Short Communication: Outbreak of *Nocardia neocaledoniensis* Mastitis in an Italian Dairy Herd

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ABSTRACT

Nocardia spp. are an uncommon cause of mastitis, and outbreaks have typically been reported in dairy farms with poor hygienic and management conditions. The outbreak described herein involved a dairy farm with 43 lactating cows that, after a long period with low bulk milk somatic cell counts (<180,000 cells/mL), experienced an increasing incidence of clinical mastitis with bulk milk somatic cell counts greater than 300,000 cells/mL. Fifteen mastitic quarters milk samples from 9 dairy cows were found to be infected by a member of the genus *Nocardia*, as identified on the basis of selected phenotypic and chemotaxonomic characteristics. The isolates were confirmed as *Nocardia neocaledoniensis* by 16S rDNA gene sequencing. Average quarter milk somatic cell count for infected udders was 863,057 cells/mL, significantly greater than the average value in noninfected quarters (189,710 cells/mL).

Key words: *Nocardia neocaledoniensis*, mastitis, immunohistochemistry

Nocardia genus is recognized worldwide as a cause of disease in human beings and animals, particularly mammals and birds. There has been a recent revolution in the taxonomy of the *Nocardia* genus. In the last few years, a wide range of biochemical and susceptibility tests, and the application of molecular techniques (for example, 16S rRNA gene sequencing), have expanded the spectrum of the *Nocardia* genus to more than 30 named species (Chun and Goodfellow, 1995; Conville et al., 2000; Roth et al., 2003).

Nocardia spp. are ubiquitous environmental saprophytes, and they are not a part of the normal flora of mammals, although they may be carried mechanically on the skin. Infection usually arises from direct inoculation of soft tissues after penetrating injuries, by inhalation of aerosols containing organisms, possibly in dust,

or through natural routes like the teats of dairy cows (e.g., during milking or mastitis treatment). Fragmentation of branching nocardial filaments late in their growth cycle results in the formation of small, unicellular, spore-like cells that are easily aerosolized from environmental substrates. Infection induces a pyogranulomatous inflammation characterized by lesions mainly reported on the skin and in the lungs, oral cavity, nervous system, soft tissues, and bones.

Nocardia spp. are an uncommon cause of mastitis, and outbreaks have usually been reported only in dairy farms with poor hygienic and management conditions. Clinical features of mastitis induced by *Nocardia* can be severe, with diffuse fibrosis and firmness of the udder, reduced milk production, altered milk containing clots of exudates and with systemic health problems, anorexia, and fever (Battig et al., 1990; Tarabla et al., 1993).

The outbreak described here involved a dairy farm with 43 lactating cows that, after a long period with low bulk milk SCC (BMSCC, BMSCC < 180,000 cells/mL), experienced an increasing incidence of clinical mastitis with BMSCC greater than 300,000 cells/mL. Quarter milk samples were collected from 43 cows. Teat ends were cleaned with chlorhexidine before sampling. The first streams of foremilk were discharged, and then 10 mL of milk was collected aseptically from each udder into sterile vials. Ten microliters of each milk sample was plated on 5% bovine blood agar and on Sabouraud's dextrose agar, incubated aerobically at 37°C, and checked daily for bacterial growth.

Pinpoint colonies were generally evident at 24 h, and by 4 d, characteristic aerial hyphae were present on the surface of colonies 2 to 3 mm in diameter. *Nocardia* colonies were dry, tightly adherent to the surface of the plates and chalky white, somewhat resembling fine cotton, when aerial hyphae were produced. *Nocardia* spp. were identified by gram staining and biochemical tests (32C Yeast Identification System, BioMerieux, Rome, Italy). Furthermore, we tested for growth at 45°C and for additional phenotypic properties as described by Saintpierre-Bonaccio et al. (2004): growth on sodium

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citrate and sodium acetate (0.1%, weight/volume), nitrate reduction and urea hydrolysis, and decomposition of casein (1.0%, weight/volume) and tyrosine (5.0%, weight/volume).

Antimicrobial susceptibility tests were performed for all isolates using a disk diffusion method with commercial disks of gentamicin (10 µg), kanamycin (30 µg), neomycin (30 µg), and streptomycin (10 µg), as previously described by Wallace and Steele (1988). A zone size of ≤10 mm was defined as the breakpoint indicating resistance to gentamicin and streptomycin after 72 h of incubation at 35°C, whereas the size for kanamycin and neomycin was defined as ≤20 mm (Wallace and Steele, 1988).

For molecular characterization, DNA was extracted from colonies, milk samples, and paraffin-embedded tissues with QIAamp DNA Mini Kit (Qiagen, Milan, Italy), according to the instructions of the manufacturer, and was subjected to PCR amplification. A 606-bp fragment of the 16S rRNA gene and a 441-bp fragment of the *hsp65* gene encoding the 65-kDa heat shock protein were amplified with primers described in Rodriguez-Nava et al. (2006) for comprehensive *Nocardia* spp. identification.

All epizootic strains were morphologically and phenotypically identical. According to molecular characterization, the representative epizootic strain showed 100% similarity to *Nocardia neocaledoniensis* for 16S rRNA and *hsp65* genes.

Among the 172 cultured milk samples that were evaluated in this study, 55.6% showed the presence of IMI. Coagulase-negative staphylococci were the predominant microorganism, being present in 29.6% of the milk samples. Fifteen mammary quarters (18.5%) from 9 cows were infected by *N. neocaledoniensis*, and *Escherichia coli* and *Streptococcus* spp. together accounted for 7.5% of mammary quarters. For each sample, the quarter milk SCC was determined with an automated fluorescent microscopic somatic cell counter (Bentley Somacount 150, Bentley Instrument, Chaska, MN).

