R-MC-3: Prototheca, Yeast, and *Bacillus* as a Cause of Mastitis

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Introduction

Mastitis in dairy herds can usually be brought under control by following the basic Program of the National Mastitis Council. Two points of this Program involve the use of antibiotics, one to treat clinical mastitis and the other to treat cows at the beginning of the nonlactating period. Antibiotic therapy, without identifying the mastitis causing organisms, is frequently the veterinarian and dairy farmer's first choice of treatment for infected cows. Cases of mastitis that are refractory to any type of treatment occur frequently also (21, 45). Very often, at the Quality Milk Promotion Services (QMPS) laboratories, bacteriological culture of milk samples from those "odd cases of mastitis" yield *Mycoplasma bovis, Actinomyces pyogenes, Streptococcus canis* (Group G *Streptococcus*), *Prototheca* or yeast. Furthermore, some milk samples may culture only Gram-positive *Bacillus* sp. The objective of this article is to discuss published material and provide new information obtained at the QMPS concerning mastitis caused by *Prototheca* sp, yeast, and *Bacillus* sp.

Prototheca mastitis

Prototheca sp are achlorophylic unicellular algae that cause mammary infections in cows (3, 10-12, 15, 16, 22-24, 29, 33, 36, 45), cutaneous, ocular, enteric and systemic infections in dogs (7, 40, 42), cutaneous and systemic infections in humans (9, 44), renal granulomatous disease in salmon parr (35), and systemic infections in rabbits, mice, rats, pigs and deer also (36). They have been isolated from bovine tissues such as lymph nodes (25, 30), supramammary lymph nodes and right horn uterus (12), gastrointestinal tract (38) and kidney (13).

Originally recovered from slime on trees, *Prototheca* sp have subsequently been isolated from a variety of environmental sources, including plants, soil, mud, streams, stagnant ponds, marine waters, cattle drinking water, bovine and pig faeces, sludge, barns and floor of a freestall barn (2, 3, 33, 28). In 1952, Lerche reported the first case in a cow that had decreased milk production and a thin watery secretion with white flakes (22). Protothecal mastitis has been unresponsive to treatment resulting in major financial losses (5, 12, 13, 16, 23, 45).

Prototheca grows on blood agar (24-36 hours) at 37°C yielding creamy-white or grayish-white, pasty, yeast-like colonies. Small colonies resembling *Cryptococcus* are produced on Sabouraud dextrose agar (25-37°C) and corn meal agar (37°C) in 24 hours. Selective *Prototheca* enrichment media (PIM, PEM) can be used to enhance isolations from environmental sources and from milk (27, 29). *Prototheca* do not have a capsule, except for *P. stagnora* (8).

Presumptive identification of *Prototheca* is based upon the characteristic morphology of the organisms as seen on culture and smears stained with Gram or methylene blue. The API 20 C system (bioMérieux Vitek, Inc., Hazelwood, Missouri) can be used to identify species (26). Two species, *P. zopfii* and *P. wickehamii*, have been reported as etiologic agents of intramammary infections in cows (10-12, 16, 22, 24, 29, 33, 36, 37). However, species level identification is not necessary when dealing with problem cows and herds.

At the four regional laboratories of the QMPS, *Prototheca* are frequently isolated from cow composite milk samples during whole herd mastitis surveys or from quarter or composite milk samples submitted to those laboratories by veterinarians and dairy farmers. Although frequently mentioned as a cause of acute mastitis with swollen, hard quarters secreting watery milk with thick clots (21, 23, 36, 45), we usually isolate *Prototheca* from cows with subclinical and chronic mastitis. Furthermore, we have observed that like *Mycoplasma bovis* (14) and *Staphylococcus aureus* (34), *Prototheca* are shed in milk intermittently by cows with intramammary infections (Pinello, C.B., R.N. González, D.J. Wilson, Cornell University, unpublished observations).

