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MOLECULAR IDENTIFICATION AND THERMORESISTANCE TO BOILING OF *NOCARDIA FARCINICA* AND *NOCARDIA CYRIACIGEORGICA* FROM BOVINE BULK TANK MILK

L.A.Z. Condas[1], M.G. Ribeiro[1*], T. Gonoi[2], T. Matsuzawa[2], K. Yazawa[2], R.G. Motta[1], M.M.J. Franco[1], F.J.P. Listoni[1]

[1]Universidade Estadual Paulista, Doenças Infecciosas de Animais Domésticos, Departamento de Higiene Veterinária e Saúde Pública, Faculdade de Medicina Veterinária, Botucatu, SP, Brasil; [2]Chiba University, Medical Mycology Research Center, MMRC, Inohana, Chiba, Japan.

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ABSTRACT

Two strains of *Nocardia* spp. were isolated from bovine milk of two individual bulk tank. Molecular identification classified the strains as *Nocardia farcinica* and *Nocardia cyriacigeorgica*. The thermorresistance to boiling of the isolates was carried out and was observed bacterial growth after boiling. Our findings indicate the potential risk of pathogen transmission to humans through contaminated milk with *Nocardia* spp.

Key words: *Nocardia* spp., bovine, bulk tank milk, thermoresistance, boiling

Somatic cell count from bulk tank milk (BTMSCC) is a function of the percentage of quarters infected by major pathogens in a dairy herd. Linear relationship between BTMSCC and the percentage of quarters infected with major pathogens was previously described (Rysanek *et al*., 2007). The identification of *Staphylococcus aureus* and *Streptococcus agalactiae* in bulk tank milk samples (BTMS) has indicate the presence of cows in the herd with infectious mastitis by these pathogens. The isolation of organisms such as coliforms, yeasts, *Nocardia* spp., and *Streptococcus* spp. (non-agalactiae) in BTMS does not necessarily indicate the occurrence of environmental mastitis, because the presence of microorganisms may be the result of direct contamination of the milk (Schoonderwoeder *et al*., 1990).

Nocardia spp. are environmental microorganisms in etiology of bovine mastitis. It inhabits the telluric environment and infects the mammary gland from different sources, such as: contaminated surroundings that the cows are exposed between milking periods in farms with poor hygienic management during milking, accumulated faecal material on the mammary gland, contaminated water used during milking, contaminated pre and post dipping solutions, or even contamination of the cannula during intra-mammary treatment (Ribeiro *et al*., 2008). *N. asteroides*, *N. farcinica*, *N. nova* and *N. brasiliensis* are recognized as the main pathogenic species in bovine mastitis (Radostits *et al*., 2007). Recently, the classification of genus *Nocardia* has been based on molecular methods, using 16S rRNA gene (Kageyama *et al*., 2004).

Despite considered an uncommon pathogen of bovine mastitis, the identification of *Nocardia* spp. from mammary infections has been increasingly notified in the last years in several countries (Ollis *et al*., 1991; Radostitis *et al*., 2007),

***Corresponding Author. Mailing address:** Univ. Estadual Paulista, UNESP, Infectious Diseases of Domestic Animals, Department of Veterinary Hygiene and Public Health, School of Veterinary Medicine, Botucatu, State of Sao Paulo, Brazil.; E-mail: mgribeiro@fmvz.unesp.br

including in Brazil (Costa *et al*., 1998; Ribeiro *et al*., 2006; Ribeiro *et al*., 2008). These studies also show the importance of investigate the epidemiology of mammary nocardiosis, in order to subsidising control and preventive actions (Costa *et al*., 1998). The milk is considered an important source of transmission of *Nocardia* to humans, mainly by the consumption of raw milk and milk products or submitted to inadequate thermic treatment (Pier and Enright, 1961).

