



Ciência Rural

ISSN: 0103-8478

cienciarural@mail.ufsm.br

Universidade Federal de Santa Maria
Brasil

Pianta, Celso; Thompsen Passos, Daniel; Hepp, Diego; de Oliveira, Sérgio José
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Ciência Rural, vol. 37, núm. 1, janeiro-fevereiro, 2007, pp. 171-174
Universidade Federal de Santa Maria
Santa Maria, Brasil

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Isolation of *Arcobacter* spp from the milk of dairy cows in Brazil

Isolamento de *Arcobacter* spp do leite de vacas leiteiras no Brasil

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ABSTRACT

Bacteriologic examinations were performed on 188 milk samples collected from cows from 11 farms for diagnosis of mastitis in three cities of Rio Grande do Sul, Brazil. Among the common causes of mastitis, the most frequent isolates were *Staphylococcus aureus*, followed by *Corynebacterium* sp, *Streptococcus uberis*, *Streptococcus dysgalactiae* and *Streptococcus agalactiae*. Bacteriologic examination of 32 milk samples from one farm didn't show bacteria known as common etiologic agent of mastitis. Six samples of *Arcobacter* spp typed by genotypic tests as *Arcobacter cryaerophilus* (five strains) and *Arcobacter butzleri* (one strain) were isolated from cows' milk of that farm. It is reported the isolation of *Arcobacter* species from the milk of cows in absence of clinical signs of mastitis. This is the first report of the detection of the microorganisms in the milk of dairy cows in Brazil. No previous reports are known from other countries.

Key words: *Arcobacter* spp, milk, dairy cows.

RESUMO

Foram realizados exames bacteriológicos em 188 amostras de leite colhidas de vacas de 11 propriedades leiteiras para diagnóstico de mastite, em três municípios no Rio Grande do Sul, Brasil. Entre as causas comuns de mastite, os isolados mais frequentes foram *Staphylococcus aureus*, seguido de *Corynebacterium* sp, *Streptococcus uberis*, *Streptococcus dysgalactiae* e *Streptococcus agalactiae*. O exame bacteriológico realizado em 32 amostras de leite de vacas de uma propriedade não demonstrou a presença de bactérias conhecidas como causadoras de mastite. Foram isoladas do leite de vacas desta propriedade seis amostras de *Arcobacter* spp, classificadas por testes moleculares como *Arcobacter cryaerophilus* (cinco amostras) e *Arcobacter butzleri* (uma amostra). É relatado o isolamento de espécies de *Arcobacter*

do leite de vacas na ausência de sinais clínicos de mastite. Este é o primeiro relato da detecção dos microorganismos no leite de vacas leiteiras no Brasil.

Palavras-chave: *Arcobacter* spp, leite, vacas leiteiras.

INTRODUCTION

Arcobacter spp are bacteria displaying motility and morphology similar to the genus *Campylobacter*, but are aerotolerant and grow at 30 °C. *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii* have been recovered from humans and livestock. *A. cryaerophilus* have been isolated from aborted pig fetuses from some countries, including Brazil (SCHROEDER-TUCKER et al., 1996; OLIVEIRA et al., 1997; 1999). *A. butzleri* have been detected from enteritis in humans (LERNER et al., 1994; LAWERS et al., 1996; LAU et al., 2002; FERNANDEZ et al., 2004; VANDENBERG et al., 2004) and also the microorganisms were isolated from poultry and pig carcasses (RIDSDALE et al., 1998; OLIVEIRA et al., 2001; 2003).

Very little is the knowledge on the presence of *Arcobacter* spp in bovine. Mastitis in cows was reported by LOGAN et al. (1982). The authors infected animals with *Arcobacter* spp, demonstrating the pathogenicity to the mammary gland. The microorganisms have been detected in bovine feces (WESLEY et al., 2000; INGLIS & KALISHUK, 2003).

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Also *A. butzleri* was isolated from 5 % of the samples of bovine ground meat in an abattoir survey (ONGOR et al., 2004).

