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FIRST ISOLATION OF MYCOBACTERIUM FARCINOGENES IN AN INDIAN CLINICAL SPECIMEN USING RAPID AND RECENT TECHNOLOGIES

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ABSTRACT
The strains of Mycobacterium (M) farcinogenes are identified in bovine farcy of Zebu cattle in eastern Africa. These cause pathogeneciy in guinea pigs, rarely affect humans. We report here, a case of first time isolation of M. farcinogenes in India a native patient of Ahmedabad. This female patient having age of 32 years old underwent laparoscopy for removal of left complex ovarian cyst in November 2016. After 20 days of surgery, she complained of swelling, pain and discharge from the port site. The patient underwent multiple dressings and multiple courses of antibiotics, but continued with the same symptoms. Abdomen examination with Ultrasonography (USG) in January 2017 showed scar with two sinus opening and discharge. The collection and tissue were sent for microbiological and molecular tests with Histo Pathological Examination (HPE). The HPE result revealed necrotic areas without granuloma but culture of fluid indicated Acid-Fast Bacilli (AFB) smear positive and negative gene Xpert, nucleic acid amplification test (NAAT testing) for evidence of NTM and further supported by LPANTMCM. Then the cultured sample was subjected to Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF) which revealed M. farcinogenes with a score of 2.04. The bacterial DNA was then extracted and its 16s Ribosomal ribonucleic acid (rRNA) gene was used for Sanger sequencing and Next generation sequencing platforms. These observations supported the strain of M. farcinogenes (DSM43637) in our sample supporting earlier genome draft of it. Thus patient was infected with a rare species of M. farcinogenes, first time isolated, identified and reported in India. This patient was treated with relevant antibiotics as per the isolate. She was responded clinically with no discharge from wound site after 45 days and was then discharged continuing the same treatment for 9 months as per treatment regimen. Clinical examination found her free from disease also.

KEYWORDS: M. farcinogenes, HPE, Cyst removal, MALDI-TOF, Sanger Sequencing, Next Generation Sequencing, Discharged culture, Female patient.

INTRODUCTION
Non-tuberculosis mycobacteria (NTM) are one of the important causes for human diseases and infections.[1] They also cause clinical syndromes in both immuno competent and immuno compromised hosts causing skin and soft tissue infections. These include osteomyelitis, lymphadenopathy, respiratory tract infections, blood stream Infections and disseminated infections.[2] Helou et al.[3] identified from bovine farcy of Zebu cattle in Eastern Africa and suggested these bacteria are allocated in the genus Mycobacterium, Taxon M. farcinogenes, distinguished into two subspecies Tchadense and Senegalense. Based on their dissimilarity,[4,7] these subspecies are raised to species level as M**.** farcinogenes and M. senegalense in the first edition of the book.[5,6] Now these two species are morphologically similar, but M. farcinogenes is relatively slower in growth. On molecular basis both are similar to M. houstonese. All three fall in a subclass M. fortuitum.

The strains of the later when compared with the former are rapid- growers as opposed to slow-growers, are more

active biochemically. These contain a characteristics mycoside C and cause a more generalized peritonitis in guinea pigs, but do show appreciable Deoxyribonucleic acid (DNA) homology with M. farcinogens.[4,7] Little is known of the relationships of M. farcinogenes and M. senegalense to establish Mycobacterium species, though the agents of bovine farcy can be distinguished by their characteristic pathogenicity for guinea pigs ability to form a stable mycelium and the malonamidase reactions. However comparative immune differences and lipid analysis did not exhibit any identification to classify as separate species.[8] The Researchers suggested more appropriate modern taxonomic methods and along with them others also are required for considering these as separate species at 16s-rDNA and 16s-23s r-DNA internal transcribed sequence level.[9,10] Hamid[11] reviewed that M. farcinogenes and M. senegalense are the causal agents of bovine farcy, where both are unique in these morphological and show also different characteristics.

There are no reported cases of M. facrcinogenes in environmental samples. No epidemiological reports are also available in its existence in domestic animals. Few locals of Sudan believe that ticks transmit these bacteria to cattle.[12] Though the zoonotic potential of the bacteria is unknown, only few reports provide its causes/infection in human.[13,14] There are also wide range of common synthetic media to grow these bacteria. Shigidi et al.[15] used diagnostic sensitivity test (DST) agar for culturing farcy organisms and mentioned that M. farcinogenes was found to grow particularly well on Muller Hinton's medium followed by modified Bennett, Tryptic Soya, glucose yeast extract and DST agars.[16,17] However, the draftgenome of M. farcinogenes NCTC 10953 was reported finally responsible for bovine farcy.[18]

Here, we report for the first time in India, the presence of M. facrcinogenes particularly in pus sample in a clinical case in Western India using specific modern technologies available in our Diagnostic Reference laboratory (Supratech Micropath Laboratory and Research Institute, Ahmedabad). AFB smear, NAAT testing, AFB MGIT culture, LPANTMCM, MALDI-TOF AND gene sequencing for the detection of specific type using 16s rRNA gene analysis.

