

Case Report

First case report of isolation of *Micrococcus lylae* from urinary catheter of a 50-year-old woman suffering from malignancy

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Abstract

Introduction: Catheter-associated urinary tract infection (CAUTI) is the most common healthcare-associated infection which tends to cause increased length of morbidity, and mortality of patients, in addition to increased bacterial resistance to antibiotics.

Methodology: In the present study, urinary catheters were collected from a 50-year-old woman suffering from malignancy, bedridden, and having urinary incontinence. These catheters were processed in laboratory for isolation of bacteria using standard procedures.

Results: Microbiological examination of the urinary catheters by biochemical, physiological, and VITEK 2 compact system revealed bacterial infection caused by *Micrococcus lylae*, a Gram-positive microorganism belonging to the family Micrococcaceae. These Gram-positive bacteria were found to be susceptible to streptomycin, erythromycin, cefotaxime, neomycin, kanamycin, vancomycin, azithromycin, chloramphenicol, and tetracycline. Bacterial species were confirmed using 16s rRNA sequencing.

Conclusions: The sequences were found to have 99% similarity with *Micrococcus lylae*. This is the first report of isolation of *Micrococcus lylae* from the urinary catheter.

Key words: CAUTI; VITEK-2; *Micrococcus lylae*; 16s rRNA; antibiotic susceptibility.

J Infect Dev Ctries 2023; 17(7):1041-1046. doi:10.3855/jidc.16477

(Received 17 February 2022 – Accepted 06 December 2022)

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Introduction

Catheter-associated urinary tract infection (CAUTI) is the most common type of hospital infection worldwide [1]. Most common organisms like *Escherichia coli* that are responsible for urinary tract infection (UTI) can also cause CAUTI. It can be multi-bacterial as many other organisms like *Proteus*, *Pseudomonas*, *Klebsiella*, *Enterobacter*, and *Enterococci* can lead to infection [2]. In the present study, *Micrococcus lylae* was detected in a urinary catheter collected from a healthcare center which was responsible for CAUTI.

Micrococcus is Gram-positive, motile or non-motile, spherical, and occurs in tetrads. It is commonly isolated from mammalian skin which is considered to be the primary natural habitat. However, reports have suggested that it is an opportunistic pathogen. Earlier the genus *Micrococcus* included *M. luteus*, *M. lylae*, *M. varians*, *M. roseus*, *M. agilis*, *M. kristinae*, *M. nishinomiyaensis*, *M. sedentarius*, and *M. halobios* [3]. However, these species now were placed in five different genera: *Dermacoccus*, *Kocuria*, *Kytococcus*, *Micrococcus*, and *Nesterenkonia*. In a recent

classification, the genus *Micrococcus* contains only two species, *M. luteus* and *M. lylae* [4]. All species of *Micrococcus* are not considered to be pathogenic but, some species have been reported to cause pathogenic infection. Gupta *et al.* reported a case of meningitis caused by *M. luteus* in healthy infants [5]. This study is the first report of *M. lylae* found in the urinary catheter.

Case presentation

In 2019, a 50-year-old woman suffering from malignant colorectal cancer at a localized stage was bedridden, had urinary incontinence and was admitted to the hospital. Screening was performed by colonoscopy and biopsy, and the doctor treated the patient with neoadjuvant therapy. Two months after the abscission, the patient suffered from severe back pain that was subsequently investigated and diagnosed as urosepsis due to catheter-associated urinary tract infection (CAUTI). Afterwards, the patient incorporated a catheter in conjunction with four new catheters that were inserted in an interval of two days to prevent complications. Altogether five catheters were implanted in the patient before further treatment. The

