. Anyway, considering the rarity of algal affections, their study is considered most functional by the International Society for Human and Animal Mycology (ISHAM) [20], so it falls in the field of medical mycology [21].

P. wickerhamii is an important human pathogen, responsible for leastwise 115 cases worldwide [22], followed by P. bovis [23], P. cutis, and P. miyajii [24–26].

Even if bovine mastitis represents the most reported form of protothecal infection in animals, the disease has been described in dogs, cats, and small ruminants [27–29]. Cases of disseminated protothecosis have been reported in fruit bats [30,31], in a deer [32], in a beaver [33], in snakes [34,35] in fish [36,37], and in horses [38,39].

P. wickerhamii was the most involved species, although P. bovis was reported in a horse. The same microalga species was responsible for disease in dogs with a more aggressive infection course, like P. ciferrii [27,40]. In this animal species protothecosis develops as cutaneous, intestinal, or systemic disease, with ocular and cerebral involvement. In addition to P. bovis, P. cutis, too, was involved in feline protothecosis [41], where the affection appears limited to skin.

More detailed information about veterinary protothecosis has been recently provided by Ely et al. [41].

Bovine protothecosis consists of clinical or subclinical mastitis. Dairy-cattle-associated Prototheca species are P. bovis, P. blaschkeae, and P. ciferrii. This latter mostly occurs in the environment [42,43], although experimental infection induced granulomatous infection of the udder [44]. P. bovis is reported as the most pathogenic species [6], but P. blaschkeae [45–47] is involved, also, while P. wickerhamii has been rarely found [48]. The species involved in bovine disease, apart from P. wickerhamii can be differentiated by phenotypic characteristics such as assimilation and temperature tolerance tests, too [18].

3. Morphology

Cells are hyaline and round, with a diameter ranging from 3 to 30 µm [49], and asexually produce sporangia (mother cells) containing from 2 to 20 endospores, variable in size and shape, depending on the alga species. P. bovis can in fact measure up to 25–30 µm in diameter [2,18], while P. wickerhamii is smaller [24,50]. In a suitable environment, sporangia break and release daughter cells. In the resting stage, Prototheca shows a thick cell wall, without cell divisions.

The cell wall of Prototheca has two layers and is composed differently from that of plants and fungi. It does not contain cellulose or chitin but has plastids bound to the cellular membrane, which hold starch deposits. These plastids may be remnants of chloroplasts from green algae [49]. Moreover, the cell wall contains a highly stable carotenoid polymer,
called sporopollenin, occurring in several algae genera, responsible for the high resistance of these organisms to cell wall degrading systems in the environment [51,52].

4. Epidemiology

The ecology of Prototheca is not fully known. These organisms are well-adapted to watery environments. They can be found in animal waste, sludge, sewage, rivers, and fountains, and prefer moist areas with high organic contents. The type of bedding interferes with water retention or in promoting drainage away from the recumbent cow, and it is reported to increase or inhibit the survival of Prototheca in the environment [53]. Environmental and climate changes driven by human activities would also promote the spreading of these algae [54]. *P. ciferrii* occurs in the environment, mostly in cow barn [55], while *P. bovis* was isolated from milk, from 20–70% of feces of healthy bovine [56], but also from drinking water, feed, and bedding of dairy farms [57–61]. Wet feeds containing high amounts of starch and oligosaccharides, (i.e., potato pulp) are also considered as a suitable medium for *Prototheca* [2]. *P. bovis* can also be recovered from milking equipment, also [61]. It was isolated from horse feces [62], from the intestine of a patient with troubles after cheese consumption [63], and recently found in a healthy human intestine [64]. Calves fed on mastitic milk containing the microorganisms are considered a further source of environmental contamination in affected herds [65]. Furthermore, microalgae can survive into the udder during the dry period, until the next milking period. Persistently infected cows continuously shed microorganisms in more than 70% of cases, while 4.9% excrete them intermittently, indicating that *Prototheca* is maintained within the herd by subclinically infected hosts [66].