MATERIALS AND METHODS

Specimen collection

A 32 years old female underwent Laparoscopy for removal of left complex ovarian cyst after normal pre operative laboratory evaluation. Stitches were removed after 10 days. After twenty (20) days of surgery, patient complained of swelling and discharge from the port site. Upon multiple dressing and multiple courses of antibiotics, discharge, swelling and pain persisted from left side port. Past, personal and family history was not significant except hypothyroidism since 3 years; she was on Tab. Thyronorm 75 mg. She presented at healthcare infectious diseases (HIDC), Ahmedabad, with anemia. Her BP, pulse rate and temperature were normal. Abdominal examination revealed presence of scar with two sinus opening and watery discharge, confirmed on sonography on Jan 2017. Incision was done and collected the fluids and tissue was sent for microbiological examination and HPE respectively. Histopathological examination showed stratified squamous epithelium with underlying tissue with areas of necrosis, mixed inflammaroty infiltrate consisting of neutrophils, lymphocytes, plasma cells, few eosinophils, few histiocytes and few foreign body giant cells with odema and vascular proliferation with no granuloma. Smear positive for AFB, but negative Gene Xpert was indirectly suggesting NTM infection. Latest diagnostic technologies were used for precise identification of the isolate at Supratech Micropath Laboratory and Research Institute, Ahmedabad.

Microbiological examinations

Potassium hydroxide (KOH) (10%) examination was done on tissue slide and was examined to identify cell types and debris. Gram stain procedure was adopted for detecting gram positive and gram negative bacteria in our sample following standard method in our laboratory. Ziehl–Neelsen stain (ZNCF) stain was also perfomed on the specimen to detect presence of AFB. Further pyogenic culture was performed by standard method following the protocol in our laboratory, whereas the fungus culture was included at two different temperatures 25^{o}c and 35^{o}c to isolate majority fungus types. AFB Culture was performed by liquid culture medium by MGIT 960. HPE Examination was done on tissue sample sections after staining with heamatoxylin-eosin (HE) and observed under microscope to notice histopathological changes.

LPANTM CM (Line Probe Assays Nontuberculous Mycobacteria Common Mycobacteria)

The genotype Mycobacterium CM test is based on the DNA-STRIP technology. The whole procedure is divided into three steps involving DNA extraction from cultured material liquid medium, a multiplex amplification with biotinylated primers and a reverse hybridization. All reagents needed for amplification, such as polymerase and primers, are included in the Amplification Mixes A and B (AM-A and AM-B) and are optimized for this test. The membrane strips are coated with specific probes complementary to the amplified nucleic acids. After chemical denaturation, the single-stranded amplicons bind to the probes (hybridization). Highly specific binding of complementary DNA strands are ensured by stringent conditions which result from the combination of buffer composition and certain temperature. Thus the probes reliably discriminate the different sequences of the bacterial species. The streptavidin-conjugated alkaline phosphatase binds to the amplicons' biotin via the streptavidin moiety. Finally, the alkaline phosphatase transforms an added substrate into a dye which becomes visible on the membrane strips as a colored precipitate. A

template ensures the easy and fast interpretation of the banding pattern obtained.

The MALDI-TOF (Mass spectrometer) is based on three functional units having ion source, mass analyzer and detection device to monitor sample ions which follow a simple protocol. The pure growth from positive MGIT culture tube was taken as a sample identification on MALDI-TOF (Bruker's microflex) to score for detection of the bacteria.[19]

Molecular analysis

DNA extraction

The pus sample taken from the patient was cultured and then cells were extracted. Sonicated bacterial cells were use for bacterial DNA extraction using manually DNA extraction Kit (Roche high pure kit, India). The concentration of DNA was determined using the Qubit 4.0 fluorometer HS DNA kit (Thermo Fisher Scientific, USA).

