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RESEARCH FOR *Mycoplasma* spp IN MILK SAMPLES FROM MASTITIC COWS

(PESQUISA DE Mycoplasma spp EM AMOSTRAS DE LEITE DE VACAS MASTÍTICAS)

(PESQUISA DE Mycoplasma spp EN MUESTRAS DE LECHE DE VACAS MASTÍTICAS)

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SUMMARY

Many species of mycoplasmas account for cases and outbreaks of mastitis in dairy herds worldwide. Mastitis due to mycoplasma in Brazil was first reported only in 1996, and its real importance in the national cases of the disease is unknown. Mycoplasmas are highly contagious agents and its control depends on sick cow detection and slaughtering. In this study, which was carried out in mid-western region of São Paulo State, Brazil, 101 and 121 samples of lacteal secretion derived from acute clinical mastitic cows and subclinical cases, respectively, were inoculated in modified Hayflick mediums. Also, all the samples underwent a molecular analysis by PCR technique with *Mycoplasma* genus specific primers. All samples were negative for Mycoplasma in the Hayflick modified medium, as well as in the PCR.

KEY-WORDS: Mastitis. Mycoplasma. PCR.

RESUMO

Diversas espécies de micoplasmas são responsáveis por casos e surtos de mastite em rebanhos leiteiros de todo o mundo. No Brasil, o primeiro relato de mastite por micoplasma deu-se apenas em 1996 e ainda pouco se sabe sobre sua importância nos casos nacionais dessa afecção. Os micoplasmas são agentes altamente contagiosos, sendo que o seu controle depende da detecção e da posterior eliminação da vaca acometida. Neste estudo, que foi realizado na região centro-oeste do Estado de São Paulo, 101 e 121 amostras de secreção láctea provenientes, respectivamente, de vacas com mastite aguda e de casos subclínicos foram inoculadas em meio Hayflick modificado. Todas as amostras de leite foram submetidas à análise molecular, por meio da técnica de PCR, para o primer genérico de *Mycoplasma.* As amostras foram negativas para micoplasma no meio de Hayflick modificado e na técnica de PCR.

KEY-WORDS: Mastite. Micoplasma. PCR.

RESUMEN

Diversas especies de micoplasmas son responsables por casos y brotes de mastitis en rebaños lecheros de todo el mundo. En el Brasil el primer relato de mastitis por micoplasma fue hecho apenas en 1996 y aún se sabe poco sobre su importancia en los casos nacionales de esta afección. Los micoplasmas son agentes altamente contagiosos y su control depende de la detección y posterior eliminación de la vaca afectada. En este estudio, realizado en la región Centro-Oeste

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del Estado de São Paulo, Brasil, 101 y 121 muestras de secreción láctea provenientes de vacas con mastitis aguda y de casos subclínicos, respectivamente, fueron inoculadas en medio Hayflick modificado. Todas las muestras de leche fueron sometidas a análisis molecular, por medio de la técnica de PCR, para el primer genérico de *Mycoplasma*. Las muestras fueron negativas para micoplasma en el medio de Hayflick modificado y en la técnica de PCR.

PALABRAS-CLAVE: Mastitis. Mycoplasma. PCR.

INTRODUCTION

Bovine mastitis remains a major challenge to the worldwide dairy industry despite the widespread implementation of mastitis control strategies. In the United States, around 40 to 50% of the cows have at least a quarter compromised (PHILPOT, 1984). A study in São Paulo and Minas Gerais states, Brazil, conducted by Costa et al. (1995), revealed 72.56% of the animals affected by subclinical mastitis.

The bovine mammary gland inflammation, like all the diseases, is multifactorial, being a result of the interaction among the etiological agent, environment and the susceptible host. Among the mastitis agents we have the physical, chemical and mainly the biological agents, like the bacteria, yeast and algae. Among the microorganisms which can be responsible for mastitis, like *Staphylococcus aureus, Streptococcus* spp, *Escherichia coli* and others, there are those from the genus *Mycoplasma* (QUlNN et al., 1994, LANGONI, 1999).

