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SHORT COMMUNICATION

Multilocus sequence analysis (MLSA) of a *Nocardia cyriacigeorgica* strain causing severe bovine mastitis in Bosnia and Herzegovina

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ABSTRACT

Nocardia cyriacigeorgica is a well-known agent of human nocardiosis and is considered an emerging pathogen, however, its identification to the species level is complex for many clinical laboratories. Available data on the clinical significance of *N. cyriacigeorgica* in veterinary medicine are sparse and mainly concern isolated reports of pyogranulomatous lesions in domestic animals. We report a case of severe bovine mastitis caused by *N. cyriacigeorgica* that did not respond to conventional antimicrobial therapy in a small holding in Bosnia and Herzegovina. After isolation of the pathogen, further identification by routine microbiological methods was not possible. Susceptibility to antimicrobials was tested using the disc diffusion method according to published recommendations. The sample was also tested by MALDI-ToF MS with inconclusive results. In addition, 16S rRNA sequence analysis, verified by multilocus sequence analysis (MLSA) using the gyrB, 16S rRNA, secA1, and hsp65 sequences, confirmed the species *N. cyriacigeorgica*. To our knowledge, this is the first report of isolation of *N. cyriacigeorgica* from a clinical case of bovine mastitis in a European dairy farm and the first MLSA method approach to distinguish a *Nocardia* spp. strain isolated from animals.

KEYWORDS

bovine mastitis, *Nocardia cyriacigeorgica*, MALDI-ToF MS, MLSA

After *Nocardia cyriacigeorgica* was first isolated from the bronchial secretions of a patient and described as a new species by Yassin et al. (2001), it became an emerging pathogen in humans (Schlaberg et al., 2008; Karan et al., 2019; Veličković et al., 2022). Although the incidence of nocardiosis is increasing, new literature data suggest that this species was misidentified before the introduction of molecular techniques into diagnostics (Mehta and Shamoo, 2020). In addition, a DNA-DNA hybridisation study of *Nocardia asteroides* drug pattern type VI (ATCC 14759 strain) and *N. cyriacigeorgica* (DSM 44484 strain) revealed that the pathogen previously referred to as *N. asteroides* is actually *N. cyriacigeorgica* (Conville and Witebsky, 2007). Information on the *Nocardia* species causing infections is clinically important because each species is different in terms of its susceptibility to antimicrobials, pathogenicity, and epidemiology (Conville et al., 2018). In veterinary medicine, there are only few data on *N. cyriacigeorgica*. So far, this bacterium has only been reported as a cause of bovine mastitis in Brazil and China (Condas et al., 2013; Chen et al., 2017). A study in Brazil found that *N. cyriacigeorgica* accounted for only 3% of *Nocardia* isolates causing udder inflammation (Condas et al., 2013). In a commercial dairy farm in China, a pathogenic strain of *N. cyriacigeorgica* was the cause of severe mastitis that could not be treated with antimicrobials (Chen et al., 2017), but the clinical significance of *N. cyriacigeorgica* in the aetiology

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of bovine mastitis under natural conditions remains less known. Taking into account the emerging pathogen status of *N. cyriacigeorgica* due to its high adaptability (Zoropogui et al., 2013) along with the difficulties to identify nocardial infections to the species level (Conville et al., 2018; Mehta and Shamoo, 2020), it is required to improve our understanding of this pathogen through reporting and classification of *Nocardia* spp. confirmed in animals. This study describes a case of severe mastitis with poor prognosis on a smallholder farm caused by *N. cyriacigeorgica*.

In early May 2022, a case of severe mastitis occurred in a six-year-old Simmental cow on a smallholder dairy farm in Bijeljina (44°46′00.1 ″N; 19°17′04.5 ″E) in north-eastern Bosnia and Herzegovina. The cow was in the first stage of lactation (~90 days after calving) and had no history of udder infections. Physical examination revealed pyrexia (41.5 °C) with depression and loss of appetite. On palpation, the udder was extremely hard with marked enlargement of the hindquarters and teats, with no pain or redness of the skin. Reduced milk production was noted, but a visual examination of the secretion did not reveal any gross changes. There were a total of nine dairy cows on this farm, housed in a traditional barn at the side of the owner's house in poor hygienic conditions. Disinfection after milking was not carried out continuously and a preventive antibiotic treatment for drying off was not administered.

