Journal of Animal and Veterinary Advances 5 (4) : 280-283, 2006

Detection of Mycoplasmal Mastitis and Determination of its Prevalence Rate in Dairy Cattle Herds in Ardabil State

Ciamak Ghazaei
Department of Veterinary, Mohaghegh Ardabili University,
Iran-Ardabil, P.O.Box 179, Iran

Abstract: Following an initial report of Mycoplasma mastitis in Ardabil state dairies, a survey of milk samples from a random group of dairy farms was conducted in Moghan area in ardabil state,Iran. Cows (n = 80) from four farms (4 cooperative farms consisting of 10550 cows/herd) with clinical mastitis and bulk milk tanks(n = 48) were monitored and sampled for clinical mastitis. A total of 80 aseptic milk samples were collected when an clinical mastitis was occurred (based on any observable signs in the udder and/or milk) and delivered to the national mastitis laboratory. Untreated samples obtained by haulers were cultured for enrichment of mycoplasmas on selective media. Samples from 4 farms obtained between February and April 2005 were examined and the incidence of positive samples was 48.75%.(39 positive sample of 80 milk samples) M.bovis presence was detected by immunoproxidase test in all of the positive sample.Detection of infection in bulk tank samples and cow milk samples was done at the high sensitivity for the techniques used, indicating that high concentrations of the mycoplasmas were to be found in such samples. Development of routine diagnostic capabilities for the diagnosis of mycoplasmal mastitis should be considered in Moghan to minimize the impact of this disease in dairy herds.

Key words: Cow, milk, mastitis, mycoplasma, mycoplasma bovis

INTRODUCTION

Initially, mycoplasmas were called Pleuropneumonia-Like-Organisms (PPLO) in view of their similarity to the pleuropneumonia agent of cattle[1-3]. The term PPLO is still used today for commercial media prepared for the isolation of mycoplasmas. The by products of growth and metabolism of mycoplasmas irritate the mammary gland tissue resulting in a marked inflammatory response characterized by acute swelling and agalactia[4,5].

Mycoplasma bovine mastitis is a highly contagious disease that results in milk loss and culling of infected animals. Frequently, this type of mastitis goes unrecognized in dairy herds and is spread in part by the sale of infected cows to unsuspecting buyers[4,6-8]. Disease detection at the herd level is usually made by isolation of mycoplasmas from either bulk tank milk or samples from cows with clinical mastitis.

Mycoplasma mastitis is an endemic problem in large dairy herds[9,4-11]. It can also appear in herds under going expansion programs. Although up to 11 different species of mycoplasma have been recognized in bovine mycoplasmal mastitis, the great majority of outbreaks are caused by Mycoplasma bovis[6,8,9]. Characteristic of this disease are its highly infectious nature, complex clinical presentation and extreme resistance to treatment[3,5,8]. Infections spread to other cows during milking, or due to improper technique when infusing acutely affected cows[12,13]. Clinical mastitis caused by Mycoplasma is characterized by a sudden onset of swelling in the udder with abnormal milk usually observed from 1 to 3 days later.

Affected cows have sudden drop in milk production with several or all four quarters appearing swollen. Milk often becomes watery in appearance and may contain small flakes of sand-like material.This is followed in a day and by abnormal milk,characterized by salmon, orange,or tancolored milk.On other hand Milk Between days 4 to 6, discharge often appears yellowish-brown, which later (between days 4 to 14) becomes very purulent("cottage cheese-like" appearance). samples often are thickened, with oatmeal-like consistency and sandy or flaky sediments may appear in the bottom of the sample. Often, the cows return to normal milk production levels but continue to shed mycoplasmas in milk intermittently. Infected cows may remain infected for life, regardless of therapy[1,2,4,6]. It is also common to see lameness and pneumonia in affected cows,and increased culling rates in the production herd.Pneumonia and lameness will also affect calves in the farm due to feeding of infected milk, or poor biosecurity practices. Strict segregation or culling of infected cows remains the only management approach for mycoplasmal mastitis[1,2,13,14]. Mycoplasma mastitis has not been recognized in Ardabil state in the past, despite

repeated attempts to culture milk samples from mastitis cases refractory to treatment. In the first months of 1998, several cases with classical signs of mycoplasmal mastitis were reported (Clinical case report). M. bovis was recovered from milk samples in these cases.Now, a survey of bulk tank samples and cow milk samples was initiated to determine prevalence of mycoplasma infection in a random population of Moghan dairy herds.

