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Case Report
First Documented Mastitis Case of *Nocardia puris* in Turkey from Two Cows: Microbiological and Molecular Identification

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Abstract

Background and Objective: Nocardia is one of the causing agents of bovine mastitis and increasing prevalence of nocardial mastitis from many countries. The aim of the present study was to characterize *Nocardia puris* isolated from two bovine mastitis cases in Turkey based on microbiological and molecular methods. **Materials and Methods:** *Nocardia* sp. was isolated from milk samples from all quarters of 1 Simmental and 1 Holstein cows submitted to Mehmet Akif Ersoy University Faculty of Veterinary Medicine, Department of Microbiology laboratory in Burdur on April, 2018 and confirmed by 16S rRNA gene sequencing. Antimicrobial resistance was assessed by disc diffusion against ampicillin, cefoperazone, ceftiofur, cephalexin cloxacillin, enrofloxacin, gentamicin, oxytetracycline, penicillin and trimethoprim sulphamethoxazole. **Results:** A total of 3 *Nocardia* spp. were isolated and molecular analysis of the obtained 16S rRNA gene sequence of 3 *Nocardia* isolates with BLAST revealed that the isolates had a 99% sequence similarity with *N. puris* strains. Based on the disc diffusion test, all *N. puris* isolates were sensitive to gentamicin, enrofloxacin, ceftiofur, oxytetracycline and sulfamethoxazole trimethoprim and resistance to ampicillin, cefoperazone, cloxacillin, cephalexin and penicillin. **Conclusion:** Overall, current results suggested that a well-defined mastitis etiology and this is apparently the first report of *N. puris* in association with bovine mastitis in Turkey.

Key words: Bovine mastitis, *Nocardia puris*, molecular identification

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Nocardia species are ubiquitous, aerobic, Gram-positive, filamentous and can be found as saprophytic components of fresh and salt-water, soil, dust, decaying vegetation and decaying fecal deposits from animals plants that classified as a member of the *Actinomycetales* order[1,2]. Pyogranulomatous suppurative processes with chronic evolution characterize infections caused by *Nocardia* species such as *N. africana*, *N. asteroides*, *N. farcinica* and *N. nova*, followed in order of importance by *N. brasiliensis*, *N. otididiscaviarum*, *N. pseudobrasiliensis* and *N. transvalensis* in human and animals[3-6]. Bovine nocardial mastitis is the most common clinical manifestation among domestic ruminants, due to the environmental manifestation[7] and is usually seen indairy herds with poor hygienic conditions[4,8].

The diagnosis of nocardiosis is usually based on direct examination since conventional cultures are complex as well as long and time-consuming[9,10]. Because the classical identification process of *Nocardia* species is complicated and incomplete, the current identification of *Nocardia* is being mainly based on molecular phylogenetic information and the reports of new species of *Nocardia* have increased[2].

The *N. puris* is a relatively rare species. Distribution of *N. puris* is considered to be geographically limited, with infections only reported in Germany, Greece, Japan[11-13]. However, Nocardia infections have not been reported from animals in Turkey.

The present study aimed to characterize *N. puris* isolated from bovine mastitis clinical cases in Turkey, based on microbiological and molecular identification.

MATERIALS AND METHODS

Milk samples from all quarters of 1 Simmental (6 years old) [from Antalya (36°32' 57.7032'' N, 31°59' 49.1784'' E)] and 1 Holstein (1 year old) [from Isparta (37°46' 4.8072'' N, 30°33' 42.8580'' E)] cows with a history of clinical mastitis from dairy farm management in manual or machine milking, submitted to Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Microbiology laboratory in Burdur on April, 2018.

Isolation and identification: Bacteriological culture was performed as described[2,14]. Briefly, the milk samples (10 µL) was streaked on 5% defibrinated sheep blood agar and maintained in aerobic conditions at 37°C for 24-48 h. Nocardial suspected colonies with dry, convex, circular, strongly adherent, powdery surface after on agar and were stained with Gram and Kinyoun stain (modified Ziehl Neelsen-MZN). Gram-positive, filamentous to coccobacillary, partially acid-fast organisms were identified as *Nocardia* spp.[1,2,14].