Frequent isolation of *Prototheca* from several herds in New York State, prompted some still unfinished studies on this type of bovine mastitis as follows (Pinello C.B., R.N. González, D.J. Wilson, Cornell University, unpublished observations):

Study No. 1

To investigate environmental relationships of *Prototheca* sp to mastitis, five dairy farms were chosen because they had cows from which *Prototheca* was isolated by the QMPS during whole herd mastitis surveys. For this study, these farms were classified as *Prototheca* infected (Herds 1 to 5). Five dairy farms without a history of *Prototheca* isolation were chosen as controls (Herds 6 to 10). Control farms had been repeatedly surveyed by QMPS with no protothecal mastitis being detected for at least 2 years. An attempt was made to select control farms similar in size, management and layout to the infected farms. At each farm, all possible water sources, bedding, and food were sampled, and representative fecal samples were obtained from cows. During the week that the environmental samples were collected, composite milk samples were aseptically obtained from all milking cows. Fecal samples were collected at each farm from 10 randomly selected cows and from all cows with previous protothecal mastitis.

Milk samples were thoroughly mixed and 0.01 ml streaked onto blood agar plates. After 48 hours of aerobic incubation at 37°C, any organisms resembling *Prototheca* sp were subcultured onto potato dextrose agar (PDA, Difco Laboratories, Detroit, MI) plates. The PDA plates were incubated at 30°C for 72 hours and isolates identified to the species level by the API 20 C system.

Between 100 and 200 ml of water samples were passed through a 1.2 mm Metricel membrane filter. Heavy particulate matter in some water samples only permitted a small volume of water to be filtered. The membrane filter was inverted on PIM medium (27), the filter removed aseptically and the residue was streaked onto the PIM with a sterile swab. Some water samples were laden with particulate matter which prevented filtration. Of these samples, 0.1 ml were plated onto PIM. Samples of feces, bedding and food materials were added to sterile distilled water. This mixture was plated with a sterile swab on PIM. The PIM plates were incubated at 30°C for 72 hours. For some samples that contained heavy fungal growth, it was necessary to replate colonies onto PIM for pure colony isolation. Colonies were counted to determine the concentration of *Prototheca* species per ml of sample.

The number of cows with protothecal mastitis in herds at the time of environmental sampling were 3 of 91, 4 of 56, 13 of 113, 4 of 119 and 1 of 58 for Herds No. 1, 2, 3, 4 and 5, respectively. In 4 of the 6 herds with protothecal mastitis, *P. zopfii* in 2 herds, P. *stagnora* and *Prototheca* sp in one herd each were isolated from fecal samples from cows with a history of protothecal mastitis and from randomly selected cows without protothecal mastitis. In the 5 herds without protothecal mastitis, either P. zopfii or *P. stagnora* were isolated from fecal samples of 5 cows in only one herd (Herd No. 9).

From the majority of the environmental samples collected from the 5 farms without protothecal mastitis, *Prototheca* sp were not isolated. These samples included water from watering troughs, milk house water, ponds, creeks, spring water, heifer barn water, swamp water, pelleted feed, ground feed (calf and cow), corn silage, mixed silage, haylage, high moisture corn, sawdust and bedding. In two farms without protothecal mastitis (No. 6 and 8), no *Prototheca* species were isolated. In the 3 remaining farms (No. 7, 9, and 10), *Prototheca* sp were isolated from stagnant water. In farm No. 7, *P. zopfii* was isolated from beneath a slurry in the grain room and from calf manure. In farm No. 10, *P. stagnora* was isolated from under the manure spreader.

Many environmental samples from the 5 farms with protothecal mastitis contained either *P. zopfii* or *P. stagnora* or *Prototheca* sp. However, *Prototheca* species were not isolated from the milk house water, milk pipeline, ponds, creeks, ditches, swamps, heifer barn water, pelleted feed (cow and calf), high moisture corn, haylage, hay and sawdust.

Study No. 2

At the beginning of 1994, a herd that was under expansion had approximately 1,500 cows in milk. The rolling mean somatic cell count (SCC) was 200,000 cells/ml and the bacterial colony count was below 10,000/ml. Toward the end of March 1994, veterinarians at the QMPS were consulted because there had been an increasing number of clinical mastitis cases that had not responded to standard intramammary antimicrobial therapy. Milk production had decreased and bulk tank milk (BTM) SCC had increased to almost 300,000 cells/ml. Several sequential BTM samples and 36 quarter or composite milk samples from cows with current clinical mastitis or a recent history of therapy failure, were submitted for bacteriological examination. *Mycoplasma bovis* was isolated from almost all the cow's samples and from all BTM samples. Furthermore, *Prototheca* were isolated from two cows (one with clinical mastitis in a mammary quarter and one with subclinical mastitis) and from three BTM samples.

