Survey of the occurrence of *Prototheca* spp. in Hungary

Content

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Introduction of *Prototheca* spp.

The *Prototheca* genus belongs to the class *Trebouxiophyceae*, order *Chlorellales,* and family *Chlorellaceae*, the same family of *Chlorella* sp., a green alga related to *Prototheca* sp. (1). Microscopical, achlorophyllic, unicellular, yeast-like algae of the genus *Prototheca* are ubiquitous in nature especially in humid and wet areas. These saprophyte microorganisms are responsible for infections in humans and different animal species. Human protothecosis mainly occurs in immunocompromised patients; however, in farm animals, these algae species can cause clinical and subclinical mastitis (2).

Mastitis-causing *Prototheca* species have recently proved an emerging disease worldwide; although its incidence is increasing permanently, its epidemiology remains largely understudied (3). The species responsible for bovine protothecosis include *Prototheca zopfii* and *Prototheca wickerhamii*. Recently, *Prototheca blaschkeae* has classified also as one of the pathogenic algae responsible for mastitis in dairy herds. The pathogenic role of *Prototheca* spp. in bovine mastitis firstly reported in 1952. There are two genotypes of the *Prototheca zopfii* species: *P. zopfii* genotype 1 (GT-I) and *P. zopfii* genotype 2 (GT-II) (4, 7). *Prototheca zopfii* GT-II was isolated from raw milk sample, while GT-I was isolated from an environmental sample (5). Previously known *Prototheca zopfii* genotype II has taxonomically reclassified as *Prototheca bovis* which is predominantly causative agent and responsible for bovine mastitis. While *P. zopfii* genotype I have been reclassified as *Prototheca ciferrii*. (5, 7, 8). Due to improper environmental conditions, insufficient milking hygiene, and the presence of infectious forms between dairy cows *Prototheca bovis* caused mastitis is emerging endemic worldwide (5, 8).

Prototheca spp. can induce either clinical or subclinical mastitis in dairy cows. If clinical form occurs, symptoms may include high fever (up to 40 °C), mammary gland oedema and pain, anorexia, and refusal to move. Subclinical protothecal mastitis is manifested by increased number of leukocytes in the udder and raw milk and can be accompanied by slight pain along with loss of appetite. Subclinical mastitis due to *Prototheca* algae decreases milk production and increases somatic cell count in raw milk, especially macrophages (5). *Prototheca* spp. remain long term in udder tissue, they can persist during the dry period and be excreted via milk in the next lactation (6).

The effectiveness of various anti-algae agents (*e.g.*, polypyrrole, antimicrobial peptides) is currently assessed *in vitro*. Detection of *Prototheca* spp. from milk and environmental samples is an essential element of the preventive measures and that of monitoring the efficiency of treatments. *Prototheca* spp. are mostly enumerated by culturing on selective media and identified and confirmed by light microscopy. Molecular diagnostic methods (*i.e.*, multiplex PCR and *cytb* gene identification) allow for species-level identification. The aim of this survey was to summarize the published data about prevalence of *Prototheca* spp. in Hungary (9). Over the past few decades, the spreading of microalgae of the genus *Prototheca*, has rapidly become an emerging threat for the dairy sector, mainly because nowadays, there is still no treatment effective towards these algae (11).

National legislation and regulation of Prototheca algae in Hungary and the EU

By this time there are no existing rules and provisions for examination of *Prototheca* algae in Hungary and the EU.

In Hungary *Prototheca* algae are responsible for bovine mastitis. These species primarily controlled and examined under general dairy hygiene and animal health regulations, with no specific national, species-level legislation targeting species (e.g. *Prototheca bovis*, *Prototheca ciferrii)*.

Infection of dairy cows with *Prototheca* spp. is increasing worldwide. *Prototheca bovis* mostly causes subclinical mastitis and increased somatic cell counts in milk. Since members of the *Prototheca* genus are resistant to most antimicrobials, culling and slaughtering of infected cows are currently the only solution to the problem (9). However, this results in significant production losses and economic damage in livestock.

