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.

Key words: bovine mastitis, *Nocardia cyriacigeorgica*, outbreak, antimicrobial resistance, pathogenicity

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-

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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 tryptone 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'-TACGGCTACCTTGTACGACTT-3'), which were used for both amplification and sequenc-

ing. 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% H₂O₂; 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-

cin (Yuduo Biological Technology Co., Ltd., Shanghai, China). In the absence of CLSI breakpoints for ampicillin, kanamycin, ceftiofur, pirlimycin, and tetracycline for *Nocardia* spp., CLSI M07-A9 recommendations for aerobic bacteria (CLSI, 2012) and VET01 recommendations for bacteria isolated from animals were used (CLSI, 2013). *Staphylococcus aureus* ATCC 29213 was used as the quality control strain.

Thirty-six pregnant 12-wk-old outbred ICR mice were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China) to determine pathogenicity of clinical *N. cyriacigeorgica* isolates. At 3 d postpartum, suckling mice were removed and the dams were inoculated. Mice were anaesthetized with tiletamine hydrochloride and zolazepam hydrochloride; 10 mg/kg injected i.m.; Zoletil 50, Virbac Laboratories, Carros, France), and inoculated with 2.5×10^4 cfu of the mid-log phase *N. cyriacigeorgica*, placed in a 1-mL sterile syringe and injected through a 32-gauge blunt

needle into the nipples of the fourth and fifth left abdominal mammary glands. Intramammary inoculation was performed with the assistance of a bench magnifier (3.0 $\times$). In negative control groups, mice were inoculated with sterile 0.1 M PBS (pH 7.4).

Clinical symptoms of challenged mice were scored as follows: 0 = normal; 1 = mildly messy fur; 2 = depression and obvious messy fur; 3 = cowered behavior and moderate depression; 4 = serious depression with prostration; and 5 = death. In both PBS and *Nocardia*-challenged groups, 18 mice were scored on d 1, whereas 15, 12, 9, and 6 mice were scored on d 3, 5, 9, and 15, respectively. On d 1, 3, 5, 9, and 15 after inoculation, 3 mice were euthanized using cervical dislocation, inoculated mammary glands were excised aseptically, and samples fixed in 10% formalin buffer and processed using standard histopathological methods. Histopathological scoring of the mammary gland of mice challenged with *N. cyriacigeorgica* and PBS

Figure 1. Phylogenetic tree of clinical *Nocardia cyriacigeorgica* isolates and other *Nocardia* ATCC strains, based on 16S rRNA gene sequencing of 1,387-bp amplicons. Bar indicates the percentage of sequence divergence.

was performed according to lesion severity: 0 = absence of histopathological changes; mild, sub-moderate, moderate, and severe were scored as 1, 2, 3, and 4, respectively. Three mice were scored on the specific day both in PBS and *Nocardia*-challenged groups, with 3 pathological sections per mouse mammary gland. Data were represented as mean $\pm$ standard deviation. Differences between *N. cyriacigeorgica* and PBS-challenged groups at the same time point were estimated using a Student's *t*-test in GraphPad Prism version 5.0 for Windows (GraphPad Software Inc., La Jolla, CA). $P < 0.05$ and $P < 0.01$ were regarded as statistically significant and highly significant, respectively.

The animal experiment was conducted in compliance with guidelines of the Beijing Municipality on the Review of Welfare and Ethics of Laboratory Animals, approved by the Beijing Municipality Administration Office of Laboratory Animals and under a protocol (CAU-AEC-2010-0603) approved by the China Agricultural University (Beijing, P.R. China) Animal Ethics Committee.

A total of 206 quarters, 81 rear quarters and 125 front quarters, of 182 (80.2%) lactating cows were affected with CM. Of the 182 cows with CM, 61 (73%) of 84 were first lactation heifers, 56 (78%) of 72 were in second parity, and 65 (92%) of 71 were cows with parity ≥ 3 . No CM cases were observed among dry cows and nonlactating heifers. Average milk production before the outbreak was 25 L/d, whereas after the outbreak, milk production decreased to 15 L/d. Bulk milk SCC was $\sim 450,000$ cells/mL before the outbreak, but increased to 800,000 cells/mL after the outbreak (milk from all affected cows was included in the bulk tank after treatment). During the CM outbreak, individual SCC of unaffected animals was not determined, but average SCC of those cows apparently did not increase.