Recent studies have demonstrated the thermoresistance of *Nocardia* strains isolated from bovine milk (Costa *et al*., 1996). The boiling of milk is one of the oldest procedures used in the elimination of pathogens from milk (Furlanetto *et al*., 2009). However there are no preliminary studies in order to investigate the efficacy of this thermal procedure in *Nocardia* strains isolated from bovine milk.

Two individual bulk tank samples in the farms located at central region in State of São Paulo were collected in sterilized flasks, adequate for microbiological culture, SCC and total bacterial cell count. The results of total bacterial cell counts of these milk samples were 300.000 CFU/mL and 172 CFU/mL, while the somatic cell counts were 1.966.000 cells/mL and 890 cells/ mL, respectively.

Both samples were submitted to microbiological culture on defibrinated sheep blood agar (5%) and maintained in aerobic conditions, at 37^{o}C, for up to five days (Quinn *et al*., 1994). Simultaneously, the same samples were submitted to culture on agar Sabouraud–dextrose, in aerobiosis, at 37^{o}C, maintained for up to 15 days (Brown-Elliot *et al*., 2006). After 48 hours, white, dry and strongly adhered colonies were obtained. At microscopy, Gram and Kinyoun stains revealed Gram positive organisms, delicate and branched mycelial structures, with coccobacilli and filamentous forms suggestive of genus *Nocardia* (Quinn *et al*., 1994; Ribeiro *et al*., 2008).

Preparation of genomic DNA samples for sequencing was performed using the guanidine thiocyanate method (Kageyama *et al*., 2004). Nearly complete 16S rRNA gene (rDNA) sequences were obtained for isolated strains. The 16S rDNA was amplified and sequenced using PCR prokaryotic 16S rDNA universal primer pairs 8F and 691R, 520F and 1100R, and 926F and 1542R. The strains were submitted to PCR using a DNA thermal cycler (TaKaRa Bio Inc., Japan) under followed conditions: 35 cycles at 94ºC for 60s for denaturation, 60ºC for 60s for primer annealing, and 72ºC for 120s primer extension. The PCR products were purified with Centri-Sep Columns (Princeton Separations Inc., USA). The DNA sequences were determined with an automatic sequence analyzer (ABI Prism 3130; Applied Biosystems Inc., Japan), using a dye terminator cycle sequencing kit (Applied Biosystems Inc.). Sequences of the 16S rRNA genes were compared against GenBank/DDBJ/EMBL database using BLAST. Sequence data of *Nocardia* type strains were retrieved from GenBank. Phylogenetic analysis was performed using software (MEGA ver. 4; Tamura *et al.,* 2007) as described previously (Shibazaki *et al*., 2011). Sequencing analysis of the 16S rDNA segments enabled classified the organisms as *Nocardia farcinica* and *Nocardia cyriacigeorgica*. The new sequences for the isolates (strain numbers IFM 11294 and IFM 11295) were deposited to DDBJ/GenBank/EMBL with accession # AB671776.1 and AB671777.1, respectively.

All isolates were exposed to "in vitro" evaluations to boiling conditions. The isolates were initially cultured in defibrinated sheep blood agar (5%) and maintained in aerobiosis at 37ºC for 72 hours. Latter, two or three typical colonies were re-suspended in 4mL of sterile Mili–Q water (ultra–pure), acconditioned in tubes with the turbidity adjusted by optical density, according to the tube 1 (one) on the McFarland scale (Bier, 1984). From the suspension, the tubes were homogenized and diluted to the decimal scale (1/10), namely: 10^{-1}, 10^{-2}, 10^{-3} and subsequently until 10^{-10}. Aliquots of 0,1mL were collected in duplicates and cultured in "Plate count agar"–PCA, using the "pour plate" technique, and maintained incubated in aerobiosis at 37ºC, for up to 96 hours. The colony-forming unit count – CFU was evaluated by the macroscopic visualization of the colonies on the culture media.