Prior to antibiotic treatment, milk samples were taken aseptically from the affected quarters into a sterile plastic cup (50 mL). Teats and udder were cleaned with warm water and thoroughly dried, and the teats were disinfected using 70% ethanol. The samples were immediately sent to the laboratory for bacteriological examination. Milk samples were inoculated on Columbia blood agar base (CM0331, Oxoid, UK) with 5% sheep blood (blood agar), MacConkey agar (CM0007, Oxoid, UK), and Sabouraud dextrose agar - SDA (BK 025HA, Biokar Diagnostics, France). The sub-cultured colonies of nutrient agar were used for catalase and oxidase assays, biochemical assays, Gram staining, and antimicrobial assays.

Disc diffusion assay was performed according to CLSI guidelines (Woods et al., 2011) and zones of inhibition were interpreted according to Lebeaux et al. (2019). Antimicrobials (BD BBL Sensi-Disc, Becton Dickinson, USA) were used at the following concentrations: ampicillin (10 µg), amoxicillin-clavulanic acid (10/20 µg), cefpodoxime (10 µg), ceftazidime (10 µg), amikacin (30 µg), tobramycin (10 µg), gentamycin (10 µg), streptomycin (10 µg), ciprofloxacin (5 µg), moxifloxacin (5 µg), trimethoprim (5 µg), trimethoprim-sulphamethoxazole (23.75 µg), chloramphenicol (30 µg), imipenem (10 µg), meropenem (10 µg) and ertapenem (10 µg). Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-ToF MS) was performed on a single colony using Bruker's direct transfer sample preparation procedure for MALDI-ToF MS (Bruker Daltonics, Germany) by a 70% formic acid extraction method prior to analysis. MALDI Biotyper software (Bruker Daltonics, USA) was used with the BDAL and Filamentous Fungi databases containing 8468 and 468 MSP entries, respectively. Subsequent PCR tests and MLSA were performed following the basic methodology for identification of *Nocardia* species (Conville et al., 2018). For DNA extraction, two isolated colonies were suspended in 100 µl of deionised water, shaken vigorously, boiled at 100 °C for 10 min, and centrifuged at 14,000 g for 10 min. The supernatant was then used as a PCR template. The presence of the 16S rRNA gene was detected according to Drancourt et al. (2000). Partial gyrB, secA1, and hsp65 genes were amplified using the primers and cycling conditions described by McTaggart et al. (2010). PCR amplicons were sent to the Macrogen Europe Laboratory, Amsterdam, Netherlands, for sequencing. Generation of consensus sequences, analysis, and quality control of the raw sequences were performed using the STADEN package (Staden et al., 2000). Using MEGA 11 software, the sequences of gyrB, 16S rRNA, secA1, and hsp65 were aligned and trimmed at defined start and end positions and then concatenated as previously described (McTaggart et al., 2010; Tamura et al., 2021). The concatenated sequence was used for phylogenetic analysis. Unrooted trees were created using the neighbour-joining algorithm after Kimura-2 parameter correction with 1,000 bootstrap repeats.

After 72 h incubation at 37 °C under aerobic conditions, pure cultures containing small, irregular, white, dry, non-haemolytic colonies were recorded on 5% blood agar. No microbial growth was observed at primary inoculation on MacConkey agar and SDA. Primary colonies from 5% blood agar were subcultured on blood agar (anaerobic and aerobic conditions), nutrient agar, and SDA. No microbial growth was detected under anaerobic conditions. Colonies sub-cultured on Columbia blood agar were extremely dry, highly adherent, and difficult to remove from the plate with an inoculating loop (Fig. 1a). In addition, growth was observed on SDA agar after 48 h under aerobic conditions at 37 °C. Colonies older than 72 h took on a yellowish-orange appearance, rose irregularly above the plate (aerial hyphae), and were much softer, i.e. cotton-wool-like. Growth on SDA was similar but not as pronounced as on the blood agar. The isolate was catalase positive, oxidase negative, bile esculin positive, glucose-fermenting, and lactose negative. Growth of subcultures was observed in thioglycollate broth under aerobic conditions. Microscopic examination revealed bacterial cell pleomorphism with Gram-positive branched bacilli as dominant forms (Fig. 1b).