removed catheters were then collected in a HiDispo™ bag (Himedia, Mumbai, India) and transported to the laboratory in sterile condition and processed by isolation on blood agar, followed by identification with the VITEK 2 system (Biomerieux, Marcy-l'Étoile, France). The VITEK 2 system indicated that there was low discrimination between two closely related bacteria. Again, four more catheters were collected from the patient and analyzed on the VITEK 2 system. The VITEK 2 system indicated similar low discrimination between *M. lylae* and *M. luteus* in all five catheters. The L- arginine dihydrolase test is an additional test recommended by VITEK 2 to confirm the identification and was performed with a standard strain of *M. luteus* NCTC 2665 as control. Data from the Bergey's manual also indicate that the only species of *Micrococcus* growing on inorganic N-agar is *M. luteus*. The two biochemical tests were carried out and the results indicated that *M. lylae* was isolated from all five catheters. Species-level identification through 16S rRNA sequencing was performed to confirm the results. The resulting sequence exhibited 99% similarity with *M. lylae*.

The antimicrobial susceptibility of the four isolated bacteria show a similar susceptibility to antibiotics like streptomycin, erythromycin, cefotaxime, neomycin, kanamycin, vancomycin, azithromycin, chloramphenicol, and tetracycline. The patient was subsequently given a dose of cefotaxime that led to clinical improvement. No further bacterial isolates were derived from the last catheter collected from the patient after the antibiotic was administered. The patient had clinically improved and was discharged from the hospital and no re-infection occurred after two months without antibiotics.

Methodology

Sampling

Urinary catheters were collected from a tertiary healthcare center in Amravati (Maharashtra, India) in the HiDispo™ bag (Himedia, Mumbai, India). These urinary catheters were further processed in a laboratory under aseptic conditions. The urinary catheters were washed with sterile distilled water and swabbed with 70% alcohol from both extraluminal sides of the catheter to remove planktonic bacteria. The catheters were then sectioned into five parts from the tip and washed with saline solution and each part was suspended in the test tube containing Ringer's solution separately. Sonication for 5 min. at 35 kHz and vortex mixing for 2 min were performed [6].

Isolation and identification

The cell suspensions obtained from the catheters were then cultured on blood agar plates. The plates were incubated at 37 °C for 24 h. The colonies were observed and identified based on morphological identification and biochemical test. For rapid and accurate bacterial identification VITEK 2 Compact (Biomerieux, Marcy-l'Étoile, France) system was used.

VITEK 2 system identification

The VITEK 2 system (BioMerieux, Marcy-l'Étoile, France) is an automated bacterial identification and susceptibility testing system [7]. It is a fluorescence-based technology that allows for kinetic analysis by reading each test every 15 minutes. The optical system combines multichannel fluorimeter and photometer readings to record fluorescence, turbidity, and colorimetric signals [8]. The bacterial strains isolated from the catheter were inoculated on a blood agar plate for 24 hrs at 37 °C before testing. Bacterial suspensions equivalent to 0.5 McFarland turbidity standard were prepared using sterile saline. Densichek (BioMerieux, Marcy-l'Étoile, France) was used for adjusting the turbidity of the bacterial suspensions. Thereupon, the identification Gram-positive (ID- GP) card was inserted in the bacterial suspension tubes and manually loaded in the VITEK 2 system.

Growth on inorganic nitrogen agar

According to Bergey's manual, all species of *Micrococcus*, except *M. luteus*, are unable to utilize inorganic nitrogen from medium [3]. After identification by the VITEK 2 system, agar containing ammonium phosphate ((NH₄)₃PO₄) was used as the source of defined ammonium phosphate was formulated by Hucher [12] to differentiate *M. luteus* and *M. lylae*. The composition of the medium was: MgSO₄, 0.200g; KCl, 0.200g; (NH₄)₃PO₄, 1g; dextrose, 10g; bromo cresol purple, 0.050g; agar (Himedia, Mumbai, India), 15g; and deionized water, 1000mL. The final pH was adjusted to 7.0 [12]. The medium was sterilized by autoclaving at 121 °C for 20 min. A loopful of isolated culture and the standard strain of *M. luteus* NCTC 2665 were streaked on an agar plate and incubated for 24 hrs at 37 °C. Growth after overnight incubation on agar medium indicates the presence of *M. luteus* and no growth indicates *M. lylae*.

