

## SIGNALS REGARDING THE ISOLATION OF CHLOROPHYL-FREE ALGAE IN THE MILK OF SOME HEALTHY COWS AND SOME WITH MAMMITIS

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### ABSTRACT

In the microbiological investigation, made on samples of normal; (n=412) and mastitic (n=482) milk, proceeded from eight dairy cows farm, in Transilvania, it was signal the presence of the algae without chlorophyl belonging to *Prototheca*, forming together with bacteria and fungi the micro flora of the mammary gland. The alga content of normal milk was signaled only in two farms (2.27%, respectively 1.21%), being established to 0.72%. In the case of mastitic milk the percentage of positive samples raised up to 3.3%, the isolating frequency being important only in one farm (9.6%), where were episodes of antibiotic resistant mastitis. The alga were well developed on gelose and Sabouraud agar, at 20-37°C, and their identification as *Prototheca*, was based on making evident the typical morphological elements, big sporangium (10-30µm) containing 4-8 daughter cells.

**KEYWORDS:** algal isolation, bovine mastitis, *Prototheca* sp.

## ISOLATION OF ALGAE IN MILK

### DETAILED ABSTRACT

The microflora of normal milk, and especially of the mastitic one is extremely diverse, being formed by a bacterial component and of another, not so well represented component, which include so called "seldom micro-organism". In these organisms are also included without chlorophyll Algae belonging to *Prototheca* sp.

The alga were isolated using current microbiological techniques, made on samples of normal milk (n=412) and mastitic milk (n=482), prelevated from 8 cows farms, totally 1,415 lactating cows, on the bases of the results obtained at the exam of each mammary quarter before drawing, using Californian test for observing he mastitis (CMT).

The bacteriological exam included inseminations on colony and simply agar, with the determination of the main morphological characters. The identification of the bacterial species type had required the utilization of the selective medium for: Staphylococci, Streptococci (blood agar), Enterobacteria (Levine medium), *Caereus bacillus* (selective agar) and *Chorine* bacterium. By the biochemical investigations was mainly used Haemolysis Test, made by the classical technique.

The mycological exam required a succession of specific investigations, resorting to some comparative tests of fungus typification, presented in the experimental protocol. [9].

The milk samples remained after insemination, were homogenised and centrifuged at 3,000 rot/min, 30 minutes, and made native preparations and smears. This were stained by the following methods: Newman (with blue methyl), Gram, May Grunwald Giemsa (MGG) and Ihone and after were microscopically examined.

The microscopically exam of the colonies obtained in gelose and Sabouraud agar emphasized morphological elements with certain taxonomic value for *Prototheca* Family which groups many species of algae without chlorophyll. In the purpose to difference the morphological elements and pointing out the typical ones were made and comparative examination of the native preparations and smears, which were stained by MGG or Polychrome Blue methods.

The processing of the data result from our researches made on representative number of normal and mastitic samples, established that the alga content of the normal milk secretion is 0.72%, and of the pathological one is 3.31%.

In the case of mastic milk, most positive samples (9.65) are taken from a farm, where frequently were developed episodes of clinically or chronically mamitis resistant to antibiotics. In other two farms was signaled a sample per each farm of mastitic milk containing algae (0.89%, respective 1.92), the isolating frequency being lower, in the same time when in the other 5 farms was not recorded the presence of this micro-organisms.

The content of *Prototheca* algae in the normal milk, established by the investigations, has just a signal value, being present just in two farms (2.27%, respective 1.21%) by the 8-th investigated farms.

In the colony, the algae were well developed on gelose and Sabouraud agar, at 20-37°C, after 24-48 hours from insemination, forming atypical colonies, colored gray-white and variable in dimensions.

The identification and including of the algae in *Prototheca* group, was based on morphological elements with certain taxonomic value, sporangia with big dimensions (10-30µm) and containing 4-8 daughter cells, well evidenced structures in the microscopically preparations stained MGG.