It is not uncommon for etiology of mastitis to go unrecognized. The number of mastitis outbreaks that have remained undiagnosed - and might have been due to *Mycoplasma* species - is unknown because either the samples of mastitic secretion are not submitted to examination or laboratory results fail to accurately determine the causative agents (BUSHNELL, 1984).

Mycoplasmas are of great historical significance in the annals of cattle disease. The first mycoplasma described on literature was *Mycoplasma mycoides* subsp. *mycoides*, agent of the contagious bovine pleuropneumonia. The mycoplasma infections have been associated with lung diseases, genital tract and articulate diseases, and other tissues, like mammary gland tissue (JASPER 1977, SIMECKA et al., 1992). The mycoplasmas belong to the class Mollicutes and to the order Mycoplasmatales (RAZIN e FREUNDT, 1984).

Mastitis due to mycoplasma was first reported in England by Da Vidson e Stuart (1960). Since that time it has been reported much in Europe, Canada, United States, Japan, Israel, Australia and New Zealand (BOUGHTON, 1979, JASPER 1987). The first recognized outbreak of mastitis due to species now called *Mycoplasma bovis*, as the most frequent cause of bovine mastitis, occurred in Connecticut in 1961 (HALE et al., 1962).

Of several *Mycoplasma* species identified as etiologic agents in mastitis in dairy cattle, *Mycoplasma bovis* appears to be the most prevalent. The species most frequently found also appear to be the most pathogenic and cause higher incidence of infection within herds (BUSHNELL, 1984, GRAND et al., 1997). Other species also related to mastitis are *M.bovigenitalium, M.canadense, M.bovirhinis, M.arginini, M.dispar, M.alkalescens, M.californicum, Acholeplasma laidlawii* and *Ureaplasma diversum* (JASPER, 1981). In Brazil the first case of mastitis due to Mycoplasmas species were reported by Mettifogo et al. (1996).

Mycoplasma mastitis is generally characterized by a sudden drop in milk production, often involving several or all four quarters. Variable degrees of edematous swelling and hypersensitivity usually accompany the mastitis and some authors report pain (JASPER et al., 1966, JASPER 1979, BUSHNELL, 1984). Mycoplasma should be suspected whenever there is an increase in severe purulent mastitis cases that resist treatment; mastitis cases which typically involve more than one quarter (often all quarters); marked loss of production; and abnormal secretions with tannish or brownish discoloration and sandy or flaky sediments in watery or serous fluid (JASPER, 1977, 1979). In the early stages, milk secretion becomes watery in appearance, with small flakes of “sandy” material. These flakes tend either to settle to the bottom, adhere to the side of the sample tube, or rise to the top. Agalactia develops rapidly as by the third to sixth day the secretion has progressed to a yellowish-brown fluid with increased fibrin and inflammatory cells. By the fourth to fourteenth day following first clinical signs, the exudates becomes very purulent and has the appearance of cottage cheese (JASPER et al., 1966, BUSHNELL, 1984). It is uncommon to find arthritis, pyrexia, anorexia or other systemic signs, with *M.bovis* infection, and the animal remains afebrile, and continues to drink and eat (JASPER, 1981, BUSHNELL, 1984).

Mycoplasma mastitis is spread primarily cow to cow through the contaminated secretions from udders of infected animals. Therefore, purchased animals shedding Mycoplasma organisms from the udder become a major source of infection for other cows. Personnel and equipment that have direct contact with milk secretion or sick cows in an infected herd and travel to other dairies, where they have similar contacts, can act as disease carriers. These include milkers, milk testers, equipment installers and veterinarians (JASPER et al., 1974, JASPER 1979, JASPER 1982, BUSHNELL, 1984, JASPER 1987).

OBJECTIVES

The objective of the present study was to research the occurrence of *Mycoplasma* genus microorganisms in lacteal secretion of subclinical and clinical mastitis cases, in cows from the mid-western region of São Paulo state.