As a contagious, antibiotic-resistant pathogen is treated within the framework of mandatory mastitis control, requiring infected, uncurable cows to be culled.

Prototheca genus is not classified as a notifiable disease (e.g., unlike foot-and-mouth disease or brucellosis).

Therefore, there is no mandatory *Prototheca* spp. eradication program and there is no obligation to register in case of *Prototheca* infection.

Authorities involved in legislation and investigation, national control bodies (who they are and what liability they incur)

In Hungary, several organizations are responsible for the classification and inspection of raw milk, ensuring compliance with quality requirements from production through processing.

The key stakeholders are the following.

Hungarian Dairy Research Institute Ltd. (MTKI Kft.)

The HDRI Ltd. is the central body for raw milk classification and certification, which tests samples in its accredited laboratory (total bacterial count, somatic cell count, inhibitory substances, fat content, protein content, lactose content).
Raw milk produced in Hungary for human consumption and processing is examined and qualified by the Raw Milk Classification Laboratory of HDRI Ltd. according to the requirements of Decree 16/2008 (15 February) FVM SZMM. The determination of fat, protein, lactose and SNF content of raw milk samples was carried out by Fourier transform mid-infrared spectroscopy (FT-MIR) method and the determination of Total Bacteria Count and Somatic Cell Count by the flow cytometry method according to the ISO 9622:2013 (IDF 141:2013) standard and according to the requirements of Annex III to Directive 3-2-1/2004 of the Codex Alimentarius Hungaricus in the 'Examination' chapter (2013) (10).
The Research and Development and Innovation Division of HDRI Ltd. has already conducted research in the topic of determining *Prototheca* from raw milk samples.

University of Veterinary Medicine Budapest (UVMB): Raw milk samples are tested by the University of Veterinary Medicine Budapest for *Prototheca zopfii*.
Table 1 shows the *Prototheca*-related microbiology laboratory services. Unfortunately, the examination method (whether growth on agar media or real-time PCR test is carried out) is not mentioned on the website (13).

Table 1: *Prototheca*-related laboratory services of the University of Veterinary Medicine Budapest

Tested Sample	**Tested Pathogen(s)**	**Price (HUF) + VAT**
Raw milk	*Prototheca zopfii* screening test	400
Raw milk	*Staphylococcus aureus* and *Prototheca zopfii* screening test	550
Raw milk	Standard microbiology test (includes *Staphylococcus aureus* and *Prototheca zopfii)*	650

(Source: 13)

HDRI Ltd. and UVMB had a joint research cooperation agreement regarding the nationwide *Prototheca* testing program. The Research and Development and Innovation Division of HDRI Ltd. has also prepared a detailed financial calculation that included screening tests (microbiological growth tests on selective agar media and real-time PCR tests as well) for

Prototheca algae at each dairy farms in Hungary. Unfortunately, due to a lack of subsidy, the joint research project was not implemented.

National Food Chain Safety Office (Nébih): Monitors compliance with food chain safety regulations and participates in the data reporting system.

Milk Interprofessional Organization and Product Board (Tej Szakmaközi Szervezet és Terméktanács): The creation and operation of the Milk Interprofessional Organization and Product Board and the sectoral self-regulatory activity implemented through it could facilitate the domestic sectoral interest conciliation and significantly contribute to the professional and legislative activities of the government. This interprofessional organization is a forum for domestic sectoral interest conciliation, it brings the producers, processors and traders closer together. The Milk Product Board was created in 1992. Recognized by the Minister of Agriculture, the Milk Product Board has been acting as a representative interbranch organization since 2013. Within the domestic dairy industry, the Milk Interprofessional Organization and Product Board accounts for 72% coverage in respect of raw material production (sales), almost 95% in respect of purchased milk while it has 70% coverage in respect of retail (14).

Animal Production Performance Testing Ltd. (ÁT Kft.): Plays a role in the certification of raw milk, particularly regarding compliance with food chain safety regulations.

Independent Accredited Laboratories: Dairy farmers / Enterprises who produce or purchase raw milk must have their milk tested at an accredited laboratory at least twice a month as part of their self-monitoring program.