Taking into consideration the risks of infection to humans, this article reports the presence of *Arcobacter* species in samples of dairy cows' milk in suspected cases of mastitis.

MATERIALS AND METHODS

One hundred and eighty eight (188) milk samples were collected from dairy cows suspected of mastitis, from 11 farms in the state of Rio Grande do Sul, Brazil, respectively from the municipalities of Montenegro (1), Ibiraiaras (9) and Novo Hamburgo (1), for bacteriologic examination, as part of a research project on bovine mastitis. They were positive by California Mastitis Test (CMT).

Each sample was inoculated onto sheep blood agar plates and MacConkey agar. The plates were incubated aerobically 24 to 48 hours. Bacterial colonies were phenotypically characterized by biochemical tests (OLIVEIRA, 2000).

Each milk sample was also inoculated into liquid EMJH (Ellinghausen McCoullough Johnson and Harris - Difco) medium at a proportion of 1mL to 9 mL of culture medium, for isolation of *Arcobacter spp.* Tubes were incubated about six days at 25 – 30°C under aerobic conditions. After this period it was used the STEELE & MCDERMOTT (1984) membrane filter method, plating the inoculum onto sheep blood agar (brain heart infusion agar supplemented with 10% defibrinated sheep blood). One drop of the growth from EMJH was filtered onto blood agar through a 0.45µm pore size cellulose acetate filter. The plates were left lids-up on the bench for about 30 minutes to dry and then inverted and incubated under aerobic conditions at 30°C for 2 days.

Suspect colonies on each plate were picked and checked by gram stain and inoculated into semi-solid EMJH (0.15% agar). Growths from semi-solid EMJH were examined under darkfield microscopy (40x magnification) for motile gram negative curved rods, as *Arcobacter spp.* Cultures were purified by streaking on blood agar and checked for oxidase, catalase and growth on MacConkey agar. Isolates were subcultured twice weekly into semi-solid BHI medium, waiting for genotypic tests.

Total DNA from each isolate characterized as *Arcobacter spp.* was extracted according to the protocol developed by BOOM et al. (1990). Briefly, 100µl of culture were lysed in 900µl of a GuSCN buffer, the nucleic acid was bound to silica particles and washed

twice with a GuSCN wash buffer and with a 70% ethanol solution and once with acetone. The tubes were dried and the nucleic acids were released from the silica particles in 50µl of water and stored at –20°C.

The primer sequences were designed to amplify a 1202-bp fragment within the coding region of the 16S rRNA gene in *Arcobacter* species. The design was based on an alignment of the 16S rRNA sequences of different *Arcobacter* species which demonstrated common conserved regions that served as targets for the primers. The forward and reverse primers used in this study were ARC-1: 5'-AGA GAT TAG CCT GTA TTG TAT C-3' ARC-2: 5'-TAG CAT CCC CGC TTC GAA TGA-3'. Specific primers for *A. butzleri* were ARC-5: 5'-TTC GCT TGC GCT GAC AT-3' and ARC-6: 5'-CTATCCAGC GTAGAA GAT G-3', amplifying a 686 bp fragment from the gene 23S rRNA (HARMON & WESLEY, 1996; OLIVEIRA et al., 2001).

The PCR reaction mixture contained 1µl of template DNA extracted from cultures, 1µM each primer, IX PCR Buffer, 1.5mM MgCl₂, 200µM each deoxynucleotide, and 1 U of *Taq* DNA polymerase in a total volume of 25µl.

The PCR amplification was performed with an initial denaturation for 3min at 94°C, followed 30 amplification cycles, each consisting of 94°C for 60 seconds, 56°C for 60 seconds, and 72°C for 60 seconds to the primers ARC-1 and ARC-2, and 40 cycles of 94°C for 20 seconds, 60°C for 40 seconds and 72°C for 60 seconds to the primers ARC-5 and ARC-6. A final extension at 72°C for 5 min was included.

Following amplification, amplification product of the primers ARC-1 and ARC-2 (genus specific) was digested with the enzyme *Ssp* 1 to discriminate between the species *A. butzleri* and *A. cryaerophilus* on the basis of the fragments generated for the restriction. The digestion from the *A. butzleri* samples forms fragments of 142, 358 and 701bp, while the *A. cryaerophilus* samples form fragments of 142 and 1060bp.