Clinical manifestations in all affected cattle, as noted by the herd's veterinarian, consisted of severe inflammation of mammary glands, firmness of the udder tissues, reduced milk production along with abnormal consistency of milk, and systemic signs of anorexia and conjunctiva hyperemia. No increase was observed in the incidence of other health events. Once daily, cows with CM were given ceftiofur sodium (Shangqiu Huakang Animal Pharmaceutical Co., Ltd., Henan province, China) as an intramammary infusion and intramuscular injections of 4×10^6 IU of benzylpenicillin potassium (Beijing Cephal Pharmaceutical Corporation, Beijing, China) and 1 million IU of kanamycin sulfate (Beijing Lishida Pharmaceutical Co., Ltd., Beijing, China) until symptoms disappeared. However, many CM cases did not cure clinically, and 23 (13%) of cows were eventually culled.

Pure growth of *N. cyriacigeorgica* was obtained on SBA plates for 12 of the 22 quarter milk samples. At least 3 types of bacterial colonies were observed on 5 SBA plates, indicating contamination, whereas 5 samples were culture negative. After 72 h incubation, *N. cyriacigeorgica* colonies showed a different appearance on SBA, Middlebrook 7H10, and GSA by visual and microscopic observation (Supplemental Figures S1A–S1F; <https://doi.org/10.3168/jds.2017-12680>). *Nocardia cyriacigeorgica* isolates were Gram-positive (Supplemental Figures S1G and S1H; <https://doi.org/10.3168/jds.2017-12680>), but were partially positive using Ziehl-Neelsen (Supplemental Figure S1I; <https://doi.org/10.3168/jds.2017-12680>). When viewed with a scanning electron microscope, *N. cyriacigeorgica* appeared as spherical spores, filaments that can fragment into bacillary and coccoid forms on 7H10 agar (Supplemental Figures S2A and S2B; <https://doi.org/10.3168/jds.2017-12680>), or *Streptococcus*-like mycelium on GSA agar (Supplemental Figure S2C; <https://doi.org/10.3168/jds.2017-12680>).

Based on BLAST analysis of 16S rRNA gene sequencing, clinical isolates were closely related to *N. cyriacigeorgica*, *N. asteroides*, and *N. abscessus* (homologies of 99–100, 98–99, and 98–99%, respectively). The phylogenetic tree, based on 16S rRNA gene sequence of 1,387-bp sized amplicons, is shown (Figure 1). The *N. cyriacigeorgica* isolates (HB1 to HB12) formed a cluster with *N. cyriacigeorgica* ATCC 14759 with a high bootstrap value of 100; furthermore, the same bootstrap value was also observed between *N. abscessus* ATCC BAA-279 and *N. asteroides* ATCC 23824. In addition, clustering of *N. cyriacigeorgica* formed a branch with the nearby clustering of *N. abscessus* ATCC BAA-279, *N. asteroides* ATCC 23824, *N. brasiliensis* ATCC 700358, and *N. farcinica* ATCC 3318, with a bootstrap value of 74 indicating a close relationship among them. The RAPD-PCR analysis of 12 isolates showed a percentage of similarity ranging from approximately 38 to 91% (Supplemental Figure S3; <https://doi.org/10.3168/jds.2017-12680>), indicating high variability of these *N. cyriacigeorgica* isolates.

All *N. cyriacigeorgica* isolates were able to grow at both 37 and 45°C on TSA plates. All isolates were strongly positive for catalase, utilization of acetamide, and reduction of nitrate to nitrite (Supplemental Table S1; <https://doi.org/10.3168/jds.2017-12680>). Results for esculin hydrolysis and acid formation from galactose and xylose fermentation on d 2 were inconclusive; however, obvious color changes were observed when incubation was extended to d 5. Additionally, isolates were negative for hydrolysis of adenine, L-tyrosine, xanthine, hypoxanthine, casein, and urea; and utilization of ci-

trate, rhamnose, sorbitol, arabinose, fructose, lactose, mannitol, inositol, glucose, and sucrose (Supplemental Table S1; <https://doi.org/10.3168/jds.2017-12680>).

All clinical isolates were uniformly resistant to gentamicin, amoxicillin-clavulanic acid, clarithromycin, ciprofloxacin, minocycline, ampicillin, rifampin, kanamycin, pirlimycin, and tetracycline. Additionally, they had high-level resistance to clarithromycin (MIC >128 µg/mL), amoxicillin-clavulanic acid (MIC ≥64 µg/mL), ampicillin (MIC ≥64 µg/mL), rifampicin (MIC >64 µg/mL), kanamycin (MIC >256 µg/mL), pirlimycin (MIC >64 µg/mL), and tetracycline (MIC ≥32 µg/mL). Also, all *N. cyriaci-georgica* isolates were susceptible to amikacin and sulfamethoxazole, whereas 10 isolates were susceptible to cefotaxime and imipenem. Finally, 3 isolates were susceptible to ceftiofur and 1 isolate was resistant to ceftiofur (Table 1).