MATERIALS AND METHODS

Bulk milk tank (n = 48) and milk samples from cows (n = 80) with clinical mastitis of 4 moghan dairy herds were obtained from specific milk hauling routes. Milk samples were plated soon after collection and delivery to the laboratory and then used for detection of Mycoplasma after development culture and immunoproxidase detection. Isolation of mycoplasma was made on petri plates containing modified Hayflick medium.

In order to culture detection composite milk samples were streaked over one-half of a plate and bulk milk tank samples over an entire plate. Plates were examined for colonies under low power on a standard microscope and Colonies was identified by their shape. Growth could be seen after 3 days of incubation at 37°C in a moist 10% CO2 incubator but 7 days of incubation was needed for the full development of colonies. On the other hand incubation were proceed 7 days before plates were diagnosed as negative.For pre-inrichment a 0.1 mL volume of each milk sample was diluted in three serial ten-fold dilutions of mycoplasma enrichment broth (Modified Hayflick broth contained 15% horse serum, DNA extract and fresh yeast).The serial dilutions were incubated at 37°C for 48 hrs to allow for enrichment of the samples. Selective inhibitors of bacterial growth were added to the mycoplasma broth and agar. Initial samples were processed using ampicillin, methicillin, bacitracin and thallium acetate. For improved control of bacterial contamination during enrichment, cefoperazone was added in addition to the above inhibitors throughout the rest of the study. For culture detection, Enhanced growth in broth followed by culture on a mycoplasma agar medium direct inoculation on mycoplasma medium plates alone.

For this, a loopful of the 1/10 dilution of each enriched sample was streaked on a modified Hayflick agar(contained 15% horse serum, DNA extract and fresh yeast) plate, which was incubated for 48 hrs at 37°C. For serological identification of the colonies,Speciation was accomplished by immunoproxidase test.The samples where mycoplasmas were identified, were subjected each sample to the immunoperoxidase test .The antisera specified for each one of the Mycoplasma spp were diluted 1:50 in a Tris salin solution (TBS) (20 mM Tris, 500 mM NaCl, pH 7.5) plus 0.05% of Tween 20 and 1% of gelatine; with the antisera diluted in this manner, 7 mm diameter discs were humidified until saturation.This same process was used to prepare the goat conjugate anti-I gG of rabbit linked to peroxidase.The saturated antisera discs were placed with forcep over the Mycoplasma spp colonies and incubated during 40 min. After this period, the saturated antisera discs were withdrawn, placing over the same site, the discs saturated with goat conjugate anti-I gG of rabbit linked to peroxidase and then were incubated during 30 min. Once the second incubation process was finished, the disks were removed and TBS was used to wash the antisera and conjugates that were not attached during the reaction.The substrate that was used was prepared with 30 mg of chlorine-naftol diluted in 10 mL of methanol , mixing this solution with a second one that contained 50 mL of TBS plus 30 μL of 30% hydrogen peroxide,according to that which has been previously described. Ten ml of this substrate were added to each Petri dish allowing an incubation time of ten minutes and then it was washed with TBS and examined with a simple or stereoscopic microscope; the colonies stained dark blue were considered positive. All the incubation processes were performed at room temperature.

RESULTS

Culture of milk samples and The micro-organism that were detected in this study were isolated from herds that had mastitis cases with a total of 80 samples and from bulk milk tank samples with a total 48. Of 4 Moghan dairy farms sampled between February and April 2005, all were detected as positive to mycoplasma by culture and of these, all positive samples were also detected by immunoproxidase test that the presence of M.bovis was confirmed. From totally 80 cow mastitic milk samples, 39 samples being as positive to mycoplasma and three farms(A, B and C) yielded positive samples and in all of the farms sampled, there were reports of clinical mastitis with characteristics compatible with mycoplasmal mastitis.Of totally 48 bulk milk samples, all about, being as positive to mycoplasma and also in these samples speciation being M.bovis. The results of the study provide clear evidence that mycoplasmal mastitis was present in Moghan dairy farms during the period of February to April 2005. The 48.75% (39/80) incidence detected based on cow milk samples with clinical mastitis that is comparable with that found in recent studies done elsewhere in the Iran (Table 1).