The herd was housed in covered freestall barns, and cows were milked in a parlor that consisted of two double-10 herringbones. When exiting the milking parlor, cows had to walk through a foot-bath located in the splashy manure-covered return alley to the freestalls. A whole herd mastitis survey was performed by the QMPS in mid-May 1994, and composite milk samples were collected aseptically from 1,450 milking cows. A BTM sample was also obtained.

Milk samples were cultured on blood agar and on Hayflick medium for mycoplasma. Eighteen of the cows, none of them with clinical signs of mastitis, and the BTM cultured *Prototheca* sp. Three cows each were in their first, third and sixth lactations, one in its fifth, and the remaining eight cows, in their second lactation. At the time of sampling, mean days in milk for those 18 cows was 162 (range= 17-320), mean milk production/cow/day was 32 kg (range= 17-50), mean linear SCC was 5.4 (range= 1.8-8.7), and the mean linear average SCC for the lactation was 4.9 (range= 2.7-7.5).

Some of the *Prototheca*-infected cows were removed from the herd, and farm owner advised to eliminate splash/puddle areas, and to clean out the return alley from the milking parlor to the freestalls more frequently. Water samples from the milking parlor tap, milking and dry cows freestalls drinking vats and from the liquid in the splash area in the return alley were collected and cultured for *Prototheca* as previously described. *Prototheca* sp was isolated only from the liquid in the return alley.

One high milk producing cow that calved in February 1994, was first detected with a protothecal intramammary infection in March 1994, continued shedding *Prototheca* intermittently until lactation ended in January 1995, calved in March 1995 and was detected still infected with *Prototheca* by mid-June 1995.

Conclusion

In the two aforementioned studies, *Prototheca* species were isolated from the environment in association with protothecal mastitis. *Prototheca* were most frequently isolated from fecal samples and water that was or which might have been contaminated by feces. Organisms were found in the feces and the environment at farms with protothecal mastitis. Cows with and without protothecal mastitis can shed *Prototheca* species in their feces. Entrance into the mammary gland by *P. zopfii* is probable through the teat orifice. Some cows became intermittent shedders of *Prototheca* species but there was no firm evidence for spontaneous recovery. *Prototheca* mastitis could be transmitted from cow to cow during milking time (3,11) with severe economic losses (12,13,16,23,36,45).

If protothecal mastitis is suspected, careful culture and identification to confirm the presence of *Prototheca* is necessary. Cases of protothecal mastitis may be misidentified as yeast mastitis because the colonies of *Prototheca* resemble yeast colonies. Careful microscopic examination of *Prototheca* colonies will reveal sporangia either empty or filled with endospores. If *Prototheca* is isolated from cow milk samples in a herd, it would be advisable to sample all lactating cows in the herd to determine if any other cows are infected (16).

Although *Prototheca* can be isolated from BTM samples on blood agar, the use of a selective *Prototheca* enrichment method is advised (29). Milk from protothecal infected cows, environmental contamination, and milking equipment are all potential sources for *Prototheca* in the BTM (3).

Since no treatment presently exists, all cows with protothecal mastitis should be removed from the herd. Culling of infected cows would reduce the risk of infection of additional cows and contamination of the environment. Attempts should be made to eliminate stagnant water and large amounts of manure from the environment. Good drainage of the areas where cows are kept would be recommended to decrease the number of organisms in the environment.

Yeast mastitis

Yeast are microorganisms which may be found on a wide variety of substrates such as soil, plants, water, nectar of flowers, fruits, exudates of trees and animals. Several species of yeast or yeast-like organisms have been reported to cause bovine mastitis. *Cryptococcus neoformans* (31) and *Candida albicans* are by far the commonest causes but other *Candida* species have also been associated with bovine mastitis (46), and more rarely may be a cause of mycotic abortion in cows (4).