In a study from Poland, Prototheca was found in bedding, barn walls, feed, and drinking water. *P. bovis* was the most represented species (47.6%), followed by *P. ciferrii* (33.3%) and *P. blaschkeae* (19.1%) [42]. *P. blaschkeae* was obtained in culture from the feces of healthy cows [28] and from fecal and environmental samples of pig farms [67]. This species was found by polymerase chain reaction (PCR) with the highest incidence among other microalga species in raw milk and cheese specimens. It is debated whether the occurrence of *Prototheca* from cheese samples depends on the raw milk or on contamination during the production and handling of cheese [68]. Furthermore, bioinformatic analysis of shotgun metagenomic sequencing data applied to wastewater samples, available from the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA) database, demonstrated the presence of *P. bovis* microalgae in two different wastewater metagenomic datasets from the USA and from Pakistan, respectively [2].

Bovine protothecosis is reported to occur worldwide, where there are large dairy herds, mostly in tropical and temperate areas [2, 21, 66]. The infection was first reported in Germany in 1952 [2], then in the United States, Canada, Belgium, France, Denmark, Germany, Brazil, Italy, Japan, China, New Zealand, Poland, Korea, and Ecuador [38, 42, 55, 69–76].

When outbreaks occur, prevalence values range from 7.5% to 36.49% [55, 57]. The infection is reported to be present in 81% of dairy herds, although less than 10% of cows seem to be affected [3, 7]. However, it is not easy to determine the real prevalence of *Prototheca* infection within a herd, being intermittent the shedding of the causative agent [5]. Predisposing factors to protothecosis are reported to be unclean or repeated intramammary infusions, and antibiotic drug treatments in the udder, where Prototheca would act as an opportunistic pathogen favored by antibiotic-induced suppression of the local flora [3, 8]. Moreover, it is also reported that the occurrence of microalgae is directly proportional to the herds’ size, probably due to the use of cooling systems increasing the amount of water in litter courses, thus favoring the development and spreading of these organisms [16].

*P. bovis* is responsible for systemic infections in compromised human patients [2], acting as a zoonotic organism. *Prototheca* spp. survive a wide range of environmental conditions as well as disinfectants [8, 9, 77]. Furthermore, *P. blaschkeae* is more resistant to salinity, and more susceptible to pH when compared with *P. bovis* [77].
5. Pathogenesis

Surprisingly, mastitis is a typical feature of bovine protothecosis, only, being *P. wickeramii* the etiological agent of caprine protothecosis, which develops as respiratory disease, probably after contact of nostrils with organisms from the environment, while ovine protothecosis has never been reported [54].

*P. bovis* is reported to be capable in biofilm production [11,12]. This polymeric matrix is produced by other organisms causing mastitis, such as bacteria and fungi.

The bovine mammary gland can be infected by *P. bovis*, following teat trauma by mechanical milking [13] and subsequent contamination of the teat orifice by environmental organic matter [14]. *Prototheca* spp. infection would induce an adaptive immune response and chronic inflammation mediated by T lymphocytes in the bovine mammary gland, while is not damaged by neutrophils, despite oxidative burst [78].