MATERIAL AND METHODS

In this study 222 milk samples of mastitic cows proceeding from three farms from the mid-western region of São Paulo state have been examined. Among these samples 121 were derived from subclinical mastitic cows, with different degrees of reactivity in California Mastitis Test (CMT); and 101 from clinical mastitic cows. Sampling was always performed adopting the hygienization washing with current water, drying with dismissable towel paper and disinfection with iodized alcohol at 5%. The milk secretion was collected in sterile glass tubes, identified and stored in isothermal boxes until their leading to the Microbiology Laboratory, in the Veterinary Hygiene and Public Health Department, São Paulo State University - UNESP.

Mycoplasma Culture

The modified Hayflick medium was used for mycoplasma isolation prepared as the technique described by Freundt (1983). The liquid medium consists on a formulation containing, as the Broth Base, hearth infusion broth (2.85g) and distillated water (90.0mL), being sterilized by autoclaving (121°C/20 minutes). After this autoclavation the supplements, which were horse serum (20.0mL); fresh yeast extract 25% (w/v) solution (l0.0mL); calf thymus DNA (Sigma Chemical Company, St. Louis, MO; Type 1. Cal. No.1501), 0.2% (w/v) solution; thallium acetate 1% (w/v) solution (1.0mL); and benzylpenicilin 20000U/mL (0.25mL) were aseptically added. The pH was adjusted to 7.8. For solid medium preparation 0.8% of Noble Agar (Difco) was added in the Broth Base before its autoclavation. The samples were inoculated in liquid and solid modified Hayflick medium in the same day. At the end of the procedure 1.8mL milk aliquots were stored in criotubes at -70°C for the PCR technique.

Afterwards aliquots of 0.lmL of the milk samples were added into tubes containing 0.9mL of liquid modified Hayflick medium, being diluted until 10^{-3} with the own culture medium. Immediately after the inoculation in liquid medium, the inoculations of the pure samples and their respective dilutions were proceeded in solid modified Hayflick medium. They were incubated under 5% CO_2 atmosphere at 37°C over 14 days. Following three-day incubation period, all the plates were daily observed under a magnifying glass. The negative result for growing mycoplasma colony was confirmed only after 14 days of incubation.

PCR

The DNA extraction protocol used was described by Fan et al. (1995), being a low cost technique which do not use toxic reagents. After, 1mL of outgrown suspension cultures diluted to 10^{-1} was centrifuged at 13000g for 10 minutes. The sediment was washed with 100µL of PBS (2.6mM NaH_2PO4; 7.4mM NaHPO4; 1.4mM NaCl; pH7.2), centrifuged at 13000g for 10 minutes. The pellet was resuspended again with 100µL of PBS and centrifuged at 13000g for 10 minutes. The final pellet was resuspended in 20µL of PBS and heated on boiled water bath for 10 minutes to break cell membranes, rapidly chilled on ice for 5 minutes. This tube was submitted again to 13000g centrifugation for 5 minutes and the suspension, where there possibly was the mycoplasma DNA, stored at -20°C for later use in the PCR experiments.

The performance of the PCR technique was done following the precaution rules described by Kwok e Higuchi (1989). The PCR assay for the detection of *Mycoplasma* spp is genus specific and was carried out as described by Van Kuppeveld et al. (1992). The expected size of amplicons was 270bp from the conserved sequence of the 16S rRNA gene, common for mycoplasma, acholeplasma, ureaplasma and spiroplasma.

The PCR assay employed the primers GPO3 and MGSO, which are complementary, respectively, to the V2 and V7 regions and are in the 798-3' and 1055-3' positions. GPO3 = 5'-GGG AGC AAA CAG GAT TAG ATA CCCT-3' and MGSO= 5'-TGC ACCATCTGTCACTCT GTT AACCTC-3'.

