The involvement of achlorophylic algae in the etiopathogenesis of bovine mastitis in a Transylvania area

Laurent Ognean¹*, Viorica Chiurciu², Meda M. Moldovan¹

¹ – University of Agricultural Science and Veterinary Medicine, Faculty of Veterinary Medicine, Department of Animal Physiology, Calea Manastur 3-5, 400372 Cluj-Napoca, Romania.
Correspondence: Tel. +40264596384, Fax +40264593792, E-mail lognean@yahoo.com

Abstract. The algae from Prototheca genus are unicellular achlorophylic microorganisms, demonstrated pathogens for humans and animals. Besides their involvement in the ethiopathogenesis of bovine mastitis, as it is widely accepted (P. zopfii, P. wicherhami, P. moriformis, P. trispora), there are already sufficient arguments about the occurrence of several forms of Prototheca infections in different animal species (skin, eye, enteric, renal and systemic in dog, systemic in rabbits, mice, rats, pigs and deer, cutaneous and systemic in humans).

Normal (n=440) and mastitic (n=524) milk samples were collected from 10 farms with lactating cows, situated in the central area of Transylvania. Milk samples were subjected to specific microbiologic investigations. It was isolated a complex microflora, including achlorophylic algae, bacteria and fungi. The algae rapidly developed on agar and Sabouraud medium, at 20-37°C, forming rich gray-white colonies. Microscopic morphological features of certain taxonomic value for Prototheca have been identified, such as the presence of large sporangia (10-30 µm) containing 4-8 daughter cells. Prototheca spp was isolated in 1.36% (X/440) normal milk sample from 3 farms (1.21 to 5.25%) and in 5.15% (X/524) mastitic milk sample from 2 farms (9.65% and 9.37%). In farms with mastitis cases, it was noticed an increase resistance to antibiotic therapy.

Keywords: Healthy and pathologic milk; Unicellular algae; Protothecosis mastitis.

Introduction

Misuse of antibiotics in mastitis therapy without the confirmation of an etiological diagnosis, based only on the reason that this disease is often produced by well-known major pathogens (staphylococci, streptococci, colibacillus etc.) are the main factors that reduce the importance of so-called rare microorganisms in the etiopathogenesis of bovine mastitis.

In the group of rare achlorophylic microorganisms are included the algae from the Prototheca genus, along with fungi, mycoplasmas, mycobacteria or leptospires (Ognean, 1997). The pathogenicity of these microorganisms needs to be associated with
the increased frequency of complicated mastitis cases that are resistant to most antibiotics, requiring clarification of the involved etiologic agents and the use of effective therapeutic solution (Aalbek et al., 1994; Ognean, 1997).

During the reports about this new taxon was shown that the unicellular algae known as "parasitic fungus", originally recovered from bark of trees, were later identified as Prototheca spp strains, disseminated in the variety of environmental sources: plants, soil, mud, streams, stagnant ponds, marine waters, cattle drinking water, bovine and pig faeces, sludge and barns (Anderson et al., 1988).

The first records about the pathogenic effects of these algae were made in 1952 by Lerche; this first case was represented by a cow, with decreased milk production and the presence of a watery secretion with white flakes.

At present it is widely accepted that mastitis caused by Prototheca spp is highly unresponsive to most treatment protocols, causing significant financial losses when it manifests in herds of lactating cows (Bodenhoff et al., 1978; Frank et al., 1969; Hodges et al., 1969).

The widespread of Prototheca spp in farms environment and their frequent dissemination by milk could be correlated with a possible endogenous source, given by the multiplication of these algae in the mammary structures (Aalbek et al., 1994; Bergann et al., 1993; Jensen et al., 1994; Tarte et al., 1992).

The high frequency of bovine mastitis in some Transylvanian farms, unresponsive to antibiotic treatments and correlated to consulted data, was the base to initiate this study. Thus our objective was to diversify the microbiologic investigations to establish the prevalence of unicellular achlorophyllic algae in the population of the microbial micro flora isolated from normal and mastitic milk.

Material and methods

Samples

The frequency of these organisms was monitored on occasion in some research focused on controlling the health of the mammary gland in 10 herds of lactating cows, research carried out in the form of regular surveys associated with milk sampling for microbiological examinations.