Products of the amplifications and digestion were visualized by 10.5% polyacrilamide gel electrophoresis and stained with silver nitrate (SANGUINETTI et al., 1994).

RESULTS AND DISCUSSION

Results of bacteriologic tests for identification of bacteria isolated from milk samples collected from 11 farms are summarized in table 1. As shown in table 1, among the common causes of mastitis, the most frequent isolate was *Staphylococcus aureus*, followed by *Corynebacterium sp.*, *Streptococcus*

Table 1 - Results of bacteriologic tests in 188 milk samples from dairy cows from the municipalities of Montenegro, Ibiraiaras and Novo Hamburgo, state of Rio Grande do Sul, Brazil

Isolate	Montenegro (70 samples)	Ibiraiaras (86 samples)	Novo Hamburgo (32 samples)	Total
<i>Staphylococcus aureus</i>	2	27	0	29
<i>Streptococcus dysgalactiae</i>	4	7	0	11
<i>Streptococcus uberis</i>	12	10	0	22
<i>Streptococcus agalactiae</i>	7	0	0	7
<i>Corynebacterium sp</i>	19	7	0	26
<i>Nocardia asteroides</i>	3	1	0	4
<i>Arcobacter cryaerophilus</i>	0	0	5	5
<i>Arcobacter butzleri</i>	0	0	1	1
Contaminants	9	18	19	46
No growth	14	16	7	37

uberis, *Streptococcus dysgalactiae* and *Streptococcus agalactiae*. Bacteriologic examination of 32 milk samples from one farm didn't show bacteria known as common etiologic agent of mastitis. Six strains of *Arcobacter* spp, typed by genotypic tests as *Arcobacter cryaerophilus* (five strains) and *Arcobacter butzleri* (one strain), were isolated from the cows' milk of that farm.

This is the first report of the isolation of *Arcobacter* spp from milk samples of bovines. It is important also because they were the only microorganisms detected in the samples, there was absence of bacteria known as pathogenic to the mammary gland. The prevalence of *Arcobacter* in healthy beef or dairy cattle is unknown.

Arcobacter spp were present into the milk samples inspite of the absence of clinical signs of mastitis. In previous studies, there is only the report of LOGAN et al. (1982) on cases of isolation of *Arcobacter* in mastitis in cows. The authors infected the animals by intramammary inoculation and each infected quarter developed an acute clinical mastitis which resolved spontaneously after 120 hours. At the present work it is not possible to confirm the pathogenic effect on the mammary gland, but the occurrence of mastitis could be subclinical.

Arcobacter spp have been associated with cases of abortion in cattle (HIGGINS & DEGRE, 1979), also isolated from preputial washing of a bull (GILL, 1988) and bovine feces (WESLEY et al., 2000). The present isolation of the microorganisms from milk enhances the difficulty to know how transmission occurs. Drinking contaminated water or untreated water is involved in transmission of *A. butzleri* (RICE et al., 1998). *A. butzleri* was detected in this study in the milk from one cow and it means a potential risk as a human foodborne agent, causing enteritis (VANDENBERG et al., 2004).

Detection of *Arcobacter* in milk alerts for the need to avoid raw milk consumption and also the prevention of environmental contamination, adopting hygienic measures for cow milking. There is also the possibility of healthy carrier cows infecting their calves. The pathogenicity of *Arcobacter* spp to calves is unknown.

CONCLUSION

The isolation of *Arcobacter cryaerophilus* (5 samples) and *Arcobacter butzleri* (1 sample) from the milk of cows in absence of clinical signs of mastitis is reported. This is the first report of the detection of the microorganisms in milk in Brazil.

ACKNOWLEDGEMENTS

We are indebted to Jane Mendez Brasil and Vilson Benedito da Silva Junior from the Bacteriology Laboratory, and we thank Bruno Nunes Castro from Biotechnology Laboratory, Veterinary School, ULBRA University.

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