For the polymerase chain reaction 50pmol of each primer was added to a microtube (0.5 mL), 2U of *Taq* DNA polymerase, 2.0mM of $MgCl_2$, 100µM of dNTP mix, 1µL of extracted DNA and ultrapure water until the final volume of 50µL. The conditions employed in PCR are 1 cycle of 94ºC for 5 minutes (initial DNA denaturation); 35 cycles of 94°C during 30 seconds (DNA denaturation), 60°C for 30 seconds (primers annealing), 72ºC for 30 seconds (DNA extension); and a final cycle of 72ºC for 10 minutes (final extension).

The products of the PCR technique (10µL) were visualized after electrophoresis in 2% agarose gel, with l0µg/mL of ethidium bromide on TAE buffer (40mM Tris-acetate; 2mM EDTA; pH8.0). A molecular weight marker with 100bp (100bp ladder Gibco-BRL) was used as size standards.

RESULTS AND DISCUSSION

Among the 222 milk samples analyzed, no one showed the growth of mycoplasma colonies, after the inoculation in modified Hayflick medium. By the PCR

technique, they were also negative using the genera specific primers GPO3 and MGSO.

The negative results for the presence of mycoplasmas, in the conditions of the present study, revealed the low incidence of these agents in bovine mastitis in Brazil when they are compared to the agents, such as *Streptococcus* spp, *Staphylococcus* spp and even *Corynebacterium* spp.

Many farms which had their animals analyzed were demonstrating characteristics in their herd that were similar to the expected ones in the presence of mycoplasmas as etiological agent of mastitis. Therefore, a lot of these animals showed chronic mastitis resistant to different treatments and macroscopic characteristics in milk which were very similar to those expected on mycoplasma mastitis.

In spite of the absence of positive results, we can not fail to consider the possibility of mycoplasmas as an etiological agent of mastitis in Brazil and that many cases with negative results in routine microbiological exams may be caused by mycoplasmas as demonstrated by Mettifogo et al. (1996, 1998) and Preito et al. (2001).

CONCLUSIONS

The result of this study has not evidenced the presence of mycoplasmas as etiological agent of mastitis as described by Mettifogo et al. (1996, 1998) and Preito et al. (2001). Although this agent seems to cause the disease only in isolated herds of our country, further researches in the field of mycoplasma mastitis are necessary as well as bring its diagnosis into practice in bacteriological laboratories as a proposal to find out its real amplitude and distribution.

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REFERENCES

BOUGHTON, E. *Mycoplasma bovis*: mastitis. **Veterinary Bulletin**, vA9, n.6, p.377-387, 1979.

BUSHNELL, R. B. Mycoplasma mastitis. Symposium on mastitis. **Veterinary Clinics of North America: Large Animal Practice**, v.6, n.2, p.301-12, 1984.

COSTA, E. O., MELVILLE, P. A, RIBEIRO, A. R., WATANABE, E. T., WHITE, C. R., PARDO, R. B. Índices de mastite bovina clínica e subclínica nos estados de São Paulo e Minas Gerais. **Revista Brasileira de Medicina Veterinária**, v.17, p.215-217, 1995.

DAVIDSON, I., STUART, P. Bovine mastitis caused by a mycoplasma. **Veterinary Record**, v.72, p.766, 1960.

FAN, H. H., KLEVEN, S. H., JACKWOOD, M. W. Aplication of polymerase chain reaction with arbitrary to strain identification of *Mycoplasma gallisepticum*. **Avian Diseases**, v.39, n.4, p.729-735, 1995.

FREUNDT, E. A. Culture media for classic mycoplasmas. In: RAZIN, S., TULLY, J. G. **Methods in Mycoplasmology**. New York: Academic Press, 1983. p.127-135.

GRAND, D., POUMARAT, F., BEZILLE, P. Mycoplasmoses bovines à *Mycoplasma bovis*. **Le Point Veterinaire**, v.28, n.180, p.23-30, 1997.

HALE, H. H., HELMBOLDT, C. F., PLASTRIDGE, W. N., STULA, E. F. Bovine mastitis caused by a *Mycoplasma* species. **Cornell Veterinary**, v.52, p.582-591, 1962.

JASPER., D. E. *Mycoplasma* and Mycoplasma mastitis. **Journal of the American Veterinary Medical Association**, v.170, n.10, p.1167-1172, 1977.