Table 1: Result of culture detection using 80 bovine milk samples from cows clinical mastitis

Farm	Sample number	Positive sample number	Ratio to totally sample in each farm (%)	Ratio to totally positive sample (%)	Ratio to totally sample (%)
Farm A	49	28	57.14	71.79	35
Farm B	14	5	35.71	12.82	6.25
Farm C	9	4	44.44	10.25	5
Farm D	8	2	25	5.12	2.5
Totally	80	39	-	-	48.75

DISCUSSION

Mycoplasma mastitis was first reported from England in 1960 and has been recognized in the United States since 1961. Since that time it has been reported from much of Europe, Canada, Japan, Israel, Australia and New Zealand. In the United States the first recognized outbreak occurred in Connecticut followed shortly there after by an outbreak involving several herds in New York. Although much of the attention in past years has been focused on California it was not until 1964 that an outbreak occurring in that state was described. It is quite likely that mastitis caused by mycoplasma existed for some time prior to its discovery, however, these early findings spawned the formation of an active mycoplasma mastitis research group in California that has added much to our current understanding of this disease. Unfortunately, too little is known about the factors which influence the frequency and distribution of this form of mastitis in dairy herds. Studies indicate that the organism is susceptible to drying and is destroyed by most disinfectants and teat dips. On the other hand, the organism will persist in unsanitary, warm, moist environments(Humidity climate) which provide the ideal conditions for its survival such as moghan area[14]. There is also evidence that the organisms can inhabit the upper respiratory tract and vagina of cows[5]. In addition, a further interesting observation is the occasional detection of mycoplasma and the virus of IBR in milk obtained from infected cows.

Culture of bulk tank and cow milk samples is a useful procedure to determine the existence of mycoplasma infected cows in a herd. All mycoplasmas are very sensitive to pH changes in the milk. Best recovery rates are achieved when fresh milk samples are plated soon after collection and delivery to the laboratory. Samples may be kept refrigerated for 3 days or frozen(in-20C) for longer periods before culturing on mycoplasma medium. With the use of bacterial growth inhibitors(ampicillin, methicillin, bacitracin and thallium acetate), the problem was overcome, although light bacterial overgrowth could make evaluation of mycoplasma colonies difficult. Addition of cefoperazone prevented all bacterial overgrowth while allowing growth of the positive control milk in all cases. The immunoproxidase test technique did have slightly high sensitivity that culture detection, as has been reported with previous methods used for the detection of Mycoplasma spp. in milk. The addition of a enrichment culture step has been shown to markedly increase the sensitivity of detection of Mycoplasma with all methods tested. In this study, pr-enrichment culture enhanced the sensitivity or the cost of diagnosis by the immunoproxidase over previously reported methods. Of the four positive farms detected in this study all were detected at the high sensitivity available. It is possible that higher incidence values could be obtained if bulk tank samples and cow milk samples were analyzed with more sensitive techniques.Inclusion of detection methods that do not require culture,also are recommended to ensure detection of Mycoplasma in milk samples containing natural or artificial inhibitors of growth of Mycoplasma. In the present study, pre-enrichment culture was performed in three serial ten-fold dilutions to ensure dilution of any mycoplasma inhibitors present in milks.

Enhanced growth in broth followed by culture on a mycoplasma agar medium has been suggested. Thurmond and co-workers in 1989 found that the combined use of direct inoculation and pre-enrichment yielded 70% more isolates of Mycoplasma from bovine milk, than the direct inoculation on mycoplasma medium plates alone. At the other study, the examination of 4,116 milk samples by both direct inoculation of milk on Hayflick agar plates and pre-enrichment in Hayflik broth produced only a 6% increase. Therefore, we agreed with Jasper that the use of pre-enrichment did not increase isolation of Mycoplasma significantly, but increased the cost of diagnosis. Most mycoplasmas isolated from bulk tank milk and cow milk samples are pathogenic but some may be Acholesplasma laidlawii, a common nonpathogenic saprophytic contaminant frequently found in the dairy environment and the teat skin. Therefore, speciation of Mycoplasma-like colonies is recommended and also Differentiating between M. bovis and other mycoplasma species is recommended to distinguish environmental contaminants from mastitis pathogens. Digitonin inhibition of sterol metabolism by mycoplasma was reported as a practical and easy method to discriminate between isolates of Mycoplasma and A. laidlawii from milk. This study provides the first report of M. bovis mastitis in Moghan dairies and documents incidence of the infection by bulk tank and cow mastitic milk samples survey methods.