*P. bovis* infection induces apoptosis of epithelial cells, as well as in the mammary gland causing severe phlogosis with macrophagic and neutrophilic infiltration, inducing release of pro-inflammatory effectors (TNF-α, IL-1β, and Cxcl-1) in murine macrophages. Increased levels of IL-8 mRNA in bovine and murine mammary epithelial cells subsequent to *P. bovis* infection induces apoptosis of epithelial cells, as well as in the mammary gland causing severe phlogosis with macrophagic and neutrophilic infiltration, inducing release of pro-inflammatory effectors (TNF-α, IL-1β, and Cxcl-1) in murine macrophages. Increased levels of IL-8 mRNA in bovine and murine mammary epithelial cells subsequent to *P. bovis* infection induces apoptosis of epithelial cells, as well as in the mammary gland causing severe phlogosis with macrophagic and neutrophilic infiltration, inducing release of pro-inflammatory effectors (TNF-α, IL-1β, and Cxcl-1) in murine macrophages. Increased levels of IL-8 mRNA in bovine and murine mammary epithelial cells subsequent to *P. bovis* infection induces apoptosis of epithelial cells, as well as in the mammary gland causing severe phlogosis with macrophagic and neutrophilic infiltration, inducing release of pro-inflammatory effectors (TNF-α, IL-1β, and Cxcl-1) in murine macrophages. Increased levels of IL-8 mRNA in bovine and murine mammary epithelial cells subsequent to *P. bovis* infection induces apoptosis of epithelial cells, as well as in the mammary gland causing severe phlogosis with macrophagic and neutrophilic infiltration, inducing release of pro-inflammatory effectors (TNF-α, IL-1β, and Cxcl-1) in murine macrophages. Increased levels of IL-8 mRNA in bovine and murine mammary epithelial cells subsequent to *P. bovis* infection induces apoptosis of epithelial cells, as well as in the mammary gland causing severe phlogosis with macrophagic and neutrophilic infiltration, inducing release of pro-inflammatory effectors (TNF-α, IL-1β, and Cxcl-1) in murine macrophages. Increased levels of IL-8 mRNA in bovine and murine mammary epithelial cells subsequent to *P. bovis* infection induces apoptosis of epithelial cells, as well as in the mammary gland causing severe phlogosis with macrophagic and neutrophilic infiltration, inducing release of pro-inflammatory effectors (TNF-α, IL-1β, and Cxcl-1) in murine macrophages.
in comparison with bacterial infections [15,85–87]. Clinical signs more frequently appear in summer and in the early lactation phase [66].

7. Diagnosis

Diagnosis of *Prototheca* infection typically relies on morphologic features of colonies, obtained in aerobic conditions on various culture media such as Sabouraud Dextrose Agar (SDA) supplemented with vancomycin (5 µg/mL) and nalidixic acid (20 g/L/mL) to prevent an overgrowth by fast-growing contaminating fungi and bacteria [88], MacConkey agar and 5% blood sheep agar, with 37 °C as incubation temperature. After 2–5 days of incubation, visible colorless or white-to-cream granular colonies (on SDA) with a compact central protrusion and non-regular margins, or small grey colonies without hemolysis (on MacConkey agar) can be distinguished [89]. The creamy-white colonies could be confused with yeasts; anyway, the presence of *Prototheca* endospores represents a characteristic aspect of microscopic identification [49]. *Prototheca* Isolation Media (PIM) and *Prototheca* Enrichment Medium (PEM) containing 5-fluorocytosine are also available for the selective growth of these agents [66]. Selective *Prototheca* development can also be obtained using media with 5.1 pH and containing acetate as the sole carbon source [90]. A presumptive, early identification could be achieved by observing colonies with a stereomicroscope after 24 h incubation. Diagnosis can also be quickly confirmed by smears or wet mount preparations, stained with methylene blue or Gram [88].

Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry proteomic analysis has been assessed to detect and identify *Prototheca* spp. Furthermore, enzyme-linked immunosorbent assay (ELISA) may be used to discriminate between infected and non-infected dairy cows, while the detection of anti-protothecal antibodies in whey and serum appears to be probative for diagnosis of protothecal mastitis [2].

Accurate identification of *Prototheca* spp. isolates is based on molecular methods. The DNA sequence-based typing of the mitochondrially encoded CYTB gene appears to be the best-established marker for both diagnostics and phylogenetics analysis of *Prototheca* algae, providing sufficient discriminatory power, accuracy, and technical feasibility to identify all currently described species. The adoption of the CYTB gene as a marker allowed to overwhelm the possible ambiguous species identification of these organisms reported by using the rDNA markers, due to *Prototheca*’s high intraspecific and intrastrain sequence variation [91].

8. Control

New approaches for control of *Prototheca* mastitis have recently been deeply revised by Bozzo et al. [16]. Attention should be paid to the herd, focusing on hygiene measures regarding the position of stables, the control of vehicles, staff, and visitors, newly introduced animals, as well as drinking water, and feed, to reduce the risk of contamination.

Infected cows should be identified by cytology and culture, then separated from healthy subjects and milked last. However, when the number of affected animals is low, culling is recommended. Indeed, currently, the only control option consists of culling of infected cows [11]. Treatments of infected quarters must be avoided [6]. Post-partum control of healthy cows is mandatory, and bulk milk from negative animals should be weekly examined for the occurrence of *Prototheca* spp. When positive, analysis should be promptly repeated and, when confirmed, cows belonging to the healthy group should be individually monitored [16]. The environmental approach would include action in stables, aisles, and the milking parlor. Considering the lack of effective drugs, the main strategy should be devoted to the control of algae amount in the environment, by enhancing hygiene measures.