Yeast colonies grown on blood agar at 37°C for 24-48 hours may be confused and misidentified as staphylococci or micrococci. The colonies of *Candida* sp generally are opaque, often white or yellowish, and at first usually smooth. Their texture is creamy or pasty, and in a microscopic smear appear to consist solely of oval to round budding blastospores. *Cryptococcus neoformans* also growths well on blood agar at 37°C usually forming colonies within 48-72 hours. Colonies initially are pale and pasty, becoming honey-brown and mucoid later. Yeast are Gram-positive, but larger than bacteria and morphologically different when compared with bacilli and cocci. The incidence of mastitis due to yeast is usually very low in dairy herds, but sometimes it can occur in epizootic proportions. It has usually been related to treatment directed toward another pathogen using contaminated syringes and canulas or contaminated antibiotic preparations (21, 31, 39). Teat injuries may predispose to the establishment of a yeast infection (31). Yeast intramammary infections were reported to be responsible for 2-3% of all clinical cases seen in a veterinary practice (41). Although the majority of the cases are mild, some intramammary infections may result in death of the affected animals (39).

Yeast infections should be suspected when there is a history of unsuccessful treatment or an intensification of clinical signs of mastitis after intramammary infusion of antibiotics (31). Frequently, there is swelling of the affected gland, sometimes fever, marked reduction in milk production, and macroscopic abnormalities of the secretion. Cases of *Candida* usually recover spontaneously; however, spontaneous recovery after antibiotic therapy for mastitis caused by *Cryptococcus* is rare (43). Although antimycotic drugs have been used for treatment of yeast mastitis (41, 43), there is no clear evidence of the effectiveness of this therapy.

Case report No. 1

SCC reached 1,300,000 cells/ml after cows and springing heifers were added to a 2,500-cow herd under expansion. Composites milk samples were obtained from approximately half of the cows in milking and cultured for mastitis pathogens. Almost 300 cows were found infected with *Streptococcus agalactiae*. Blitz therapy was decided by the owners and herd veterinarian, and antibiotic treatment with a commercial product was given to all four quarters of 495 cows carrying subclinical or clinical infection with *Strep. agalactiae* or cows that had not been cultured, but had California Mastitis Test (CMT) scores of 2 or 3. Treatment was performed by a team of 9 farm employees. One week after the treatment, BTMSCC was 400,000 cells/ml.

Approximately 10 days after treatment, severe acute mastitis developed in 30 cows and milk production dropped dramatically. Affected udders were swollen, hard and painful, and a majority of those animals showed clinical signs of systemic disturbances like fever (40-41°C), increased heart rate and marked anorexia. Mammary secretions were either thick and yellowish or had many flakes, and some contained blood. Bacteriological culture and microscopic examination of the secretions revealed the presence of yeast. Cows were segregated and milked last. Symptomatic treatment was administered and frequent milking out

of the affected cows was performed as possible. Then, new cases of yeast mastitis appeared almost every day for about 2 weeks; newly affected cows were added to the segregated group.

Spontaneous clinical recovery occurred in the majority of the affected cows within two months. Random samples of milk from these cows were cultured and found negative for yeast. However, CMT scores of those milk samples were 1 or 2. Several cows still scored 1 or 2 to the CMT 90 days after the observed onset.

Case report No. 2

A 40-cow herd had several clinical cases of mastitis that were treated by the owner, apparently with commercial intramammary infusions. Some of the cows developed fever and two ceased milk production. The owner called a local veterinary practitioner who examined the cows and observed abnormal secretions in all treated quarters. The veterinarian obtained several milk samples, administered some supportive therapy, and told the farmer to stop the antibiotic treatment. Milk samples cultured by the veterinarian yielded yeast-like fungi, and he advised the farmer to milk the affected cows last, improve sanitation, and not to treat cows without his supervision.

The farmer was blaming either the sawdust he had recently purchased for bedding or the silage he was feeding to the cows as the cause of the mastitis outbreak. The affected cows did not improve, one cow died, and additional cows developed the same type of mastitis. The veterinarian collected more milk samples, and also sawdust and silage samples and with the owner, consulted about the problem with QMPS. These milk samples were cultured on blood agar and on Sabouraud dextrose agar. All cultured milk samples yielded a yeast-like fungi that the author of this report had not seen before and was unable to classify. However, the organism could not be recovered from sawdust and silage.

As more cows appeared everyday with clinical mastitis in at least one mammary quarter and he was suffering substantial milk losses, the farmer, in desperation, started to treat the cows with intramammary infusions he prepared with a variety of drugs such as amphotericin B, Polymyxin B, miconazole, thiabendazole, and cycloheximide. Neither the veterinarian nor QMPS knew how this drugs were obtained by the farmer.