During the investigations, each udder quarter was examined by clinical methods and by using the Californian test (CMT), differentiating healthy from mastitic quarters. The next step consisted in collecting milk samples for microbiological investigations, under strict aseptic conditions (Ognean, 1997) resulting in the formation of two groups: one with normal milk samples (n=440) and one with pathologic milk samples (n=524).

The two combined included 964 milk samples, from 6 dairy farms and 4 small farms of lactating cows from the central area of Transylvania, samples that underwent a series of bacteriological and mycological investigations to isolate and identify the main taxon components of normal and pathological milk micro flora.

The bacteriologic exam included culture in bouillon and simple agar and the identification of the main morphological features of the obtained cultures, respectively their classification by type of colonies. Identification of some bacterial species and genres required the use of selective media, specific biochemical investigations and the application of some procedures, already tested in a previous research (Ognean, 1997).

Mycological examination also followed an experimental protocol based on specific investigations and standardized tests for typing fungi (Ognean, 1997). After culturing, milk samples were homogenized and centrifuged at 3,000 rotation/minute for 30 minutes and from the resulted deposit were made native preparations and smears which were stained and examined microscopically.
In the case of native preparations we used a vital staining method with blue toluidine and for smear straining we used Newman (with methylene blue), Gram, May Grunwald Giemsa (MGG) and Ihone methods (Ognean and Rotaru, 1998).

The identification of typical morphological features of the genus *Prototheca* at the microscopic examination of native preparations and stained smears, from the milk deposit and/or grown cultures, was the base for the identification of the algae without chlorophyll.

**Results**

The performed microbiological investigations have revealed complex aspects regarding the structure of the isolated micro flora from normal and mastitic milk samples, among which also those related to bacterial and fungal components that are not covered by this study, but are found in the context of some explanations. Microbiological investigations of milk samples taken from healthy and mastitis cows revealed a *Prototheca* spp carrying normal milk of 1.36% and a prevalence of these algae in the pathologic milk of 5.15%. Distribution of positive samples in farms showed varied percentages, homogeneous and slightly lower in the case of healthy milk samples (1.21 to 5.25) compared with those recorded for pathological milk samples (0.89 to 9.65) (table 1).

The analysis of this representation shows the predominance of positive cases with *Prototheca* spp. (5.15%) in the samples from infected quarters, respectively pathological milk.

Most prototheca mastitis cases were diagnosed in 2 of the investigated farms (9.65% and 9.37%), in which the clinical mastitis cases resistant to antibiotic therapy also predominated.

Presence of these algae was also observed in other 3 farms, but with lower frequency (0.89-3.4%). In a half of the investigated farms, the algae were not found in normal or pathologic milk micro flora (table 1). Milk secretion from quarters with clinical prototheca mastitis, confirmed by culture and microscopic examinations, was significantly reduced and presented important organoleptic changes, the aqueous feature and rich content of white-grey flakes being relevant (figure 1).

The examination of the culture revealed an abundant growth of the algae on the usual microbiologic medium, especially on agar-agar and Sabouraud agar. The colonies developed very well on solid mediums, at 20-37°C, after 24-48 hours after culturing, presenting similar morpho-cultural features with those of yeast cells, variable size and a gray-withe colour (figure 2).

Microscopic examination of native preparations and stained smears, performed from milk deposit and culture mass, showed the presence of some large (10-30 µm), vegetative formations (sporangia), containing 4-8 daughter cells. These specific structures were easy recognised in vital preparations and also on the stained smears and so, microscopic investigations provided clear morphological details when using vital preparations and/or panoptic stain.

For this reason we considered relevant the details observed in the vital preparations, which highlighted the morphologic elements representative for the identification of *Prototheca* genus algae. As shown in the figure 3, these cellular structures have a round or oval shape, encasing more round elements with endosporic aspects or daughter cells, which need to be differentiated from certain atypical cells present in milk, as the lipophaghe character macrophages (figure 4).