Comparative results from mycoplasma culture and immunoproxidase test confirmed that M.bovis was present in high concentrations in bulk tank samples and cow milk samples. Even though the incidence of infection with M. bovis appeared to be low, M. bovis mastitis is a significant risk for dairy farms and appropriate surveillance measures should be considered. At present, there is no routine diagnostic capability for mycoplasmal mastitis within moghan. Acquisition of such capability should be considered. The most important environmental factors relate to sanitation in regards to milking practices and milking machine function. Cow-to-cow transmission may occur via the milker's hands, the use of sponges or multiple use cloths for udder preparation, use of ineffective teat dips or the improper application of teat dip and by inadequate sanitary measures employed when treating cows, to name a few. Careful attachment and detachment of milking units resulting in a minimum of vacuum disturbance, control of liner slippage and routine maintenance of milking machine equipment components are essential features of proper milking technique and machine function and demand a conscious effort in mastitis control[1,8,13,14].

Control measures to be adopted when a herd is diagnosed with mycoplasma mastitis have been suggested and include the following:

- Sample cows with clinical mastitis for Mycoplasma sp.
- Culture all cows for mycoplasma. This is done on composite samples (one sample includes milk from all four quarters).
- Remove all cows with positive mycoplasma milk cultures from the main milking strings of the herd. The following alternatives may be considered:
- Monitor the herd by sampling the tank milk after each string is milked once each week until negative cultures have been obtained two times from all samples. Collect cow samples from all strings associated with positive tank samples.
- Continue weekly samples on total tank milk until four consecutive samplings are negative. Then collect and test on a monthly basis for several months.
- Test each clinical mastitis quarter for mycoplasma until the herd is free of mycoplasma. Do not return cow to main strings unless test is negative for mycoplasma. Remove positive cows as in point 2.
- Test each fresh cow before admission to milking string. Remove positive cows (point 2).
- Keep mastitic cows separate from fresh cows at all times.
- Always milk any known mycoplasma cows last or in a separate milking set-up. Milkers should never milk clean cows after milking infected cows without changing clothes and disinfecting hands.
- Teat dipping with an effective teat dip should be rigorously followed.
- Where possible, disinfect teat cup clusters in a clean disinfectant solution after milking cows suspected of having mycoplasma infection.
- Always test purchased cows for mycoplasma prior to admission to regular herd. Several outbreaks have followed purchase of herd additions.

ACKNOWLEDGMENTS

This study was supported in part by a grant from the veterinary research sponsored by Mohaghegh Ardabili university.

REFERENNCES

1. Anderson, K.L., 1993. Update on bovine mastitis. Philadelphia, Saunders, 6: 11-27.
2. Blood, D.C., O.M. Radostitis and J.A. Henderson, 1989. Vet. Medicine. Ed., 7: 501-513.
3. Chimi, A., 1996. Veterinary bacteriology and bacterial disease. Nashr publication, pp: 54-68.
4. Jasper, D.E., 1981. Bovine mycoplasmal mastitis. Advances in Vet. Sci. Comparat. Medicine, 25: 122-155.
5. Jasper, D.E., T.T. Boothby and C.B. Thomas, 1987. Pathogenesis of bovine mycoplasma mastitis. Isr. J. Med. Sci., 6: 625-627.
6. Hale, H.H., C.F. Helmboldt, W.N. Plastridge and E.F. Atula, 1962. Bovine mastitis caused by a mycoplasma Species. Cornell, Vet., pp: 52-582.
7. Schalm, A.W., N.C. Jain and E.J. Carrol, 1971. Bovine mastitis. Lea and Febiger, Philadelphia, pp: 206-243.
8. Tabatabaye, A.H. and R. Firuozi, 2001. Disaese of Animals Due to Bacteria. Tehran University Press, pp: 469-480.
9. Bayouni, A., T.B. Faruer, B. Bushnell and Olivera, 1988. Enzootic mycoplasmal mastitis in alarge dairy during an eight-year Period. Javma, 192: 902-905.
10. Louise ruhnke, H., D. Thewley and F .C. Nelson, 1976. Bovine mastitis in ontaria due to mycoplasma agalactiae subsp. Bovis. Can. J. Comparat. Med., 40: 142-147.
11. Mackie, D.P., H.J. Ball and E. Logan, 1986. Mycoplasma californicum mastitis in the dry dairy Cow. Vet. Rec., 4: 350-351.
12. Thomas, C.B., P. Willeberg and D.E. Jasper, 1981. Case control study of bovine mycoplasma mastitis in California, Ameri. J. Vet. Res., 42: 511-515.
13. Philpot, W.N., 1979. Control of mastitis by hygiene and therapy. J. Dairy Sci., 62: 168-175.
14. Carrol, E.J., 1977. Environmental factors in bovine mastitis. J. Amer. Vet. Med., 10: 1143-1147.