Antimicrobial susceptibility test: All the *Nocardia* isolates were submitted to antimicrobial susceptibility test by means of disc diffusion method using amoxicillin clavulanic acid (30 µg; AMC), ampicillin (10 µg; AMP), cephalexin (30 µg; CL), cefoperazone (75 µg; CFP), ceftiofur (30 µg; FUR), cloxacillin (30 µg; OB), enrofloxacin (5 µg; ENR), gentamicin (10 µg; CN), trimethoprim sulphamethoxazole (25 µg; TS), oxytetracycline (30 µg; OT), penicillin (10 units; P) according to the guidelines from Clinical and Laboratory Standards Institute (CLSI) on Mueller-Hinton agar (Oxoid Ltd., Hampshire, UK) according to instructions[15].

Molecular identification: Molecular identification of isolates were done with 16S rRNA sequence analysis (ABI 3130; Applied Biosystems, USA) using universal primers 27F(5-AGAGTTTGATCCTGGCTCAG-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 were constructed by employing the neighbor joining method using MEGA (www.mega software.net) and MUSCLE (www.ebi.ac.uk/Tools/msa/muscle/) software, based on the 16S rRNA gene sequences. Genetic distance were calculated using a Kimura's 2-parameter model. Tree robustness was assessed by bootstrap resampling (100 replicates each)[16].

RESULTS

Isolation and identification: A total of 3 (two was from Holstein, one was from Simmental) *Nocardia* spp. were isolated. Each strain was isolated from an individual mammary of cow and was named as A, B and C, respectively. Nocardial suspected colonies with dry, circular and white colour as chalk and attached firmly to the medium were difficult to emulsify (Fig. 1). Microscopically, all isolates were Gram-positive, filamentous to coccobacillary and weakly acid fast with MZN.

Antimicrobial susceptibility: Based on the disc diffusion method, susceptibility of *Nocardia* spp. isolates (n = 3) to select antimicrobials were assessed. A, B and C isolates had

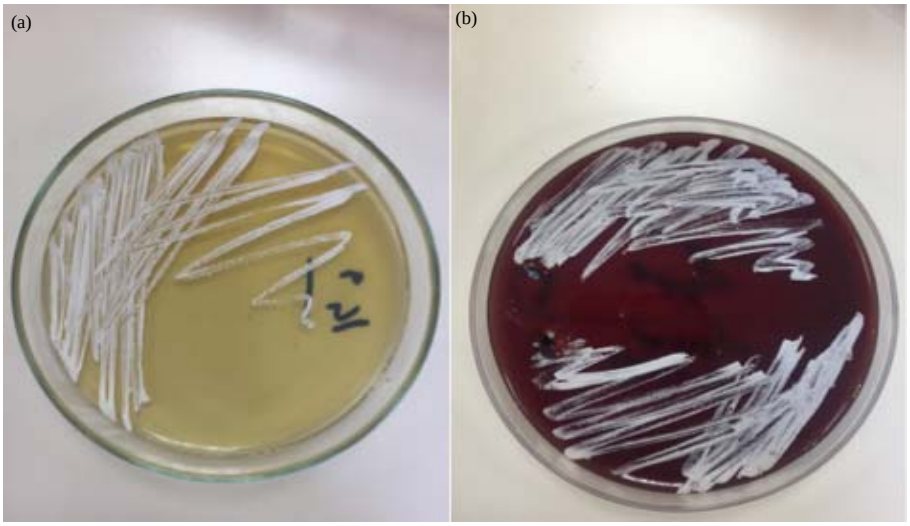

Fig. 1(a-b): (a) *Nocardia puris* cultures on nutrient agar (Oxoid, UK) and (b) 5% defibrinated sheep blood agar (Oxoid, UK)