The statistical analysis of the individual and average data obtained at microbiological investigation of 440 milk samples collected from healthy mammary quarters and 524 from mastitis mammary quarters revealed the presence of the achlorophilic algae from *Prototheca* genus in 6 respectively 27 cases, showing a low carrying level for normal milk (1.36%) and relative low for the pathologic milk (5.15%) (figure 5).
Table 1. The frequency of algae identification from *Prototheca* genus in normal and mastitic milk, collected from 8 dairy cow farms from Transilvania

<table>
<thead>
<tr>
<th>Farm</th>
<th>Investigated samples</th>
<th>Positive samples</th>
<th>Investigated samples</th>
<th>Positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nr</td>
<td>nr</td>
<td>%</td>
<td>nr</td>
</tr>
<tr>
<td>1</td>
<td>88</td>
<td>2</td>
<td>2,27</td>
<td>145</td>
</tr>
<tr>
<td>2</td>
<td>74</td>
<td>-</td>
<td>-</td>
<td>112</td>
</tr>
<tr>
<td>3</td>
<td>68</td>
<td>-</td>
<td>-</td>
<td>73</td>
</tr>
<tr>
<td>4</td>
<td>82</td>
<td>1</td>
<td>1,21</td>
<td>52</td>
</tr>
<tr>
<td>5</td>
<td>47</td>
<td>-</td>
<td>-</td>
<td>45</td>
</tr>
<tr>
<td>6</td>
<td>22</td>
<td>-</td>
<td>-</td>
<td>24</td>
</tr>
<tr>
<td>7</td>
<td>16</td>
<td>-</td>
<td>-</td>
<td>16</td>
</tr>
<tr>
<td>8</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>15</td>
</tr>
<tr>
<td>9</td>
<td>55</td>
<td>3</td>
<td>5,45</td>
<td>96</td>
</tr>
<tr>
<td>10</td>
<td>28</td>
<td>-</td>
<td>-</td>
<td>42</td>
</tr>
<tr>
<td>Total</td>
<td>440</td>
<td>6</td>
<td>1,36</td>
<td>524</td>
</tr>
</tbody>
</table>

Figure 1. Watery character with grey-white flakes in a sample of milk, intensely positive at culture and microscopic examinations for *Prototheca* spp. Figure 2. The morphologic aspect of the *Prototheca* spp. colonies and the abundant grown on Sabouraud agar at 5 days after culturing. Figure 3. Mastitis milk containing cells with *Prototheca* spp. Endospores (C-e), along with many fat sferules and cellular debris (vital staining x 200). Figure 4. Healthy milk, containing fat sferules (g) and a metaplasic monocyte-macrophage in a lipophage (M-I) (vital staining x 400).
Discussions

The results of our study along with other actual research denote the extinction of the pathogenic *Prototheca* strains on the mammary gland, protothecosis mastitis being identified in various geographic areas: USA, Australia, Denmark, Israel, Canada (Aalbek, 1994; Enders et al., 1992; Dion, 1979).

Regarding the overall composition of the isolated microflora from mastitic milk, our results are comparable with those obtained by different researchers in the field, which proved that the polymicrobial associations in milk secretions are predominant (Aalbek et al., 1994; Jensen et al., 1994; Cox et al., 1974). In this context, the analysis of the obtained data revealed the frequent association of bacteria and fungi and/or with unicellular algae. In this regard we mention only the frequency of the main bacterial and fungal taxa which were isolated: staphylococci (27.1%), streptococci (16.3%), micrococcus (13.0%), diplococci (14.1%), bacilli Gram positive (5.4%) and negative bacteria (3.2%), saprophytic * Bacillus* species (4.3%), mixed bacterial flora (11.9%), nonspecific bacterial flora (4.3%), *C. tropicalis* (20.8%), *C.rugosa* (8.5%), Calbicans (8.0%), *C.krusei* (6.9%) and and other yeasts species.

Concerning the isolation and the identification of the achlorophylic algae and their inclusion in *Prototheca* genus, we only punctuate the simplicity and ease of procedures, with the abundant grown of the cultures on the microbiologic usual media, especially on agar. The microscopic examination of vital preparations and stained smears, performed from milk deposit and/or cultures also has particular relevance for the identification of morphologic elements representative for this genus.

