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Short communication: Molecular characteristics, antimicrobial susceptibility, and pathogenicity of clinical *Nocardia cyriacigeorgica* isolates from an outbreak of bovine mastitis

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ABSTRACT

The occurrence of nocardial mastitis, mostly in the context of outbreaks, has been reported in many countries. However, there is a paucity of reports regarding detailed characterization of *Nocardia cyriacigeorgica* from bovine mastitis. Thus, herein we report characteristics, antimicrobial susceptibility patterns, molecular identification, and pathogenicity of *N. cyriacigeorgica* isolated from an outbreak of clinical mastitis in a dairy herd in northern China. A total of 182 (80.2%) lactating cows had clinical mastitis with severe inflammation and firmness of the udder, reduced milk production, and anorexia, with no apparent clinical response to common antibiotics. Out of 22 mastitic milk samples submitted to our laboratory, 12 *N. cyriacigeorgica* were isolated and characterized using standard microbiological analysis, 16S rRNA gene sequencing, random amplified polymorphic DNA PCR analysis, biochemical assays, and antibiotic susceptibility testing. Additionally, in vivo experiments were done to determine pathogenicity of these clinical mastitis isolates. All isolates were resistant to ampicillin, amoxicillin-clavulanic acid, ciprofloxacin, minocycline, rifampicin, and aminoglycosides (type VI pattern). Additionally, intramammary inoculation of mice with *N. cyriacigeorgica* caused chronic inflammatory changes, including hyperemia, edema, and infiltration of lymphocytes and neutrophils, as well as hyperplasia of lymph nodules in mammary glands. Therefore, we concluded that *N. cyriacigeorgica* was involved in the current outbreak of mastitis. To our best knowledge, this is the first report to characterize *N. cyriacigeorgica* isolated from cases of bovine mastitis in China.

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Short Communication

Multidrug-resistant *Nocardia* spp. have been reported to cause nocardiosis in humans and dairy cattle (e.g., Condas et al., 2013; Hashemi-Shahraki et al., 2015). *Nocardia* is a genus of aerobic and saprophytic actinomycetes, gram-positive, branching, filamentous, and modified acid-fast bacilli. These environmental pathogens are ubiquitous in air, soil, water, and decaying organic matter (Brown-Elliott et al., 2006).

Mastitis is the most common clinical manifestation of nocardial infections in livestock (Condas et al., 2013). *Nocardia* spp. are considered a causative agent of bovine mastitis, with occurrence reported in several countries (Cook and Holliman, 2004; Pisoni et al., 2008; Ribeiro et al., 2008). Clinically, nocardial mastitis is characterized by a suppurative or granulomatous inflammation of the mammary gland, with severe clinical manifestations, diffuse udder fibrosis and firmness, reduced milk production, and altered milk appearance, as well as systemic involvement, including anorexia and fever (Pisoni et al., 2008; Kogure et al., 2010; Condas et al., 2013). *Nocardia* spp. are usually resistant to most antimicrobials commonly used in veterinary medicine (e.g., gentamicin, amoxicillin-clavulanic acid, ampicillin, ciprofloxacin, kanamycin, and ceftriaxone), leading to chronic IMI resulting in drying up of the quarter and culling of the cow (Radostits et al., 2007). Interestingly, *Nocardia* spp. have a variety of antibiotic susceptibility patterns, which can be used for phenotypic identification (Brown-Elliott et al., 2006).

The most prevalent species of *Nocardia* associated with bovine mastitis are *Nocardia farcinica*, *Nocardia asteroides*, *Nocardia nova*, and *Nocardia brasiliensis* (Radostits et al., 2007; Condas et al., 2013). So far, apparently, only 1 report is available regarding isola-

tion of *N. cyriacigeorgica* from bovine mastitis (Condas et al., 2013), whereas nocardiosis caused by *N. cyriacigeorgica* is more frequently reported in humans (Hashemi-Shahraki et al., 2015; Xiao et al., 2016). The aim of present study was to investigate the biochemical characteristics and phylogenetic relatedness using 16S rRNA gene sequencing, antimicrobial susceptibility patterns, and pathogenicity of *N. cyriacigeorgica* isolated from a severe outbreak of bovine mastitis.