Arginine dihydrolase test

The VITEK 2 system is unable to discriminate between *M. lylae* and *M. luteus*. To differentiate between the two species arginine dihydrolase test was

performed based on the method of Thornley [9]. The composition of the medium was: peptone, 1g; sodium chloride (NaCl), 5g; dipotassium hydrogen phosphate, (K_2HPO_4) 0.3g; phenol red, 0.01g; L-arginine monohydrochloride ($C_6H_{14}N_4O_2 \cdot HCl$), 10g; agar 3g; (Himedia, Mumbai, India), pH 7.2 [10]. The medium was sterilized by autoclaving at 121°C for 20 min. Slants were prepared and allowed to solidify at room temperature. The isolated organism and the standard strain of *M. luteus* NCTC 2665 were inoculated by stab and were incubated at 37 °C for 24 hrs. After overnight incubation presence of arginine dihydrolase was indicated by a dark pink or red color in a positive test while in a negative test, the color remained unchanged or became yellow [11].

Antibiotic susceptibility

Antibiotic susceptibility testing of isolated microorganisms was performed using the Ezy MIC strip test (Himedia, Mumbai, India) on Mueller Hinton agar plates. 0.5 McFarland standard of isolated culture was swabbed over the surface of the Mueller Hinton agar plates. The Ezy MIC test strip (Himedia, Mumbai, India) was placed over the surface of the Mueller Hinton agar and incubated at 37 °C for 24 hrs.

DNA isolation

DNA was isolated by suspending the bacterial pellet in Tris-EDTA (TE) and incubating at 98 °C for 1 min. The lysate was used as template DNA for polymerase chain reaction (PCR).

16s rRNA analysis

The PCR amplification was carried out using 16s rRNA primers 8F (AGAGTTTGATCCTGGCTCAG) and 1492R (AAGTCGTAACAAGGT) [29]. PCR was performed in a 20 µL reaction mixture containing genomic DNA, 2µL of 2 mM dNTPs, 0.7 µL of 10 pmol/µL solution of each primer, 2µL of 10X *Taq* buffer, and 2.5 unit of *Taq* DNA polymerase. The PCR conditions used were: initial denaturation at 95 °C for 120s followed by 40 cycles at 95 °C for 40 s, 50 °C for 40 s, and 72 °C for 90 s. PCR products were analyzed by electrophoresis on 1.2% agarose gels containing 0.5 µg/mL ethidium bromide and were visualized on a gel documentation system (Syngene, Mangalore, India).

The PCR products were purified and sequenced bidirectionally by Sanger's dideoxy sequencing using ABI 3500 genetic analyzer (Applied Biosystem, Foster City, US) with ABI Big Dye™ Terminator Cycle sequencing kit (ThermoFischer Scientific, Massachusetts, USA) at the Central Instrumentation

Cell (CIC), Sant Gadge Baba Amravati University (SGBAU), Amravati, Maharashtra, India. The reverse complement of the obtained DNA sequence was obtained using a bioinformatic tool as it plays a key role in maintaining the orientation of our 5' and 3' ends since DNA is antiparallel. The sequence similarity was searched using the nBlast tool [27]. The sequence was submitted to the National Center for Biotechnology Information (NCBI) gene bank (Accession no. MW356812).

Subsequently, nucleotide sequences of urinary tract associated pathogens were collected using GeneBank (NCBI) in a Fast Adaptive Shrinkage Threshold Algorithm (FASTA) format file along with codes for nucleotides and amino acid sequences [30]. The phylogenetic tree of the isolated bacterial strains was created using neighbor joining method with MEGA XI program [28].

Results

Urinary catheters collected from a tertiary healthcare hospital were processed in the laboratory and the bacterial isolates were grown on blood agar plates. The colonies were circular, convex, yellow, or creamy-white. Gram's characteristics appeared to be purple, and arranged in tetrads or irregular clusters. When observed on blood agar, they showed β-hemolysis and appeared as creamy white colonies. VITEK 2 test was performed based on Gram's stain characteristics for the detection of the microorganisms. The results obtained from VITEK 2 identification showed low discrimination between *M. lylae* and *M. luteus*. Low discrimination results or slashline occur when the generated biotype is insufficient to distinguish between two or more species. Slash line selections are considered technically non-discriminatory and require additional monitoring and testing to resolve a single classification. In the case of low discriminating identification, two or more options have been listed in the order of their probability calculations. However, in the case of microorganisms with low discrimination, additional tests are recommended to allow further differentiation.