The above-mentioned organization could take responsibility for *Prototheca* testing in Hungary.

National statistics prevalence, time-series data—where available—for trend analysis, etc.

Mastitis caused by *Prototheca* spp. results in significant economic losses, and based on available data, it is becoming increasingly common in domestic dairy herds. The pathogen first appeared in Hungary around 1998–1999; Szilárd Jánosi and colleagues were the first who reported the algal infection at three Hungarian dairy farms (12).

National statistics are only available at the website of Animal Production Performance Testing Ltd. Gödöllő, Hungary. This company inform monthly their partners about the presence of *Prototheca* spp. in raw milk samples. **Figure 1** shows the prevalence of *Prototheca* sp. in raw milk samples between 2025 and 2026.

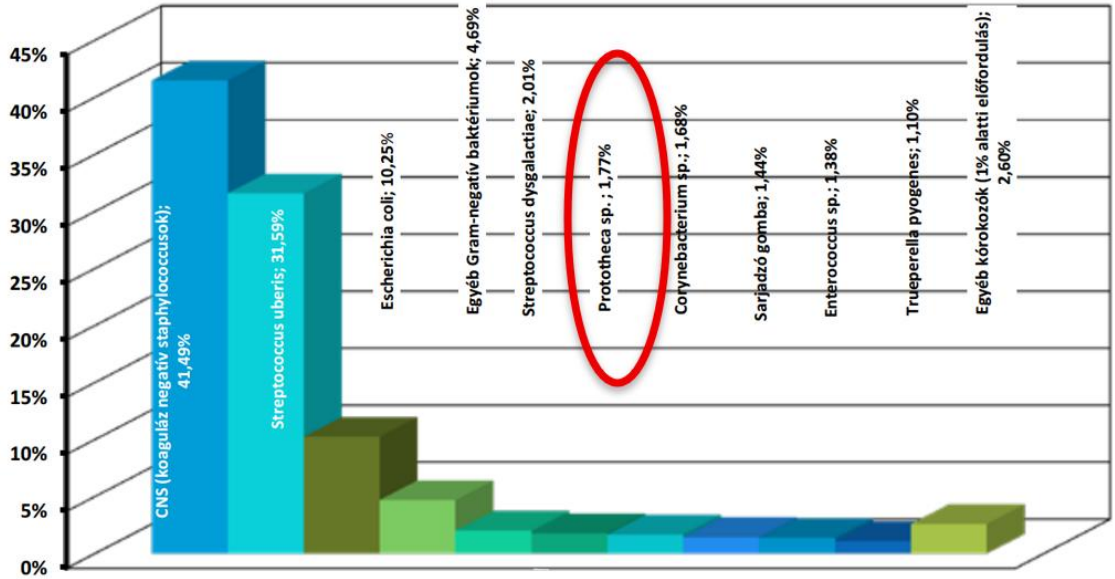

Fig. 1. Annual proportion of the major classes of pathogen microorganisms (percentages) isolated from raw milk samples during comprehensive microbial analysis.

Measurements were carried out by Animal Production Performance Testing Ltd. in Hungary. Milk samples were tested between March 1, 2025, and February 28, 2026 (15).

Prototheca alga was the sixth frequent pathogen in raw milk samples. 1,77% of the tested samples were positive. The examination method is not mentioned in this newsletter.