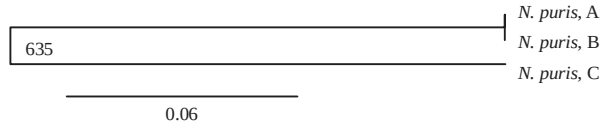

Fig. 2: Phylogenetic relationships of isolates belonging to *Nocardia puris* on the basis of the 16S rRNA gene sequences. Bootstrap values of the species-specific clusters are also shown

common susceptibility (CN, ENR, FUR, OT, TS) and resistance (AMP, CL, CFP, OB, P) patterns although, separated with C strain showed resistance to AMC.

Molecular identification: Analysis of the obtained 16S rRNA gene sequence with BLAST revealed that the isolates had a 99% sequence similarity with *N. puris* strains. The phylogram in Fig. 2 showed the close relationship of *N. puris* isolates. A discrete genetic cluster was clearly formed supported by a bootstrap value of 635.

DISCUSSION

Bovine nocardial mastitis is known since 1956 and *Nocardia asteroides* established pathogenicity and has been detected as a sporadic cause of mastitis since then[17,18].

The early recognition of Nocardial mastitis using microbiological and molecular identification an *in vitro* susceptibility drug tests are critical to increase the response to treatment or to prompt early culling of infected animals[4,8,19].

In the current study, a total of 3 *Nocardia puris* were isolated from 8 milk samples of two bovine mastitis cases and apparently the first description of these species causing bovine mastitis in Turkey. Interestingly, it was noticed that there is only one study about *Nocardia puris* mastitis based on the literature investigations[7]. In the last decades, *N. asteroides*, *N. farcinica* and *N. nova* were the most frequently identified species (from bovine mastitis) diagnosed by phenotypic methods[8]. Infections due to *N. puris* have been considered to be associated with abscess, endophthalmitis and choroiditis, researchers were previously reported infectious cases due to this bacterium which were isolated from human[11-13,20,21].

Among 2 of the cases, 16S rRNA gene sequence data confirmed the presence of *N. puris* in milk samples. The 16S rRNA gene is highly conserved with variable regions that allow characterized by rapidly available results, improved accuracy and taxonomical meaningfulness of all *Nocardia* species[2,19]. The identification of *Nocardia* spp. based on sequencing is reported by others, regarding 16S rRNA gene sequencing and numerous sequences available in public databases[7,10]. Due to the phylogenetic tree, the highest 16S rRNA sequence similarities of strains were observed with sequences of A, B, C. This data supported that similarity of phylogenetic characteristics could be associated with isolates obtained from geographically close regions. Otherwise, it was thought that similar biosecurity deficiencies of both farms because the most common manifestation of nocardial mastitis due to environmental transmission[7].

Antimicrobial therapy of nocardial mastitis may produce temporary clinical relief and cessation of shedding but no permanent cures. Control involves removal of infected animals[1,4,8,19]. In present study, the antimicrobials selected were those most frequently used in the treatment of bovine mastitis[4] and animal nocardiosis[22,23]. Total of 3 isolates were resistant to ampicillin, cephalexin, cloxacillin, cefoperazone, penicillin, however limited data that were obtained couldn't allow differed from other studies[4,23]. Furthermore, therapy for Nocardia mastitis is usually ineffective due to the intracellular location of the microorganism and virulence factors that induce pyogranulomatous processes[8,19,22].

CONCLUSION

The present study showed that *N. puris* was detected for the first time from bovine mastitis cases in Turkey by using both conventional and molecular diagnostic methods. These results highlighted the phenotypic identification methods of *Nocardia puris* should be supported by molecular methods and it is indicated that 16S rRNA sequencing is useful for determining relationships between isolated strains from Nocardial mastitis cases.

SIGNIFICANCE STATEMENT

The impact of *N. puris* marked on its identification, resistance to antimicrobial agents and molecular techniques may assist in more efficient protect and control of bovine mastitis.

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