Based on the results obtained from the VITEK 2 system, the biochemical test was performed to differentiate *M. lylae* and *M. luteus*. *M. lylae* and *M. luteus* differ based on the L-arginine dihydrolase test that was performed in the laboratory. *M. lylae* showed a negative result, that is, the color of the medium did not change or turn yellow; while *M. luteus* gave a positive result, that is, the color of the medium changed to a dark pink color. This test was conducted in the

laboratory and after 24 hours of incubation, the color remained the same, and subsequent incubation led to yellow colonies while the standard strain of *M. luteus* produced pink color. It was concluded that *M. lylae* had been identified.

An additional test was performed to differentiate between *M. lylae* and *M. luteus* based on their inorganic nitrogen requirements. *M. lylae* showed no growth on the inorganic nitrogen agar medium, while *M. luteus* showed growth on the inorganic nitrogen agar medium. After 24 hours of incubation, no growth was observed on the agar medium, whereas in another plate, growth was observed because of the standard strain of *M. luteus*.

16s rRNA analysis was used for species level identification. DNA was isolated from the bacterial cultures and a 16s region was amplified with the amplicon size of ~1400 bp. The PCR product was sequenced in both directions with Sanger’s dideoxy sequencing using ABI 3500 genetic analyzer. 16s rRNA nucleotide sequences (1400 bp) from bacteria isolated from the urinary catheter were aligned using ClustalW. The sequences were compared to the nucleotide sequences described in the gene bank using the nBLAST alignment tool. The nucleotide sequences showed similarity up to 99% with species *M. lylae*. A phylogenetic tree was produced based on the 16s rRNA sequences from the bacterial samples. This phylogenetic tree included the isolated bacterial samples along with the other 16s rRNA deposited sequences in a neighbour-joining method analyzed using MEGA XI. Based on the phylogenetic analysis it

Figure 1. The neighbor-joining phylogenetic tree includes common urinary tract pathogens as outgroup and *Micrococcus lylae* and *Micrococcus luteus* as neighbours. The percentage of replicate trees (1000 replicates) is shown next to the branches. Evolutionary analyses were conducted using MEGA7.



was found that *M. lylae* and *M. luteus* belonged to similar clade and were closely related to each other (Figure 1).

After identification of the microorganism by VITEK 2 compact and another biochemical test the isolated culture was identified to be *M. lylae*. The antimicrobial activity of *M. lylae* was analyzed using the Ezy MIC strip test method. *M. lylae* was susceptible to streptomycin, erythromycin, cefotaxime, neomycin, kanamycin, vancomycin, azithromycin, chloramphenicol, and tetracycline, and resistant to oxacillin, gentamicin (Table 1).

Discussion

Urinary catheters were gathered from a tertiary healthcare facility and processed further using a conventional microbiological method. A blood agar plate was used for preliminary identification, and it revealed colonies that were round, convex, yellow, or creamy-white in appearance. Tetrad-like cocci were observed, based on Gram's characteristic. VITEK 2 test was performed based on Gram's characteristic and the result showed low discrimination between *M. lylae* and *M. luteus*. The laboratory report generated by VITEK 2 recommended supplemental tests that allow analysis and identification of isolates with low discrimination. Based on the recommendations, an L-arginine dihydrolase test was performed and the culture was identified as *M. lylae*. The inorganic nitrogen agar test, also confirmed the identification of *M. lylae*. For species-level identification, 16s rRNA analysis was performed and the PCR product was sequenced in both directions. The nucleotide sequence aligned using ClustalW and nBlast showed 99% similarity with *M. lylae*. Based on the result of phylogenetic analysis, MW356812.1 was found to be closely related to AJ312751.1. The bootstrap value also supported this conclusion.