**KEYWORDS:** algal isolation, bovine mastitis, *Prototheca* sp.

## INTRODUCTION

The mastitides are between the bovine illnesses with the most complex etiology, including a large number of pathogenic agents by a high taxonomy variety [6, 8, 10]. The high frequency of bacterial mammary infections, produce by important pathogenic agents (Staphylococci, Streptococci, E. coli, etc.), together with the difficulty of clearing up the etiologic diagnosis, produce the total unjustified tendency of minimization of involving some micro-organism seldom present in the etio-pathogenesis of bovine mastitis. In this group are included the *Prototheca* alga group, without chlorophyll, beside fungi, mycoplasma, leptospire, mycobacterium [9].

## MATERIAL AND METHODS

In the purpose of determination the microflora structure of normal and pathological milk secretion were made complex micro biological investigations, on a representative number of milk samples (n=894), drawn during the period 1990-1998, from 8 dairy cows farm, located in the central part of Transilvania.

By periodical investigations made on those 5 farms and 3 small farms were checked a total number of 1415 cows in lactation, from which were drawn 412 samples of normal milk (n1) and 482 samples of pathological milk (n2)

The drawing and the manipulation of the milk samples were made respecting the sterile conditions imposed by microbiological investigations.

The samples were divided into two categories: normal milk and mastitic milk, on the bases of the results obtained at the exam of each mammary quarter before drawing, using Californian test for observing the mastitis (CMT). Thus, the milk secretion samples were difference by the intensity of the reaction in negative (-), representing the normal milk and positive (+, ++, +++), corresponding to the mastitic milk.

For the isolation and identification of the main taxonomic agents components of the milk micro flora were made complex microbiological investigations, bacteriological and mycological.

The bacteriological exam included inseminations on colony and simply agar, with the determination of the main morphological characters in the cultures

obtained in the liquid and solid medium and their classification after the colony type. The identification of the bacterial species type had required the utilization of the selective medium for: Staphylococci, Streptococci (blood agar), Enterobacteria (Levine medium), *Caereus bacillus* (selective agar) and *Chorine bacterium*. By the biochemical investigations was mainly used Haemolysis Test, made by the classical technique.

The mycological exam required a succession of specific investigations, resorting to some comparative tests of fungus typification, presented in the experimental protocol for the fungus isolation and identification [9].

The milk samples remained after insemination, were homogenized and centrifuged at 3,000 rot/min, 30 minutes, and made native preparations and smears. This were stained by the following methods: Newman (with blue methyl), Gram, May Grunwald Giemsa (MGG) and Ihone and after were microscopically examined.

The isolation and identification of the *Prototheca* alga was facilitate by the abundant development on the usual microbiological medium, especially on those with agar.

The microscopically exam of the colonies obtained on gelose and Sabouraud agar emphasized morphological elements with certain taxonomic value for *Prototheca* Family which groups many species of algae without chlorophyll. In the purpose of difference the morphological elements and pointing out the typical ones, were made a comparative examination of native preparations and smears, which were stained by MGG or Polychrome blue methods.

## RESULTS AND DISCUSSION

The microbiological research made on 894 samples of milk, drawn for 8 dairy cows farm, located in the central part of Transilvania, emphasize different aspects concerning the structure of the micro flora isolated from the normal and pathological milk. Under the composition aspect, we have noticed the diversification of the milk micro flora by the presence of the algae, together with the bacteria and fungi. The frequency of the identified microbial species presented essential differences in the case of

normal and mastitic milk. In accordance with the data published by the anterior researchers [6,7,8,9] the micro flora of normal and mastitic milk is extremely complex, including different species of bacteria, fungi and algae, usually which form poly-microbial associations.

The researchers mentioned above revealed aspects about the bacterial and fungi components, reason

why in this paper we will specially referred to a component not so well known, represented by *Prototheca* algae.

The processing of the data result from our researches made on representative number of normal and mastitic samples, established that the alga content of the normal milk secretion is 0.72%, and of the pathological one is 3.31%. (Table 1)

Table 1: The identification frequency of *Prototheca* algae group in ordinary and mastitic milk processed from 8 dairy cow farms in Transilvania