In a commercial privately owned dairy herd located in Tangshan city, Hebei province, in the north of China, composed of 550 Holstein-Friesian cattle, including 227 lactating cows, an outbreak of severe clinical mastitis (**CM**) occurred June 11 to 18, 2013. The lactating cows were fed a TMR and were housed in a bedded-pack barn with inadequate bedding and a surface covered with wet manure. Before the outbreak, there was 10 d of continuous rain and daily peak ambient temperatures were 22 to 29°C. Lactating cows were milked thrice daily by 4 milking staff at 0600, 1400, and 2000 h in a 2 × 12 parallel milking parlor. The milking machine was inadequately maintained, with excessive vacuum pressure (47.8 kPa). Both pre- and postmilking teat disinfection was done by dipping with 0.15% iodine solution. One towel was used for 2 cows. At drying off, all cows were given intramammary treatments with Orbenin EDC (Cloxacillin benzathine, Zoetis, Parsippany, NJ). The herd did not use any self-compounded drugs. During the outbreak, 22 quarter milk samples were aseptically collected from 22 cows with CM that had not recently received antibiotic treatment. Samples were shipped on ice to the mastitis laboratory at the College of Veterinary Medicine, China Agricultural University, Beijing.

Milk samples (10 μL) were streaked onto tryptose soy agar (**TSA**; Aoboxing Bio-Tech, Beijing, China) supplemented with 5% defibrinated sheep blood (also known as sheep blood agar, **SBA**) using aseptic cotton swabs and incubated for 24 to 72 h at 37°C. *Nocardia*-like colonies were sub-cultured on SBA, Middlebrook 7H10 agar supplemented with 0.5% glycerol, and 10% Middlebrook ADC enrichment (7H10, Becton Dickinson and Company, Franklin Lakes, NJ) and Gause's synthetic agar (**GSA**, Land Bridge Technology Co., Ltd., Beijing, China). Staining properties were tested by Gram and Ziehl-Neelsen staining (Solarbio Science and Technology, Beijing, China). Spores and mycelium were investigated with a light microscope (Olympus, Tokyo, Japan) and a scanning electron microscope (Hitachi S-3400N, Tokyo, Japan).

Molecular identification of clinical isolates was done with 16S rRNA sequence analysis, using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGCTACCTTGTTACGACTT-3′), which were used for both amplification and sequencing. Sequences were analyzed in the National Center for Biotechnology Information database using the Basic Local Alignment Search Tool (**BLAST**). The phylogenetic tree, based on 100 bootstrap replicates, was constructed by employing the neighbor joining method using MEGA 5.2 software (http://www.megasoftware.net), based on the 16S rRNA gene sequences trimmed to 1,387 bp of clinical *Nocardia* isolates and some other *Nocardia* species ATCC strains downloaded from the National Center for Biotechnology Information (Figure 1). Genetic distances were calculated using a Kimura's 2-parameter model. *Mycobacterium bovis* ATCC BAA-935 (CP009449.1) was used as an outgroup species.

Random amplified polymorphic DNA (**RAPD**) PCR analysis was performed with 12 *N. cyriacigeorgica* isolates using the RAPD primer RBA 15A 5′-AAGAGCCCGT-3′ according to previously described protocols (Trivedi, 2015). Amplified DNA fragments were separated on a 1.5% agarose gels, stained with ethidium bromide, and visualized by gel documentation system (Alpha Imager EC, San Leandro, CA). The similarity between strains was clustered by using the Dice coefficient and the unweighted pair group method with arithmetic averages (UPGMA) of the PFGE profiles (Liu et al., 2015). Growth of *N. cyriacigeorgica* isolates was assessed on duplicate TSA plates, incubated at 37 and 45°C for 5 d. Biochemical tests were performed according to the manufacturer's instructions (http://www.tianhe138.com/download5.aspx?InfoVer=500&ClassID=20&PID=5), which included hydrolysis of adenine, L-tyrosine, xanthine, hypoxanthine, casein, urea (Sigma, St. Louis, MO); utilization of citrate, esculin, rhamnose, sorbitol, L-arabinose, fructose, galactose, lactose, mannitol, inositol, xylose, glucose, sucrose, and acetamide; and production of catalase and nitrate reductase (Hangzhou Binhe Microorganism Reagent Co. Ltd., Hangzhou, China). In addition, adenine, hypoxanthine, L-tyrosine, xanthine, and casein decomposition were also performed (Gordon et al., 1974), and results were assessed after 7 and 14 d. Negative controls, without *Nocardia*, were similarly processed. For the catalase test, a loop of colony was transferred to a drop of 3% H_2O_2; the reaction was recorded as positive if bubbles appeared immediately.

Antimicrobial susceptibility patterns of all isolates were determined against 15 antimicrobial agents using the MIC method (broth microdilution), according to CLSI M24-A2 (CLSI, 2011) recommendations. Antimicrobial agents included amoxicillin-clavulanic acid (5:1), amikacin, cefotaxime, ciprofloxacin, minocycline, sulfamethoxazole, tetracycline (BBI Life Sciences Corporation, Shanghai, China), imipenem, rifampicin, clarithromycin, and ceftiofur (Sigma), gentamicin, ampicillin, kanamycin (Amresco, Solon, OH), and pirlimy-