Control of bedding, although considered as a more expensive option in respect to culling, should consist of changing the type of bedding. Spruce shavings appear more effective in preventing the growth of *Prototheca* spp., in respect of manure, sand, and...
Bedding should be maintained dry, checking the status, mainly in moist seasons [92].

Milking hygiene comprehends the cleaning of the udder, especially the teat tip to avoid environmental microalgae from gaining access to mammary tissue. The contamination, in fact, can happen by exposure to manure or from direct contact with cow to cow. For these reasons, milking equipment must be maintained clean, using disinfectants, as well teats disinfected by dipping prior and after milking. Dust and feces can, in fact, transmit the organism from the environment and milk proteins and fat after milking [2].

It is difficult to identify the best disinfectant from the review of literature, varying dilutions, and times of exposure. Among conventional preparations chlorhexidine and iodine appear as the most suitable options [9,10,93–95], proven to be active at lower concentrations than that recommended by the manufacturer [95]. Quaternary ammonium salts and dodecylbenzenesulfonic acid resulted in active, too [10]. Moreover, alkylidiaminoethyl-glycine, hydrochloride, sodium hypochlorous acid, sodium hypochloride, and guanidine have been successfully employed, as well as peracetic acid and polyhexamethylene biguanide [9,96]. Furthermore, these latter, scored very effective against biofilm-producing *P. bovis* strains [9,97].

Recently, dinitroanilines used as herbicide compounds acting against plant tubulins such as dinitramine and chloralin, showed strong inhibitory effects on *P. blaschkeae*, while dinitramine only scored active versus *P. cferr* and *P. bovis* [98]. These agents inhibit the mitotic spindle formation and possess a selective action for different eukaryotic clades. Dinitroanilines do not exert any toxic effect on mammalian cells. This novel approach follows a report dating back to 1964 [99] regarding the use of aminotriazole and is based on the statement that green microalgae are more closely evolutionary related to plants rather than animals and fungi [98].

Natural compounds have been in vitro checked. A methanolic extract of *Clematis vitalba* was effective against both *P. bovis* and *P. wickerhamii* [100], with MICs varying from 1.4 μg/mL to 11.6 μg/mL while a leaf extract of *Camellia sinensis* scored active to *P. wickerhamii* [101].

Several essential oils have been tested for their anti-*Prototheca* action and EOs from *Melaleuca alternifolia* and *Citrus bergamia* [102], from *Cinnamomum zeylanicum* and *Thymus vulgaris* [103], and from *Citrus paradisi* [104] exerted a significant in vitro activity.

Physical tools consist of the use of antimicrobial photodynamic therapy [105] and of blue light to control *Prototheca* biofilm in the environment, mostly on surfaces [106], with promising results, as well as cold atmospheric plasma [107,108].

Another physical tool is milk sterilization. *P. bovis* and *P. blaschkeae* are in fact heat tolerant and are completely inactivated at 100 °C, only [109]. For this reason, pasteurization cannot be considered, not significantly affecting the survival of these species. Their ability to form cell clumps would hamper a complete exposure of microalgae to heat [110].

Infected milk should not be administered to calves, to avoid fecal environmental contamination following the passage through the animals’ intestines.

Finally, infected cows must be separated from healthy ones in calving areas [16].

### 9. Treatment

To date, no therapy protocol has been proven as fully effective in controlling *Prototheca* spp. infections in dairy cows.

When only a quarter is involved, cauterization can be performed, but, although the other quarters can yield a higher amount of milk [111], attention should be paid to avoid residual secretion from diseased quarter spread microorganisms in the environment [92].