Starting at 3 d after inoculation, mice inoculated with *N. cyriaci-georgica* increasingly had signs of messy fur and anorexia, were sluggish, and had slightly cowered behavior in comparison to control mice ($P < 0.01$; Figure 2A). Inoculated mammary glands had no prominent visual signs; however, gross examination during autopsy revealed edema, hyperemia, and formation of nodules (diameter, 2–3 mm) in mammary glands at d 5. Histopathologically, mammary epithelial cells had vacuolated degeneration on d 1 (Figure 2C). On d 3, necrosis and severe exfoliation of epithelial cells appeared in the mammary alveolar tissues (Figure 2D), with infiltration of inflammatory cells, edema, and congestion present in the mesenchymal tissues from d 5. Furthermore, inflammatory cells and necrotic epithelial cells were also observed in mammary ducts and in atrophic alveoli (Figure 2E, 2F, and 2G). Additionally, inflammatory cells appeared in blood vessels in mammary gland and lymphoid tissues, and some mastocytes were detected in mammary glands on d 9. Degeneration and necrosis was observed in mammary epithelial cells (Figure 2G). The grossly apparent nodules were lymph nodes with swelling and hyperplasia (Figure 2H), with germinal centers with abnormal inflammatory cells and vacuolation in the necrotic areas. Mean histopathological scores of mouse mammary glands are presented (Figure 2B). In comparison to the control group, histopathological changes were observed in the mammary gland of mice challenged with *N. cyriaci-georgica* ($P < 0.01$), including infiltration of inflammatory cells, necrosis of epithelial cells, and hyperplasia of lymph nodes (mentioned above).

In this study, 12 clinical *N. cyriaci-georgica* isolates from an outbreak of CM were characterized. Accurate identification of *Nocardia* spp. is necessary for clinical epidemiological studies. Bovine nocardial mastitis has been reported to be strongly associated with poor man-

Table 1. Antibiotic susceptibility patterns of 12 clinical mastitis *Nocardia cyriaci-georgica* isolates by MIC

Drug ¹	MIC (µg/mL) of <i>N. cyriaci-georgica</i> isolates												CLSI break point ²			Resistance rate (%)
	HBN1	HBN2	HBN3	HBN4	HBN5	HBN6	HBN7	HBN8	HBN9	HBN10	HBN11	HBN12	R	I	S	
GEN	32	32	32	32	32	32	32	32	32	32	32	64	≥16	8	<4	100
KAN	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256	≥32	—	≤12	100
AMK	4	4	4	8	4	4	4	4	8	8	4	4	≥16	—	<8	0
AMC	64	128	128	128	128	128	128	128	128	128	64	128	≥32	16	<8	100
AMP	64	64	128	128	64	64	64	64	64	64	64	64	≥32	—	<0.25	100
CTX	8	16	4	8	8	8	8	8	8	16	4	8	—	16–32	<8	100
CFT	8	32	8	8	16	8	16	8	8	16	8	8	≥32	16	<8	8.3
CLR	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128	≥8	4	<2	100
CIP	16	16	16	16	16	16	16	16	16	16	16	16	≥4	2	<1	100
MIN	8	16	16	16	16	16	8	16	16	16	16	16	≥8	2–4	<1	100
TET	32	32	32	16	32	32	32	32	32	16	16	32	≥16	8	<4	100
SMZ	4	8	4	4	4	4	8	8	4	8	4	8	≥64	—	≤32	0
RIF	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	≥2	—	<1	100
IPM	4	8	4	4	8	4	4	4	4	4	4	4	≥16	8	<4	0
PIR	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	≥4	—	<2	100

¹GEN = gentamicin; KAN = kanamycin; AMK = amikacin; AMC = amoxicillin-clavulanic acid; AMP = ampicillin; CTX = cefotaxime; CFT = ceftiofur; CLR = clarithromycin; CIP = ciprofloxacin; MIN = minocycline; TET = tetracycline; SMZ = sulfamethoxazole; RIF = rifampicin; IPM = imipenem; PIR = pirlimycin.
²R = resistant; I = intermediate; S = susceptible.

agement practices and unhygienic measures in dairy herds (Ribeiro et al., 2008; Ali et al., 2014). Factors including improper disinfection of teats before and after milking and intramammary infusions with contaminated instruments or pharmaceuticals predisposed to nocardiosis (Ollis et al., 1991; Cook and Holliman, 2004). The dairy herd in the present study had poor hygiene and insufficient milking management and mastitis control practices, with high isolation rates in

CM samples of other bacteria including *Bacillus cereus* (41%), *Escherichia coli* (36%), *Staphylococcus chromogenes* (23%), and *Streptococcus dysgalactiae* (14%). Unfortunately, samples from bedding and the milking parlor were not collected for culture, and because the farm stopped dairy activities shortly after the outbreak, no samples could be obtained retrospectively. Given the lack of similarity in RAPD-PCR analysis (Supplemental Figure S3; <https://doi.org/10.3168/jds.2017-12680>),