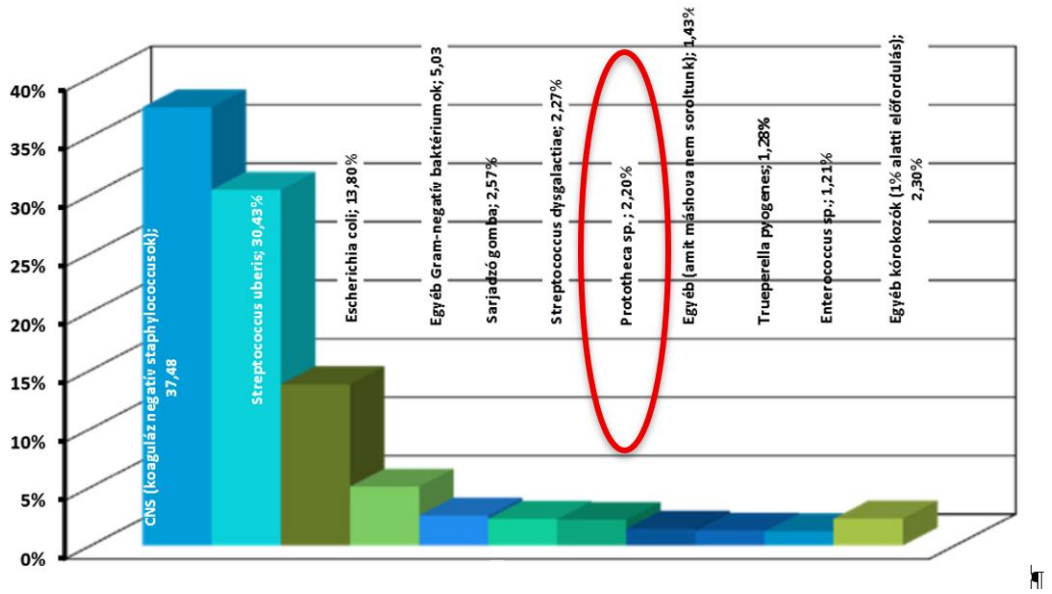

Fig. 2. Annual proportion of the major classes of pathogen microorganisms (percentages) isolated from raw milk samples during comprehensive microbial analysis.

Figure 2 represent the result of the measurements were carried out by Animal Production Performance Testing Ltd. in Hungary. Milk samples were tested between May 1, 2024, and April 30, 2025. (16).

Prototheca alga was the seventh frequent pathogen in raw milk samples. 2,20% of the tested samples were positive. The examination method is not mentioned in this newsletter.

The results of raw milk microbiological tests conducted by the microbiology laboratory of the Department of Animal Hygiene and Animal Health and the Mobile Clinic at the University of Veterinary Medicine Budapest have consistently identified the algae as the fourth or fifth most common udder pathogen between 2013 and 2019 (21). Results are presented in **Table 2.**

Table 2 Prevalence of *Prototheca zopfii* alga in Hungarian raw milk samples

Year	Ranking	Percentage of prevalence (%)
2019	4.	5,5%
2018	5.	4,5%
2017	5.	3,4%
2016	5.	3,3%
2015	4.	7,3%
2014	4.	7,1%
2013	4.	7,8%

Note: In recent years, the three most common pathogens have typically been Coagulase Negative staphylococci, *Streptococcus uberis* and *Escherichia coli* in Hungary (21).

Previous research and research findings (list of relevant research activities or publications, key findings)

Introduction

It has long been known and widely accepted among professionals that high-quality and healthy milk can only be produced by healthy dairy cows. Milk being a pivotal dietary staple, offers a multitude of nutrient substances to human health and it is produced primarily in dairy herds in Hungary. A characteristic of the dairy cattle sector is that producers maintain relatively large herds, which is why a modern, preventive approach to herd health is of emphasized importance (6, 21).

Mastitis is a significant disease among animal illnesses; it belongs to the category of so-called multifactorial diseases, as the development of the disease involves not only the presence of pathogens but also the animal's immune system and the environment—including its microbial load and climatic characteristics—which play a significant role (6, 21).

In case of bovine mastitis, even in its subclinical form, the number of somatic cell count in the milk emerges above the admissible limit; these cells are primarily various types of white blood cells derived from the blood (6, 21).

The increased somatic cell count is the result of the body's natural self-defence mechanism. The more serious the mastitis is, the higher the somatic cell count is present in the milk, which also indicates the severity of the infection. Milk secreted from udders affected by mastitis is not suitable for human consumption, and in the absence of effective pasteurization technology, it is also unsuitable for feeding calves due to the presence of pathogenic microorganisms.

Additionally bovine mastitis reduces the fat-, casein-, calcium- and potassium content of raw milk, which is detrimental from both a nutritional and processing standpoint (6, 21).