JASPER., D. E. Bovine mycoplasmal mastitis. **Journal of the American Veterinary Medical Association**, v.175, p.1072-1074, 1979.

JASPER., D. E. Bovine mycoplasma mastitis. **Advances in Veterinary Science and Comparative Medicine**, v.25, p.121-159, 1981.

JASPER., D. E. The role of *Mycoplasma* in bovine mastitis. **Journal of the American Veterinary Medical Association**, v.181, n.2, p.158-164, 1982.

JASPER., D. E. Bovine mastitis due to *Mycoplasma* **Revue Scientifique et Technique de l'Office International des Epizooties**, v.6, n.3, p.801-807, 1987.

JASPER., D. E., JAIN, N. C., BRAZIL, J. H. Clinical and Laboratory Observations on Bovine Mastitis due to Mycoplasma. **Journal of the American Veterinary Medical Association**, v.148, n.9, p.1017-1029, 1966.

JASPER., D. E., AL AUBAIDI, J. M., FABRIANT, J. Epidemiologic observations in Mycoplasma mastitis. **Cornell Veterinary**, v.64, p.407-415, 1974.

KWOK, S., HIGUCHI, R. Avoiding false positives with PCR. **Nature**, v.339, p.237-8, 1989.

LANGONI, H. Complexidade etiológica na mastite bovina. In: ENCONTRO DE PESQUISADORES EM MASTITES, 3., Botucatu, SP, 1999. **Anais...**, Botucatu, p.3-18.

METTIFOGO, E., NASCIMENTO, E. R., MÜLLER., E. E., NASCIMENTO, M. G. F., FREITAS, J. C. Mastite bovina por *Mycoplasma bovis*. **Revista Brasileira de Medicina Veterinária**, v.19, n.1, p.22-25, 1996.

METTIFOGO, E. MÜLLER., E. E., PARDO, E., BUZINHANI, M., YAMAGUTI, M. First isolation of *Mycoplasma bovigenitalium* from bovine mastitis in Brazil. In: INTERNATIONAL CONGRESS OF MYCOPLASMOLOGY, 12., Sydney, 1998. **Anais…**, Sydney.

PHILPOT, W. N. Economics on mastitis control. Symposium on mastitis. **Veterinary Clinics of North America: Large Animal Practice**, v.6, p.233-245, 1984.

PREITO, L. G., MÜLLER., E. E., FREITAS, J. C., MEITIFOGO, E., BUZINHANI, M., YAMAGUTI, M., SALVADOR., R. Mastite bovina por *Mycoplasma bovis* em rebanhos leiteiros. **Pesquisa Veterinária Brasileira**, v.21, n.4, p.143-145, 2001.

QUINN, P. J., CARTER, M. E., MARKEY, B. K., CARTER, G. R **Clinical Veterinary Microbiology**. London: Wolfe, 1994. 648p.

RAZIN, S., FREUNDT, E. A. The Mycoplasmas. In: KRIEG, N.R, HOLT, J.G. **Bergey's manual of systematic bacteriology**. Baltimore: Williams & Wilkins, 1984. v.1, p.740-792.

SIMECKA, J. W., DAVIS, J. K., DAVIDSON, M. K., ROSS, S. E., STÃDTLANDER, C. T. K., CASSEL, G. H. Mycoplasma diseases of animals. In: MANILOFF, J., MCELHANEY, R. N., FINCH, L. R., BASEMAN, J. B. **Mycoplasmas: molecular biology and pathogenesis.** Washington: American Society for Microbiology, 1992. p.391-415.

VANKUPPEVELD, F. J. M., VANDERLOGT, J. T. M., ANGULO, A. F., VAN ZOEST, M. J., QUINT, W. G. V., NIESTERS, H. G. M., GALANA, J. M. D., MELCHERS, W. J. G. Genus and species-specific identification of mycoplasmas by 16SrRNA amplification. **Applied and Environmental Microbiology**, v.58, p.2606-2615, 1992.