Figure 2. Clinical scores (A), histopathological scores (B), and histopathology of mammary tissues of mice challenged with clinical *Nocardia cyriacigeorgica* isolates (hematoxylin-eosin staining; C to H). Swollen mammary epithelial cells with vacuolated degeneration on d 1 of infection (C). Severe exfoliation appeared in the mammary alveoli on d 3 (D). Multiple inflammatory cells (black arrow) in interstitial and necrotic cells in atrophic alveoli on d 5 (E). Interstitial edema and lymphoid tissue in the mammary gland including lymphocytes, neutrophils, granulocytes, macrophages, and necrotic cells, on d 9 (F). Large numbers of inflammatory cells (black arrow) in mammary tissues and blood vessels on d 15 (G). Lymph node with dispersed necrotic areas in the germinal center on d 15 (H). Results are presented as mean $\pm$ SD. $**P < 0.01$ was considered highly significant. Color version available online.

we concluded that the *N. cyriacigeorgica* isolated likely originated from the environment without cross-infection among cows. Perhaps bedding or the water used at milking (or both) were contaminated with *N. cyriacigeorgica*, as cows with this pathogen contaminate the soil, contributing to transmission (Pisoni et al., 2008). That the bedding was very dirty and not frequently removed may have caused repeated challenge of cows with this pathogen (Saubolle and Sussland, 2003). Furthermore, the herd did not use self-compounded drugs that could have been contaminated with *Nocardia*, and it is therefore unlikely that this was the source of the infection.

Nocardia cyriacigeorgica, *N. asteroides*, and *N. abscessus* used to be classified as *N. asteroides* complex (Brown-Elliott et al., 2006). Moreover, *N. cyriacigeorgica* used to be known as *N. asteroides* (Brown-Elliott et al., 2015). The 16S rRNA gene sequencing of *N. cyriacigeorgica* isolates had a homology of 98 to 99% to *N. asteroides* and *N. abscessus*, and all 3 had a close relationship in the phylogenetic tree. Although molecular methods, particularly 16S rRNA gene sequencing, are frequently used for taxonomy and identification of *Nocardia* spp. (Conville and Witebsky, 2007; Pisoni et al., 2008; Brown-Elliott et al., 2015), as done in the present study, biochemical characteristics and antimicrobial susceptibility patterns can also be used to verify the species.

The antibiotic susceptibility pattern of the clinical *N. cyriacigeorgica* isolates was type VI (resistant to ampicillin, amoxicillin-clavulanic acid, clarithromycin, and ciprofloxacin and susceptible to cephalosporins, amikacin, linezolid, and imipenem). This pattern is identical to the drug pattern of *N. asteroides*, whereas *N. abscessus* is reported to have a type I pattern (susceptible to ampicillin and amoxicillin-clavulanic acid; Brown-Elliott et al., 2006). Because *N. cyriacigeorgica* isolates were susceptible to amikacin, cefotaxime, ceftiofur, sulphamethoxazole, and imipenem, some could theoretically be used to treat mastitis caused by this pathogen, if diagnosed early. Due to the prolonged treatment with the low cure rate, human food safety, and public health concerns associated with drug residues, cattle suffering from nocardial mastitis should preferably be culled, which will also decrease the spread of nocardial infection (Cook and Holliman, 2004; Radostits et al., 2007).

Inoculation with clinical *N. cyriacigeorgica* isolates caused chronic inflammation of murine mammary glands. In addition, these mice had systemic symptoms and the contralateral mammary gland also became infected, implying that *Nocardia* may spread via blood circulation. This was apparently the first report to determine pathogenicity and histopathology of clinical

N. cyriacigeorgica isolates from bovine mastitis. Furthermore, very few studies reported histopathology of mammary tissues infected by other *Nocardia* spp. (Pisoni et al., 2008). In concordance to our study, Pisoni et al. (2008) also detected necrosis, infiltration of neutrophils and lymphocytes, and eosinophilic colonies of *Nocardia*-like organisms in bovine mammary tissues infected by *N. neocaledoniensis* during an outbreak.

To the best of our knowledge, this is the first report on the isolation, biochemical characteristics, and phylogenetic relatedness using 16S rRNA gene sequencing and antimicrobial susceptibility patterns of *N. cyriacigeorgica* isolated from an outbreak of CM in a dairy herd. The clinical *N. cyriacigeorgica* isolates caused considerable damage to murine mammary glands, indicating that *N. cyriacigeorgica* has potential to cause bovine mastitis.

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