Antimicrobial testing by disc diffusion method revealed that the isolate was sensitive to imipenem, tobramycin, gentamycin, amikacin, trimethoprim, trimethoprim-sulfamethoxazole, ciprofloxacin, and moxifloxacin and resistant to ampicillin, amoxicillin-clavulanic acid, chloramphenicol, cephalosporins (cefpodoxime and ceftazidime), carbapenems (ertapenem and meropenem) and streptomycin. Although the broth dilution method has been performed in numerous studies, it is not always straightforward for testing the susceptibility of *Nocardia* spp. to antimicrobials (Conville et al., 2012). *N. cyriacigeorgica* isolates generally share a common pattern of susceptibility, however, there is considerable variation in these patterns within the species,

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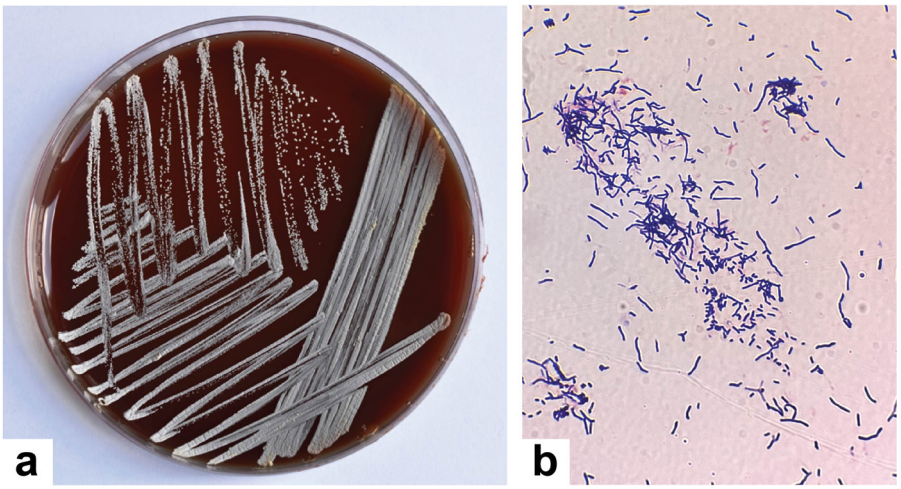

Fig. 1. Colonies on 5% blood agar: white, dry, non-haemolytic (a); Gram staining: Gram-positive branching bacilli (b)

which is also confirmed by our results (Condas et al., 2013; Brown-Elliott et al., 2015; Lebeaux et al., 2019).

Species identification within the genus *Nocardia* is not possible using routine microbiological and biochemical methods, which is a significant problem for low-budget and non-reference laboratories (McTaggart et al., 2010). In our case study, MALDI-ToF MS resulted in low identification and unreliable results for the bovine strain of *N. cyriacigeorgica* studied due to the limitation of spectra used for *Nocardia* species in the current database. *N. cyriacigeorgica* was the best matched sample with an identification value of 1.61, but values were set at 1.70–1.99 for low-confidence identification and 2.0–3.0 for high-confidence identification. The use of MALDI-ToF MS is limited for accurate identification of rare *Nocardia* species, as the method depends on the library databases used, sample preparation, incubation time, culture media, and the fact that some spectra vary between isolates or strains of the same species (Brown-Elliott et al., 2015; Conville et al., 2018; McTaggart et al., 2018). However, as *N. cyriacigeorgica* is a common human pathogen, human isolates can be readily identified using the above method (Girard et al., 2016).

Analysis of the 16S rRNA segment (1,491 bp) confirmed the species *N. cyriacigeorgica* based on its ≥99.6% sequence identity to the reference sequence in GenBank using BLAST, as previously described (Petti et al., 2008). We used the EzBioCloud 16S-based ID software to confirm the species as *N. cyriacigeorgica*, by using our partial 16S rRNA sequence with the top hit strain being *N. cyriacigeorgica* DSM 44484, with 100% identity and 98.2% completeness. Furthermore, the results of MLSA-based phylogenetic analysis confirmed that the isolated bacterium was indeed *N. cyriacigeorgica* (Fig. 2). All sequences obtained in this study have been submitted to GenBank and are available under the following accession numbers: OP108372, OP146097, OP146098, and OP146099. Multilocus sequence analysis is a powerful tool in taxonomic and phylogenetic studies of prokaryotes and is a reference method for the identification of *Nocardia* species (McTaggart et al., 2010; Brown-Elliott et al., 2015). Phylogenetic trees constructed using MLSA generally show higher discriminatory power within *Nocardia* spp. compared to trees based on the 16S rRNA gene sequences only (Wei et al., 2019). Therefore, we performed the 16S rRNA sequence analysis and further analysed the result using MLSA. The *N. cyriacigeorgica* isolate from this study formed a cluster with *N. cyriacigeorgica* strain OAHPP13691 from a human clinical sample with a maximal bootstrap value of 100. Unfortunately, *N. cyriacigeorgica* sequences from animal strains were not available in GenBank for MLSA.