In healthy milk the somatic cell count (SCC) is less than 400.000 cells per mL, although in the presence of infectious agents, especially pathogenic bacteria, SCC can be extremely high. Cytological appearance of inflammation in mammary gland depends on the origin and the time-course of the inflammatory response. According to predominant cell type there can be suppurative, pyogranulomatous, granulomatous and lymphocytic inflammation. *Prototheca* spp. as facultative pathogen creates chronic inflammation in mammary gland with high SCC and reduced milk production (6).

Mastitis causing pathogens are mostly typically bacteria, which explains why the disease is primarily treated with antibiotics. However, mastitis caused by achlorophyllated unicellular algae (*Prototheca* species) is spreading rapidly worldwide, including Hungary. Mastitis caused by *Prototheca* spp. infection generally does not present with distinct symptoms; in most cases it occurs only in a subclinical form, although an acute form is also known. The pathogenic algae are resistant to the most antimicrobial agents. Routine mastitis treatment is often initiated with some type of antibiotic without preliminary laboratory diagnosis of the causative agent or resistance testing. Most cases the used antibiotics are ineffective that is why treatments are often repeated, antibiotic use in the affected herds can be quite significant (18, 21).

However, this is completely inconsistent with current professional efforts, as the European Union's top strategic priority is to reduce the use of antibiotics in food-producing livestock. This is clearly driven by efforts to reduce the development of antibiotic resistance, which poses an increasing threat to humanity (18, 21).

Due to these efforts, both food producers and consumers are increasingly preferring antibiotic-free foods, which poses a serious challenge for livestock farmers, who must therefore seek alternative solutions. However, such solutions are currently lacking. It is also worth noting that the ecological footprint of industrial livestock farms (especially dairy herds) could be reduced through optimized use of medications (18, 21).

The first occurrence of Prototheca mastitis in Hungary - Preliminary results

In Hungary, no cases of algal mastitis had reported until 1998-1999. Firstly, in the years 1998-1999 in 32 large-scale dairy herds altogether 223 cases of bovine mastitis by *Prototheca zopfii* were identified by Szilárd Jánosi (17). In the following years algae were isolated from several cows in more than 50 dairy herds. The ratio of algal mastitis increased from 2 % to 4.5 % in years 1999 and 2001, respectively. Each of these dairy farms were in Hungary, at a continental type, temperate zone climate. Both the sporadic and epidemic forms of *P. zopfii* mastitis were observed. Three of these dairy herds with a high incidence of algal mastitis were closely studied (farm survey). All the herds affected by the epidemic form had poor hygienic conditions and suffered from several managerial faults, but no specific predisposing factors could be identified. The *P. zopfii* infection usually resulted in a chronic subclinical, or a mild clinical, inflammatory process in the udder followed by a dramatic loss in milk production and permanent increase in somatic cell count. The histopathological findings could be characterized, as a progressive interstitial mastitis associated with alveolar atrophy. The self-recovery rate was very low. *Prototheca zopfii* is concluded as a common mastitis pathogen of the dairy cows in Hungary as well (17).
Mastitis caused by *Prototheca* spp. leads to significant economic losses, and based on the available data, it is likely becoming increasingly frequent in Hungarian dairy herds.
Treatment of *Prototheca* spp. caused mastitis with conventional antibiotics is not possible, so in the absence of effective treatment, otherwise valuable animals must be culled.
An urgent task is the development of an effective treatment protocol, which includes prevention strategies based on an understanding of predisposing factors, as well as the development of effective veterinary pharmaceuticals (21).

Unfortunately, Hungarian Dairy Research Institute Ltd. does not have reliable data that would allow us to determine the exact prevalence of the disease caused by *Prototheca* spp. nationwide. Nowadays, the available data come from diagnostic laboratories (e.g. Animal Production Performance Testing Ltd. (ÁT Kft.) whose primary purpose is to assist veterinarians in their diagnostic work by analysing the samples they receive, rather than to assess the national prevalence of the disease. The samples they examine typically come from animals suspected of having some form of udder disease; therefore, the prevalence determined based on these tests cannot be considered as a representative national average incidence rate (9, 21).