For all statistical procedures, means and their standard errors (SEM) were computed by using statistical software (SPSS 15.0, SPSS Inc., Chicago, IL). Results are expressed as mean values ± standard errors of the mean. Statistical analysis was performed by using 1-way ANOVA, and significant differences were declared at $P < 0.01$.

The mean quarter milk SCC for infected udders was $863 \pm 107.2 \times 10^3$ cells/mL (range 116 to $3,712 \times 10^3$ cells/mL). This was significantly greater ($P < 0.01$) than the mean value in noninfected quarters ($189.7 \pm 18.6 \pm 10^3$ cell/mL, range 8 to $1,129 \times 10^3$ cells/mL).

All epizootic strains showed the same susceptibility pattern. They were resistant to streptomycin and kana-

Figure 1. Immunohistochemistry of bovine infected mammary gland. In the necrotic tissue, *Nocardia neocaledoniensis* colonies, strongly positive for Grocott stain, are easily recognizable. Magnification bar represents 50 µm.

mycin but were susceptible to neomycin and gentamicin.

It is obvious that the in vitro activity of antimicrobial agents is a major criterion for the choice of therapy. The experiences of Gillespie and Timoney (1981), Sears (1986), and Tarabla et al. (1993), however, suggested that the prognosis is unfavorable with antibiotic therapy and that there is a risk of spread of infection to the rest of the herd. Consequently, the affected animals in this herd were culled. At necropsy, the infected quarters were characterized by a diffuse nodular firmness and with thickened linings of teat sinuses and gland cistern. Supramammary and inguinal lymph nodes were edematous but did not contain focal necrotic lesions. Neither gross nor microscopic lesions were observed in other organs or tissues.

Samples from mammary glands and supramammary lymph nodes of slaughtered infected cows were fixed with formalin and embedded in paraffin. Histological examination of 4-µm-thick sections, stained with hematoxylin-eosin, Gram and Grocott, revealed large, poorly encapsulated, multifocal to coalescing pyogranulomatous lesions. Single pyogranulomas were characterized by a center of necrotic debris and neutrophils, intermixed with epithelioid elements and rare giant cells. Surrounding the necrosis, several eosinophilic colonies of *Nocardia*-like Gram and Grocott-positive bacteria (Figure 1) surrounded by epithelioid cells were clearly recognizable. Lymphocytes and plasma cells, intermixed with fibroblasts, constituted the outermost layer of the lesions. In the lymph nodes, only hyperplastic

Figure 2. Immunohistochemistry of bovine infected mammary gland. Positive colony of *Nocardia neocaledoniensis*, surrounded by inflammatory cells, is observed in a mammary alveolus. Magnification bar represents 50 μ m.

changes were observed, and Gram staining resulted negative.

Immunohistochemistry was performed using the avidin-biotin-peroxidase complex (ABC) method (Hsu et al., 1981). The polyclonal serum against *Nocardia* was used as primary antibody, 1:60,000 diluted in Tris-buffered saline. As a negative control, for each immunohistochemical test, sections from the mammary gland and lymph nodes were incubated with a polyclonal antibody directed against *Mycoplasma mycoides*. The immunohistochemical reaction was developed with 3-3'-diaminobenzidine. Sections were then counterstained with Mayer's haematoxylin, dehydrated through graded alcohols, clarified in xylene, and mounted in balm. With immunohistochemistry, bacterial colonies were always strongly positive with a good granular brownish reaction (Figure 2). Moreover, numerous positive single bacterial bodies were also recognizable in the necrotic center. Conversely, all the sections of lymph nodes examined gave negative results.

Outbreaks of nocardiosis are rare and have usually involved only a limited number of animals with particular risk factors in the same herd, particularly the use of blanket dry cow therapy, especially neomycin-containing products supplied in multidose vials (Ferns et al., 1991; Ollis et al., 1991; Stark and Anderson, 1990). The difficulties of diagnosis, however, can lead to an underestimation of the real prevalence and incidence of cases. The source of infection in this herd has never been located with certainty, but epidemiological investigation points to inadequate hygienic procedure during administration of intramammary therapy as the most

likely cause. Segregation of infected cows, mainly during milking, and their culling have been shown to prevent the further spread of infection within the herd (Sears, 1986).

Effective control of mastitis due to *Nocardia* spp. is based on early detection, for which definitive and early diagnosis are critical. The need for early detection has encouraged the development of faster and more sensitive diagnostic techniques. Because infected animals could yield negative results on aerobic culture for several weeks before *Nocardia* can be isolated and because, in a mixed culture, other organisms could plausibly mask the slower growing of *Nocardia*, diagnostic tests other than bacteriological exams should be considered when *Nocardia* is suspected.

Moreover, *Nocardia* spp. identification requires laborious, time-consuming phenotypic and chemotaxonomic methods. Molecular methods offer an alternative, particularly when bacterial cultures are not possible. The advantages of molecular identification of bacteria using 16S rRNA sequencing are rapid turnaround time, reliable identification, and taxonomic meaningfulness. The 16S rRNA gene of *Nocardia* is highly conserved with constant regions that are identical for all *Nocardia* spp. and variable regions that are species-specific (Brown-Elliott et al., 2006).

To our knowledge, this is the first report of detection of *N. neocaledoniensis*, a novel actinomycete isolated from soil, as responsible for bovine mastitis. Progress in the epidemiological analysis of cases of nocardiosis will be achieved when improved diagnostics for these infections (molecular and serological diagnosis) are available, when the genetic diversity of *Nocardia* spp. isolates is fully understood, and when molecular typing is systematically performed in populations with increased cases of nocardiosis.

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