Farm name	Ordinary milk samples			Mastitic milk samples		
	Investigated No	Positives No	%	Investigated No	Positives No	%
1 Researches Farm SCPCB Tg. Mures	88	2	2.27	145	14	9.65
2 Production Farm SCPCB Tg. Mures	74	-	-	112	1	0.89
3 Chintau Farm-IAS Cluj	68	-	-	73	-	-
4 SDE Farm Cluj	82	1	1.21	52	1	1.92
5 Duvana FarmSCPCB Cluj	47	-	-	45	-	-
6 Remetea Micro-Farm Mures	22	-	-	24	-	-
7 Sincrai Micro-Farm Mures	16	-	-	16	-	-
8 Sinpaul Micro-Farm Cluj	15	-	-	15	-	-
Total	412	3	0.72	482	16	3.31

In the case of mastitic milk, the majority of the positive samples (9.65) are proceeded from a farm, where frequently were developed episodes of clinical or chonical mamitis resistant to antibiotics. In other two farms were signaled samples per each farm of mastitic milk containing algae (0.89%, respectively 1.92), the isolating frequency being lower, at the same time when in the other 5 farms was not recorded the presence of this micro-organisms.

The content of *Prototheca* algae in the ordinary milk, established by the investigations, has just a signal value, being present only in two farms (2.27%, respective 1.21%) by the 8 investigated farms.

In accordance with the actual opinions unanimously expressed by the researcher in the field [3, 9, 10, 12] the micro-flora of the mammary secretion is extremely diverse, being mainly composed by poly-microbial associations. In this context can be framed the data obtained by us, which emphasized the frequent association between bacteria and fungi, sometimes together with algae. The bacterial

component of the signaled micro flora had included: Staphylococci (27.1%), Streptococci (16.3%), Micrococci (13.0), Diplococci (14.1%), Gram positive bacilli (5.4%), Gram negative bacteria (3.2%), germs belonging to *Bacillus anthracites* family (4.3%), mixed flora (11.9%) and unspecific flora (4.3%), and the mycotic component was dominated by some species of *Candida*: *C. tropicalis* (20.8%), *C. rugosa* (8.5%), *C. albicans* (8.0%) and *C. krusei* (6.9%).

In the stained smears microscopically exam, made in the milk sediment were evidenced, in the case of 3 samples, typical morphological elements for the identification of the algae belonging to *Prototheca*. This were well revealed by the MGG stain, presenting cellular structures rounded or oval in shape, which include many spherical components with aspect of endospore or daughter cells.

In the colony aspects we have noticed an abundant development of the colonies in the usually micro biological media, preferring the gelose and

Sabouraud agar. The colonies similar with the yeast colonies, with variable dimensions and gray-white color, have well developed in agar at 20-37°C, after 24-48 hours after insemination.

The microscopically exam of the native preparations and smears made from the colonies shown the presence of some forms looking like sporangia, with the big dimensions (10-30µm) and containing 4-8 daughter cells. These structures were easily evident also in native preparations and in the stained ones; in our researches we have obtained clear morphological details, using MGG stain method.

*Prototheca* Family includes many species of algae without chlorophyll with ubiquity spreading: soil, water, mud, bovine faeces etc. Their presence in the milk can also have an endogenic origin, from the mammary gland level [1, 2, 8, 11, 12].

The first signals about the algae belonging to *Prototheca* Family pathogenesis belong to Lerche, which in 1952 described in Germany a bovine mastitis case produced by *P. zopfii*. This represented, in fact, the first information about the production of alga infections in mammals [2]. Starting with the results obtained by Lerche, Schiefer and Gedef (1968) reproduced the experimental mammary infection in the cow with *P. moriformis*; the mastitis symptoms appear after two days from infection, with a purulent character.

More consequent information about the etiopathogenic importance of the algae in the bovine mastitis appears only after 25 years after the first recorded. So that Schonborn and Seffner (1977) made ample investigations in bovine mastitis produced by *P. trispora*, and in 1978 Gedek and Weber described an enzootical episode of mastitis with *Prototheca* in lactating cows. The authors emphasized the transmitting of the infection in focus and the ethiological agent resistance at usual therapies, especially with antibiotics, bringing as an argument the ending of the respective episode, which required the slaughtering of 22 cows from 88 infected.

During the last two decades it's noticed an extension of the researches concerning the pathogenic action of *Prototheca* species for the mammary gland, being signaled mammary infections in different areas: U.S.A, Australia, Denmark, Israel, Canada [1, 2, 3,

10, 11]. The results of this researches underline the invasive character of the infection, in which the vegetative forms of *Prototheca* are penetrate in alveoli, endothelium and interstice, producing important destruction and purulent granulomatoses inflammations, accompanied by hypo- or even agalactia.