The development of chronic infections, as well as the resistance of microalgae to conventional drugs, could be ascribed to the formation of a pyogranulomatous phlogistic process [94]. For these reasons, several antimicrobials have been checked, although in vivo studies on cows are not reported [98] and any empirical treatment resulted in unsuccessful [66,112]. Some information can be gathered from literature concerning the treatment
of human protothecoses, although the algal species involved are different. Nystatin and amphotericin B scored in vivo effective against *P. wickerhamii*, when involved in localized disease [24]. However, nystatin and filipin showed higher MIC values, and resistant strains to all checked drugs were recorded [113,114].

Conventional antimycotic drugs were in vitro checked against *P. bovis* [109,113–115], with amphotericin B considered quite efficient, also in the presence of biofilm-producing *P. bovis* strains [12]. However, in a multicentric study, involving 144 isolates of *P. bovis* and *P. blaschkeae*, strong differences in sensitivity to amphotericin B and some azoles were reported based on the geographic source of strains [114]. Ketoconazole appeared to have some antialgal activity [58,114]. Antibiotics such as kanamycin, gentamicin, pimaricin, colistin sulfate, and netilmicin showed some anti-*Prototheca* effects, with *P. bovis* less sensitive than *P. blaschkeae* and *P. ciferri* [113,115–117]. Anyway, *Prototheca* strains from Brazil show higher MIC values, when compared to Italian ones [117].

To bypass these drawbacks, research on novel drugs has been enhanced.

The use of essential oils for topical infusion in a dense excipient has been suggested [103]. *Cinnamomum zeylanicum* scored as the most effective when tested against *P. bovis*, followed by *T. vulgaris*, *Syzygium aromaticum*, and *Salvia sclarea*, reporting MIC values ranging from 0.02% to 0.25%. Tea tree oil and bergamot oil scored effective at 0.06% and 0.3%, respectively [102–104]. Finally, *Litsia cubeba* and *Origanum vulgare* also had an anti-algal effect at 0.75% and 1% against *P. bovis* and *P. blaschkeae*, respectively [104]. This EO concentration can be used to treat udder without damaging epithelial cells.

Iodopropylcarbamate used alone and/or in combination with AMB appears as a promising choice to treat bovine mastitis by *Prototheca* [118], as well 3-bromopyruvate [115]. Moreover, these drugs were safe for bovine cells.

Bovine peptides BMAP-28, Bac5, and LAP can kill *Prototheca* sp. and Bac5 and LAP are able to act via non-lytic mechanisms [119]. More recently, other antimicrobial peptides, such as pexiganan, h-Lf1–11, LL-37, and cecropin B exerted a good anti-algal activity [120]. Conversely, little is ascertained about the concentration of these compounds in tissues and fluids. Although they are reported as physiologically stable, allowing to predict a clinical application, in vivo effectiveness should be determined [121].

Silver nanoparticles are considered promising tools for the treatment of *Prototheca* spp., for bovine mastitis, lacking cytotoxicity at microbicide concentration [122,123].

Photodynamic therapy, mediated by aminolaevulinic acid administration, scored effective against *P. wickerhamii*, although repeated treatments are needed to inactivate the organism. Furthermore, *P. wickerhamii* antibacterial and antifungal susceptibility profile is not affected, unlike other agents such as *Candida albicans* or *Scedosporium* spp. [124], displaying further differences between fungi and *Prototheca*.

10. Conclusions and Prospects

*Prototheca* microalgae, although still considered an uncommon etiologic agent, represent an insidious intruder threatening cattle herd health and determining productive losses. Increasing numbers of clinical cases globally identified would indicate these microalgae as emerging pathogens; they can be isolated from a wide variety of environmental and non-environmental sources, due also to the ability in producing biofilm. This ability to spread and contaminate a huge variety of substrates, as well as their high resistance to elevated temperatures, renders *Prototheca* prevention a very hard task. In addition, early infection signs are subtle and difficult to detect. The poor response to conventional antimycotic drugs represents an additional challenge when facing this infection. Although it would seem unrealistic to completely eradicate the risk of cows encountering these microalgae, the adoption of proper on-farm protocols and management, with the highest attention to hygiene measures, would be beneficial in reducing the magnitude of this problem. Thus, keeping the attention focused on early diagnosis, together with the development of new, alternative, and effective agents and formulations, would be strongly advised to prevent, treat, and control *Prototheca* infections.
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