Mastitis management – udder health

At first sight, the frequency of *Prototheca* algae occurrence in milk samples may seem low; however, the pathogen causes very serious economic losses in infected herds.
The role of the algae pathogen can range from sporadic infections in affected farms to herd-wide infection (18).
If these pathogenic algae are not detected early, or if the necessary measurements are not taken, up to 30–40% of the dairy herd may become infected. The *Prototheca* spp. caused mastitis can manifest in both clinical and subclinical forms.

The symptoms observed in clinical mastitis are not usable for diagnostic purposes; therefore, infected animals cannot be identified based on these symptoms alone.
However, it may be indicative that routine antibiotic udder treatments are ineffective, as the inflammation persists in a subclinical form for a long time after the clinical symptoms may have subsided, with occasional clinical flare-ups (18).
After a certain period, the affected quarter of udder often begins to atrophy, milk production decreases significantly, and eventually this quarter may come dry completely.
Currently, there is no registered medication on the market that is effective against the algae and approved for use in food-producing animals for the treatment of protothecal mastitis. Therefore, infected animals are isolated and then culled, which significantly increases the economic losses caused by the algae, just as do the completely unnecessary and ineffective udder treatments that are often administered over a prolonged period.
In 2011 Kovács and Ózsvári investigated infection of *Prototheca zopfii* in dairy herd which consisted of 600 Holstein Friesian cows.
At this studied farm economical losses caused by *P. zopfii* mastitis were calculated nearly 25 million HUF (89,000 EUR) annually. The annual loss per cow averages more than 41,000 HUF (151 EUR)! It is worth noting that the clearly identifiable cost of treatment accounted for only10.4% of the total losses (18).

Table 3 Changes is milk production and composition of control (healthy) and the *Prototheca* zopfii- positive groups.

Group	**Number of cows**	**Average milk production (kg per day)**	**Average somatic cell count (cells per mL)**	**Average fat content (%)**	**Average protein content (%)**	**Average lactose content (%)**
Control / Healthy	376	23,93	476.000	3,84	3,50	4,74
P. zopfii -positive	152	21,49	1.495.000	3,78	3,54	5,5

As **Table 3** shows the *Prototheca zopfii* infection rate was 28,78 % at this studied dairy herd (n =528). It is clear, that the average daily milk production of *P. zopfii*-positive cows decreased by an average of 2.44 kg/day compared to the healthy dairy cows.
When comparing the average somatic cell count of the two groups, a significant increase could observed in *P. zopfii*-positive animals (**nearly one and a half million**), although the average SCC of *P. zopfii*-negative animals was also about half a million cells per mL. At the same time, it is interesting that the sugar content of the milk produced by infected cows was significantly higher, by 0.76 percentage, than that measured in the cows of the control group. There was no significant difference in milk fat and milk protein content. These results clearly demonstrated the pathogen's impact on milk quality and production levels (18).

Occurrence of Prototheca spp. in the environment

Prototheca zopfii genotype I and II (*P. ciferrii and P. bovis*) were the most frequently detectable species in the environment of dairy farms. From drinking water, watering troughs, feed, manure, and mud could be detected *Prototheca*-positive environmental samples. Wet feeds rich in starch and oligosaccharides, such as potato pulp, may also serve as a medium for *Prototheca* spp. (19). In milking equipment, in its pipelines, on teat cup rubbers can even survive these microalgae even if routine disinfection procedure with chlorine solution is completed (19).
Marques et al. (2010) demonstrated that only heat treatment at 100°C for 1 second destroyed the mastitis-causing species *Prototheca bovis* (formerly *P. zopfii* genotype II) and *P. blaschkeae*

with 100% efficacy. During pasteurization at 75°C for 20 seconds, 12% of *P. bovis* algal cells and 23% of *P. blaschkeae* cells remained viable. Based on the above results, monitoring the spread of *Prototheca* species is recommended during both milk production and milk processing (20).