The unknown yeast-like organism was then identified as *Trichosporum beigelii*. Later, culture of milk samples from three recently calved cows yielded *Prototheca* sp. The farmer decided to sell the cows to slaughter and go out of business.

As a conclusion, yeast may gain entrance into the mammary gland of dairy cows from the environment carving their way through teat injuries or tissue damage caused by a previous bacterial infection, or may follow intramammary treatment. This is more frequently seen when a bulk bottle of treatment is used, and doses are drawn out of the bottle different times. Old needles stuck through stoppers or dusty stoppers make this more likely. Therefore, care during infusion and a single dose sterile treatment tubes should be used. The majority of the yeast infections are self-limiting, and cows usually return to milk production. As several species of *Candida* were isolated at the same time from cows with intramammary infections and teat cups of milking machines (46), a potential risk exists for yeast infections to be transmitted from cow to cow at milking time.

Bacillus sp mastitis

Many species of aerobic, sporogenous bacilli have been identified and classified; most stain Gram-positive, although Gram-variable strains may occur. Of these species, *Bacillus anthracis* is the only significant pathogen. Other species are considered to be commensal and nonpathogenic under most circumstances. *Bacillus* sp are widely distributed in nature and most species exist in soil, water, dust, air, feces and on vegetation. Spores are resistant to heat and chemical disinfectants and *B. cereus* and *B. licheniformis* are of importance to dairy hygienists as spoilage organisms in pasteurized milk and cream (20).

During bacteriological examination of milk samples obtained for mastitis diagnosis, colonies of different sizes and colors of *Bacillus* sp are frequently seen on blood agar plates as contaminants. These organisms may occur in milk because at the time of obtaining the sample: 1) they exist in the teat canal and teats are not

properly flushed before collection; 2) contamination from chores performed in tie-stall barns by farm personnel such as feeding the cows or cleaning; 3) teat ends are still humid with 70% alcohol. Furthermore, at two QMPS laboratories, *Bacillus* sp were isolated from cotton and gauze pledgets soaked in 70% alcohol used for teat disinfection, and from the containers in which field personnel was carrying the pledgets to the farms.

Bacillus cereus has been identified as an uncommon cause of mastitis (1, 6, 17-19, 20, 32). The first case reported (6) was attributed to the introduction of the organism during treatment of a chronic intramammary infections when a single plastic syringe was used by a dairy farmer to infuse the quarters with an antibiotic solution. Later, *B. cereus* mastitis occurred in several California herds involved in efficacy trials of a proposed dry-cow therapy product (19). In several British herds (18), *B. cereus* mastitis caused the death of cows with hemolytic systemic symptoms. Chaff used as bedding was reported as the source of one outbreak of mastitis in which multiple cases occurred (18). In another British herd, *B. cereus* was identified as the cause of gangrenous mastitis in which three cows died. The source of infection appeared to be "heated" brewers grains (1). In Canada, acute gangrenous mastitis was diagnosed in two dairy cows and in tissues from several cows which died acutely with signs of toxemia (32). *Bacillus cereus* was isolated from the affected mammary glands (32).

Our experience at the QMPS with *Bacillus* sp mastitis is limited. In one herd, a first lactation cow had chronic mastitis in two mammary quarters with frequent flare-ups. The herd veterinarian had treated the cow with two different antibiotics, three weeks between treatments. The cow had shown mild fever and milk production had practically ceased in both quarters. Three consecutive composite secretions from the affected quarters per week were obtained during a three-week period. Pure culture of *B. subtilis* were obtained from almost all the samples. The cow was sent to slaughter. In another herd, five cows were treated for contagious mastitis with a home mastitis remedy, and within a day all the cows showed severe udder swelling. Udder secretions, and discarded, still in use and unused bottles of the antibiotic home preparation were sent to the QMPS. Yeast from four cows and *B. cereus* from the remaining animal, were isolated from the milk samples. Neither of these mastitis agents were isolated from the bottles.

Few colonies of *Bacillus* sp on blood agar plates represent contamination. However, *Bacillus* sp should be considered as a cause of intramammary infection in a cow if culture of sequential milk samples is pure and strong with concomitant high SCC or clinical signs of udder disease. If colonies are large, flat, grey and with a wide zone of clear hemolysis, *B. cereus* should be suspected.

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