The decimal dilutions that presented bacterial growth of 10^5 CFU/mL, which corresponded to the 10^{-3} and 10^{-2} dilutions, were used as inocula. Subsequently, 1mL from all the isolates containing 10^5 CFU/mL were pipetted to glass tubes closed with hydrophobic cotton and submitted to a elevation of temperature, in a bath, until it reached 100ºC (boiling 1). For temperature measurement, a sterile thermometer was placed in one of the tubes with reference strain. The same procedure was repeated for all isolates (also containing inocula with 10^5 CFU/mL), maintaining the tubes in the bath 1 minute after they have reached 100ºC (boiling 2). After these, the procedure was executed again, maintaining the tubes in the bath for 5 minutes after they have reached 100ºC (boiling 3). Aliquots of 0,01 mL from the tubes from all three thermal treatment groups (boiling 1, 2 and 3) were submitted to microbiological culture in defibrinated sheep blood agar (5%), in aerobiosis at 37ºC, in order to evaluate the re-isolation of strains after thermal treatment.

The thermic treatment showed that *Nocardia cyriacigeorgica* strain survived to boiling at 100ºC, while isolate of *Nocardia farcinica* survived for one minute at 100ºC.

In the individual bulk tanks from which *Nocardia* spp. was isolated, the SCC was higher than recommended by the Brazilian regulations of 600.000 cells/mL (Brasil, 2011). A comparative analysis showed that 200.000 cells/mL of CCS in individual bulk tanks corresponds to 15% of infected quarters in a herd, and 700.000 cells/mL represents two thirds or more quarters infected by a pathogen (NMC, 2001). Schoonderwoerd *et al*. (1990) reported *Nocardia* genus in milk from affected quarters and from the respective individual bulk tanks (205.000 cells/mL of SCC), indicating that the origins of the agent in the tanks were the affected quarters, or contaminated utensils used in the storage of milk and the tanks. This association between SCC in the bulk tank and the *Nocardia* mastitis indicate the importance of evaluating the SCC in bulk tank in order to evaluate the milk quality. Furthemore, the presence of *Nocardia* species in bulk tank milk indicate the need of increment on dairy farms of control measures against environmental agents of bovine mastitis.

N. farcinica was referred as one of the main bovine mastitis causal agent in Brazilian dairy herds. However, in this study the identification of the pathogen was performed by phenotypic evaluation (Ribeiro *et al*., 2008). The present report described for the first time the molecular identification of *N. farcinica* in bulk tank milk from dairy herds.

N. cyriacigeorgica has been identified recently in other countries around 10% to 22% of the *Nocardia* species causing disease in humans patients (Muñoz *et al*., 2007; Tremblay *et al*., 2010; Tan *et al*., 2010). In Brazil, Chedid *et al*. (2007) studied 22 cases of immunosuppressed patients affected by nocardiosis, and 27.27% of these cases were caused by *N. asteroides* complex, to which the *N. cyriacigeorgica* species belongs. The *N. cyriacigeorgica* was recently classified based on molecular techniques (Kageyama *et al*., 2004). Due to this new taxonomic classification, there are no recent descriptions of infection by this specie in humans and animals in Brazil. Although the role of animals in the transmission of *Nocardia* to humans remains unclear, our findings indicate the potential risk of transmission of *Nocardia* from cows to humans by milk.

The evaluation of thermoresistance of isolates showed that the pathogen resists to boiling. Other studies have submitted isolates to inferior temperatures for longer periods, such as 45ºC and 50 ºC for up to eight hours, with bacterial growth after thermal treatment (Komaid, 2001). Likewise, *N. asteroides* isolated from bovine milk was submitted to pasteurization conditions also with bacterial growth (Costa *et al*., 1996). *Nocardia serbivorans* was described to have survived to boil (Erikson, 1955).

The present report showed that *Nocardia* genus has the similar behaviour of a thermoduric microorganism. Our findings indicate potential risk to human consumption of raw, pasteurized and boiled milk contaminated by *Nocardia* spp., especially for the immunosuppressed human patients.

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