Spreading of Prototheca spp. infection

The *Prototheca zopfii* alga can be found in many places in the dairy farms. Moreover, it can survive there for extended periods. Results showed that *Prototheca* spp. were able to survive for months in milk, water, and the environment, even at temperatures as low as -23 °C or under dry conditions. For this reason, new udder infections must be constantly anticipated in the barn environment, especially when the teat canal is not fully closed, e.g., immediately after milking and the days following dry-off, as well as around the time of calving. Similarly, deficiencies in the hygienic and thorough preparation of the udder during milking can play a significant role (19).

Recommendation for national (and V4-level) legislation and/or oversight, as well as research areas.

Over the past two decades, increased attention has been paid to the genus *Prototheca*, a yeast-like alga whose frequency of occurrence is increasing. The spread of *Prototheca* spp. is a major concern for the dairy industry, primarily due to the complexity of diagnosis and the lack of effective treatment methods (22).

HDRI Ltd. recommend these solutions for the elimination and detection of this emerging mastitis pathogen microalgae:

1. **Review the available data, results, examination methods of *Prototheca* spp.**

Researcher of HDRI Ltd. will review the Hungarian results about *Prototheca* spp. infection in dairy herds. A detailed list will be prepared about researcher in Hungary who has published scientific articles about *Prototheca* spp. According to the available results HDRI Ltd. is about to mark a map about the dairy farms where *Prototheca* spp. examination has already been conducted. Focus will be kept on the examination methods, sample matrixes, sampling methods. The aim of this first point is to prepare data for a meta-analysis and discover the most problematic dairy herds in Hungary.

2. **Development of examination methods**

HDRI Ltd. has already examined raw milk samples for the presence of *Prototheca* spp. with two different methods. The first method involved the selective enumeration of *Prototheca* species on Prototheca Isolation Medium. The PIM agar as a selective and differentiating culture medium has been proved to be appropriate diagnostic tool in the detection of *Prototheca* spp. from raw milk samples. The disadvantage of this culturing method is the long incubation time and one week required to report results (9).

The second method encompasses a faster, reliable and more accurate molecular diagnostic method real-time Polymerase Chain Reaction (real-time PCR).

Results can be confirmed by microscopically assessment, however the specificity and sensitivity values of is much lower than culturing, but it could help with the identification of inoperative microbes.

The new methods should be introduced in the routine laboratory practice. Most of the laboratories are testing the presence of *Prototheca* genus by non-accredited methods. The aim is to obtain the accredited status of the *Prototheca* detection methods.

There are commercially available rapid, high-sensitivity real-time PCR diagnostic tool which is designed to identify major mastitis-causing bacteria and *Prototheca* spp. in bovine milk within 3 hours.

A multiplex PCR system (m-PCR) has been developed by Vasco-Julio et al (23) to accurately differentiate the five most important pathogenic *Prototheca* species, including the three species associated with infection in dairy cattle (*P. ciferrii, P. blaschkeae*, and *P. bovis*) and the two species responsible for human infections (*P. wickerhamii* and *P. cutis*). The method is low-cost since it employs a simple "heat-shock" method in a TE buffer for DNA extraction. Furthermore, it requires only primers, a Taq polymerase, an agarose gel, and a molecular weight marker for identification. The method was based on published *Prototheca* cytochrome B sequences and was evaluated using reference strains from each of the five *Prototheca* species. The validity of the method was confirmed by identifying 50 strains isolated from milk samples. The specificity was tested *in silico* and with experimental PCR trials, showing no cross-reactions with other *Prototheca* species, as well as with bacteria, fungi, cows, algae, animals, or humans. The method could detect mixed infections involving two or three *Prototheca* species, providing a rapid test that delivers results within three hours (23).

3. **In any cases when somatic cell counts is above 400.000 per mL in raw milk sample, it should be immediately tested for *Prototheca* spp.**

Nationally recommended to determine the alarm level of somatic cell counts (cells per mL) in raw milk samples. Above the permissible limit of 400.000 SCC /mL it is suggested to examine the *Prototheca* spp. content from each raw milk sample.

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