On the same farm, two Simmental cows had been culled for severe mastitis in the past two years (2020–2022). Bacteriological examination of milk samples taken from these animals prior to culling showed the presence of coryneform bacteria, but no definitive aetiological diagnosis was made at that time. Therapy, which included topical (intramammary) and systemic application of amoxicillin, gentamicin, and enrofloxacin, proved clinically unsuccessful, in contrast to the *in vitro* disc diffusion results. The exact doses and duration of therapy were not known to the authors.

As part of the follow-up, the veterinarian administered parenteral therapy with trimethoprim-sulfamethoxazole (Sulfakel, Kela Laboratoria, Belgium) according to the manufacturer's instructions. According to the veterinarian, the treatment was not successful and the cow was culled. The owner did not inform the authors in time, so it was not possible to perform a histopathological examination of the udder. It was recommended that the barn and milking equipment be disinfected, that the milk of all cows on the farm be examined bacteriologically, that disinfection with iodine be carried out after milking, and that the unaffected animals be treated with antibiotics during the dry period (Mihajlović et al., 2022). The owner refused to have the milk of all cows on the farm bacteriologically tested. No new cases of mastitis were reported from the farm.

Studies have shown that *N. cyriacigeorgica* is frequently isolated from soil samples (Carrasco et al., 2020), so the case of mastitis described here may be due to environmental

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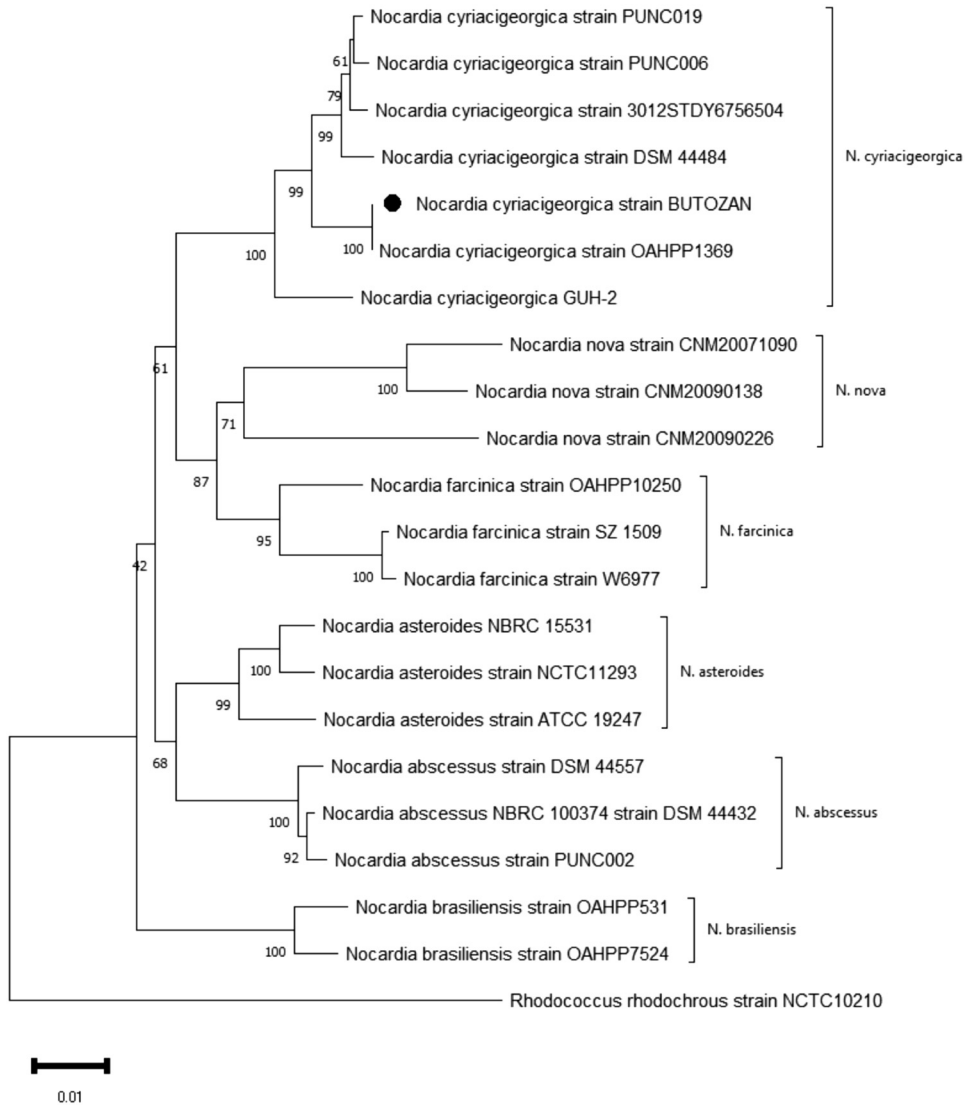