Table 1. Ezy MIC Strip test of *Micrococcus lylae*.

	Antibiotics	Ezy MIC Strip Test
1	Vancomycin	S
2	Azithromycin	S
3	Chloramphenicol	S
4	Tetracycline	S
5	Oxacillin	R
6	Gentamicin	R
7	Streptomycin	S
8	Erythromycin	S
9	Cefotaxime	S
10	Neomycin	S
11	Kanamycin	S

S: Sensitive; R: Resistant.

The genus *Micrococcus* has nine species, according to Bergey's Handbook of Systematic Bacteriology. 3]. Many *Micrococci* (except *M. luteus* and *M. lylae*) have been renamed based on phylogenetic and chemotaxonomic analysis [14].

The new names for these organisms are *Dermacoccus nishinomiyaensis*, *Kocuria varians*, *Kocuria roseus*, *Kocuria kristinae*, *Kytococcus sedentarius*, *Arthrobacter agilis*, and *Nesterenkonia halobia* [15]. Currently, *M. lylae* and *M. luteus* are the only species in genus *Micrococcus*. The earlier genus of *Micrococcus* was not considered to be pathogenic [14]. However, strains of *Micrococcus* tend to cause various infections. *Kocuria kristinae* strains are responsible for central venous catheter-related bacteremia in patients with ovarian cancer [22], infective endocarditis [21], catheter associated urinary tract infection [23] and acute leukemia [24]. Apart from *Kocuria* spp., there are reports of *M. luteus* causing meningitis [5], pneumonia [25] and a patient with systemic lupus erythematosus (SLE) with brain abscess caused due to *M. luteus* infection [26] has also been reported. So far, no case of a catheter associated urinary tract infection with *M. lylae* has been reported.

In the reported case of *M. luteus*, the organism was found to be susceptible to antibiotics including penicillin, erythromycin, streptomycin, kanamycin, tetracycline, novobiocin, chloramphenicol, neomycin, vancomycin and plomycin B. In another case of *M. lylae*, it was found to be resistant to methicillin and susceptible to erythromycin, streptomycin, penicillin, novobiocin, tetracycline, chloramphenicol, neomycin, vancomycin, kanamycin, and polymyxin B [19]. Our isolated *M. lylae* from the catheters was sensitive to streptomycin, erythromycin, cefotaxime, neomycin, kanamycin, vancomycin, azithromycin, chloramphenicol, tetracycline, and resistant to oxacillin and gentamicin.

Infections caused by *M. lylae* have not been reported until now and the bacteria was considered harmless. This report highlights that *M. lylae* should be considered as an emerging pathogen as its clinical spectrum has shifted from immunocompromised to immunocompetent patients.

Conclusions

Bacteria were isolated from urinary catheters collected from a 50-year-old woman in a tertiary healthcare hospital and were processed by standard microbiological techniques. Identification by VITEK 2 compact had low discrimination. The low discrimination results from VITEK 2 compact

recommended supplementary tests to differentiate between *M. lylae* and *M. luteus*. The additional tests identified *M. lylae* as the bacteria isolated from catheters. Antibiogram of *M. lylae* found it to be susceptible to streptomycin, erythromycin, cefotaxime, neomycin, kanamycin, vancomycin, azithromycin, chloramphenicol, and tetracycline. The isolated cultures were further analyzed by 16s rRNA analysis and sequenced bi-directionally by Sanger's dideoxy sequencing for species-level identification. The sequences obtained had 99% similarity with *M. lylae*. A sequence of 1400bp length has been submitted to the NCBI with the accession number MW356812.1

To the best of our knowledge, we describe the first case of *M. lylae* isolated from a urinary catheter. Thus, *M. lylae* must be considered as pathogenic bacteria that can lead to malignancy.

Acknowledgements

The authors thank Rajiv Gandhi Science and Technology Commission, Mumbai for their financial support.

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Conflict of interests: No conflict of interests is declared.