In the last decades concerns about milk microflora diversity, including these algae and fungi have increased considerably (Aalbek et al., 1994; Higgins et al., 1989; Jensen et al., 1994; McDonald et al., 1984; Pore et al., 1987; Tarte et al., 1992). Thus, to argue the increasing spread and involvement in the etiopathogenesis of cow mastitis of these unicellular algae we present a summary of the research developments in the field. All consulted data show that first remark about the pathogenicity of the *Prototheca* genus algae was carried out by Lerche (1952) and consisted in a case description of a cow with mastitis caused by *P. zopfii*, in Germany. In fact this notification also represented the first information about the occurrence of some infection with achlorophylic algae in mammals. Based on the results obtained by Lerche, in 1968 Schiefer and Gedek manage to reproduce experimentally the mammary infection with *P. moriformis* in cows and noted that two days...
after infection, mastitis symptoms with purulent character have triggered.

After 25 years from the first notification, it starts to appear convincing information about the etiopathogenetic importance of these algae in bovine mammary pathology. Thus, it prove to be relevant the results of some extensive investigations performed by Schönborn and Seffner in 1977 on bovine mastitis caused by *P. trispora*. These studies were completed in 1978 by Gedek and Weber, which described an enzootic episode of *Prototheca* mastitis in lactating cows. About the evolution of this episode, which ended with the sacrifice of 22 cows from 88 with the infection, it was relevant the transmission of the infection in outbreak and the resistance of the etiologic agent to usual antibiotic treatments.

Many consulted data show the current trend for diversify the research, because the increase of the pathogenic potential of *Prototheca* spp strains for various animal species.

Thus, the increase isolation of algae from the most various biological samples: sheep and horse faeces, urine of chronic nephritis dogs (Arnold et al., 1972), cattle supramammary lymph nodes (Migaki et al., 1969; Rogers, 1974), gastrointestinal tract and dog kidney (Frank et al., 1969; Smith, 1980). However we can conclude that the most *Prototheca* species with proved patogenity (*P. zopfii, P. wicherhami, P. moriformis, P. trispora*) are predominantly involved in production of cows mastitis (Bodenhoff et al., 1978; Frank et al., 1969; Hodges et al., 1969; Ognean, 1997) and rarely in the production of other infection forms in humans and animals: cutaneous, ocular, enteric, renal and systemic in dog (Buyukmihci et al., 1975; Tyler et al., 1980; Van Kruiningen, 1970); cutaneous and systemic in humans (Cox et al., 1974; Venezio et al., 1982); systemic in rabbits, mice, rats, pigs and deer (Spalton, 1985).

Conclusions

*Prototheca* genus comprise species of unicellular and achlorophyllic algae with proved pathogenicity for humans and animals, being involved mainly in the production of mastitis in lactating cows (*P. zopfii, P. wicherhami, P. moriformis, P. trispora*) and rarely other infection forms in humans and animals (cutaneous, ocular, enteric, renal and systemic in dogs, systemic infections in rabbits, mice, rats, pigs and deer, respectively cutaneous and systemic infections in humans).

The results of the microbiologic investigations performed on normal (n=440) and mastitic (n=524) milk samples, collected from 10 groups of lactating cows from the central area of Transylvania, revealed the complex structure of the isolated micro flora, which also contained achlorophyllic algae, along with bacteria and fungi.

In the group of normal milk samples from healthy mammary quarters a carrying level with *Prototheca* spp. of 1.36 % was identified, the algae being isolated only in 3 of 10 investigated farms (1.21-5.25%).

The frequency of positive *Prototheca* samples was of 5.15% in pathologic milk collected from mastitis mammary quarters, predominantly in two of the investigated farms (9.65% respectively 9.37%), where were associated with forms of clinical mastitis unresponsive to antibiotic treatments.

Algae cultivation proved to be very easy, with very good development on agar and Sabouraud agar, at 20-37°C, with the formation of rich grey-whitish colonies after 24-48 hours.

Algae identification and classification in *Prototheca* genus, was based on the morphologic elements of certain taxonomic value, a particular relevance had the identification of large sporangia (10-30 µm) containing 4-8 daughter cells by vital and panoptic stained microscopic preparations.

References