Fig. 2. Multilocus sequencing analysis (MLSA)-based phylogenetic tree, constructed from 1946-bp concatenated gyrB-16S rRNA-secA1-hsp65 sequences of clinical *Nocardia* isolates and type strains from GenBank. The *N. cyriacigeorgica* strain, isolated in this study is marked with a black circle. High bootstrap values, indicated at branching points, support the reliability of the tree topology. Scale bar corresponds to 1% genetic distance

transmission of the pathogen as a consequence of poor hygienic conditions (Pisoni et al., 2008). Nocardial mastitis is often severe and characterised by pyogranulomatous inflammation with poor prognosis, justifying the culling of affected animals after unsuccessful therapy (Pisoni et al., 2008; Chen et al., 2017), as was the situation in our case. According to the literature, nocardial mastitis is most often caused by *Nocardia nova*, *N. asteroides*, *Nocardia farcinica,* and *Nocardia brasiliensis* (Ribeiro et al., 2008; Condas et al., 2013).

Interestingly, *N. cyriacigeorgica* is a pathogenic species highly likely to evolve to a more virulent human pathogen (Zoropogui et al., 2013). In that sense, it is no surprise that the isolate from our study clustered with a *N. cyriacigeorgica* strain from a human sample. Moreover, the results of MLSA presented by Vautrin et al. (2021) show that environmental and clinical strains of *N. cyriacigeorgica* belong to the same cluster, underscoring that all strains of this bacterium are potential threats to human health. Regarding data on human nocardiosis caused by *N. cyriacigeorgica* in Bosnia and Herzegovina, there are no published cases of the disease, however, there are reports from the region suggesting that the incidence of nocardial infection is rising (Karan et al., 2019; Veličković et al., 2022). Nocardiosis is an emerging saprozoonosis increasingly important in terms of public health. However, the possibility of direct transmission of *Nocardia* spp. from animals to humans is low and, apart from a cattle inspector's case, which occurred under undetermined circumstances, is limited to case reports of bite or scratch wounds inflicted by dogs or cats (Smit et al., 2003; Mahendra and Dave, 2016). Nevertheless, the occurrence of these infections in animals, although rare, should be documented and the requirement of the One Health approach emphasized.

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To the authors' knowledge, this is the first report of isolation of *N. cyriacigeorgica* from a clinical case of bovine mastitis in a European dairy farm and the first MLSA method approach to distinguish a strain of *Nocardia* spp. isolated from animals. In contrast to most European countries, the majority of lactating cows in Bosnia and Herzegovina are kept under traditional housing conditions, often with low hygiene status, which may be a predisposing factor for environmental mastitis infections. Further studies are needed to analyse the possible environmental and hygienic aspects leading to the occurrence of nocardial mastitis and the importance of the genus *Nocardia* in the pathogenesis of infections in domestic animals. It would also be interesting to focus on the study of virulence genes in these animal strains and help fill the large gaps in sequence information in public genetic databases. In general, all confirmed cases of nocardiosis, whether human or animal, should be identified down to the species level to allow a better understanding of the prevalence, geographical spread, and consequences of this disease.

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