Also, it is necessary to reveal the diversification of the pathogenic action given to *Prototheca* Family, signaled in other species of animals, the algae were usually isolated from faeces, as is the case of sheep and horses, or from the urine of the dogs with chronically nephritis [2]. Nowadays the following species of *Prototheca* are considered pathogenic; *P. zopfii*, *P. wicherhami*, *P. moriformis* and *P. trispora*, which are exclusively involved in the producing of the mastitis in cows [6, 9].

## CONCLUSIONS

By microbiological investigations made on a representative number of normal (n=412) and mastitic (n=482) milk samples, from eight dairy cows farm, located in the central part of Transilvania was isolated a complex micro flora containing algae, without chlorophyll belonging to *Prototheca* group, together with bacteria and fungi.

The normal milk content in *Prototheca* had just a signal value, being established to 0.72% and being present just in two farms (2.27%, respectively 1.21% by those eight.

In the case of mastitic milk, in 3.3% of the samples were identified algas, majority (9.65%) proceeded from Research Farm SCPCB Tg. Mures, in which were signaled clinical and chronically antibiotic-resistant mastitis.

In the colony, the algae were well developed on gelose and Sabouraud agar, at 20-37°C, after 24-48 hours from insemination, forming atypical colonies, colored gray-white and variable in dimensions.

The identification and including of the algae in *Prototheca* group, was based on morphological elements with certain taxonomic value, sporangia with big dimensions (10-30µm) and containing 4-8 daughter cells, well evidenced structures in the microscopically preparations stained MGG.

## REFERENCES

- [1] Aalbek B., Stenderup J., Jensen E.H., Valbak J. Nylin B., Huda A. (1994): Mycotic and Algal Bovine Mastitis in Denmark, *Acta Path. Microbiologica Immunologica Scandinavica*, 102 (6): 451-456
- [2] Bergmann A. (1993): Experimental Prototheca Mastitis in the Cow and its Treatment with Teramisol Hydrochloride. *Berliner und Munchener Tierarztlieche Wochenschrift*, 106: 257-260
- [3] Conant N.F., Smith D.T., Baker R.D., Callaraway J.L., Martin D.S. (1969): *Manual of Clinical Mycology*. W. B. Saunders and co., Philadelphia
- [4] Enders F., Weber A. (1992): Vorkommen und bedeutung von algen der gattung Prototheca bei tieren, *VET*, 2:16-21.
- [5] Jensen H.E., Aalbaek B. (1994): Pathogenity of Yeasts and Algae Isolated from Bovine Mastitis Secretions in a Murine Model. *Mycoses*, 37(3-4): 101-107
- [6] Ognean L., Spinu M., Timen S., Cozma V., Roman M., Merlescu L.; Negrea O. (1992): Investigatii etiopatogenice in diferite forme de mastite la vacile din rasa Baltata Romaneasca. *Buletin IACN - Zmv*, 46: 237-246
- [7] Ognean L., Statov C., Merlescu L., Oana L. (1993): Particularitati ale diagnosticului micologic in mastite la vaci. *Buletin USACN - ZMV*, 47: 165-173
- [8] Ognean L. (1997): *Mamitele micotice la bovine*, Ed. Casa Cărții de Știință Cluj-Napoca
- [9] Ognean L., Spanu M., Cozma V., Statov C., Borda D. (1996): Valoarea tehnicilor micologice actuale utilizate in identificarea fungilor izolati din lapte. Simpozionul "Actualitati in patologia animalelor domestice" USAMV - FMV Cluj-Napoca, 22: 90-96
- [10] Quinn P.J., Carter M.E., Markey B., Carter G.R. (1994): *Clinical Veterinary Microbiology*. M. Wolfe
- [11] Tarte K, Becerra T., Alvarez O.S., Villareal A. (1992): Mastitis Bovina Clinica causada por algas del genero Prototheca. *Veterinary Bulletin*, 62(4): 326
- [12] Ungureanu C., Minciuna V. (1983): *Afectiunile glandei mamare la vaci*. Edit. Ceres, Bucuresti

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