Sanger DNA sequencing

The 16S rRNA gene was designed to determine properties of designed primers[20] and also we are using Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to check our region of interest is covered or not. Primers were synthesized at 100pM (Picomole) scale and cartridge purified (Eurofins India). Polymerase Chain Reaction (PCR) amplification was carried out using rTaq Premix Taq Version 2.0 PCR master mix with 8F and 1492R, 16SrRNA gene specific primers at 10pM concentration under standard conditions. PCR amplifications were all carried out in duplicate alongside a known normal control cell line sample. Successful PCRs were purified prior to sequencing using ExoSAP-IT reagent (Affrymetrix-USA) according to the manufacturer's protocol. Purified PCR products were Sanger sequenced in both forward and reverse orientations using the same primer sequences used for PCR at 10pM final concentration using BigDye® v3.1 according to manufacturer's cycling conditions. BigDye® v3.1 sequencing reactions were then purified using Ethylenediaminetetraacetic Acid (EDTA) and sodium acetate according to the manufacturer's protocol and analyzed on 3500 Genetic Analyzer (Applied Biosystems USA). Sanger sequencing data was analyzed using Codon Code Aligner v5.0.2, (Codon Code Corporation Centerville, USA) and NCBI blast the entire sequence.

Next generation sequencing (NGS)

For targeted NGS analysis, 16S rRNA gene primers were used. PCR was performed using 5-15 ng/ µl bacterial DNA and rTaq Premix Taq Version 2.0 PCR master mix (TAKARA) for initial denaturation at 95°C for 10 mins, followed by 35 cycles of denatuation at 95°C for 30 sec, annealing at 52°C for 4 min and elongation at 72°C for 2 mins. Final elongation at 72°C for 10 mins. and holding period at 4°C. About1.4 kb PCR amplicons was purified using (1.4x) Agencourt AMPure XP reagents (Beckman Coulter, USA) to remove the primer dimer. For End repair and fragmentation use 100ng/ µl of DNA concentration and incubate the end-repair reaction for 20 minutes at room temperature using the Ion plus fragment kit (Thermo Fisher-USA). Amplicons purified using Agencourt AMPure XP reagents. The amplicons were ligated to adapters with barcodes of the Ion Xpress™ Barcode Adapters kit (Thermo Fisher-USA) for 15 mins at 25°C then 72°C for 5 min and hold at 4°C. Adapter ligated amplicon libraries were purified using Agencourt AMPure XP reagents (Beckman Coulter, USA). The library concentration was determined using an Ion Library Quantitation Kit (Thermo Fisher-USA), then each library was diluted to 8 pmol and the same amount of libraries was pooled for one sequence reaction. Next, emulsion PCR was carried out using the Ion OneTouch™ 2.0 System and Ion 540™ OT2 Reagents (Thermo Fisher-USA) according to the manufacturer's instructions. Template-positive Ion Sphere™ Particles were then enriched with Dynabeads MyOne™ Streptavidin C1 Beads (Thermo Fisher-USA) using an Ion OneTouch™ ES system (Thermo Fisher-USA). Purified Ion Sphere particles were loaded on 540 (Compatible with Ion S5 System) Chip. Massively parallel sequencing was carried out on Ion S5 sequencer (Thermo Fisher, USA) using Ion S5 Sequencing kit according to the manufacturer's instructions. Sequencing was performed using 500 flow runs that generated approximately 200 bp reads.

Data analysis

The data generated from the Instruments are transferred to the Standalone Torrent Suite (version 5.2) analysis server as Direct agglutination test (DAT) files. This DAT files contains electrical signals generated during sequencing flow. The Torrent Suite software (version 5.2) coverts this electrical signals into numerical one and store them in 1.wells files during the signal processing step. BaseCaller will read the information stored in 1.wells file and covert them into sequence of bases and computes the quality scores of each base. The next step is barcode filtration and adapter, low quality reads, low signal bases trimming. The final data is stored in an unaligned Binary Alignment Map (BAM) file which can be used for further alignment processes. The unaligned BAM files are downloaded from the Torrent Suite software (version 5.2) and converted into FASTQ format file to run with BWA aligner (version 0.7.12-r1039). Burrows-Wheeler Aligne (BWA) aligner is used to align the raw reads to human reference genome hg19 to remove human reads from the data. The unaligned non-human reads are then extracted from the BWA output file and stored in BAM format file. Newly generated BAM files are then uploaded to the standalone Ion Reporter software (version 5.2) for identifying the presence of bacterial genome present in the data. The uploaded BAM files are run through the Metagenomics 16S w1.1workflow of Ion Reporter (version 5.2) for the identification of species present in the data.

Simultaneously, the reads in a BAM files are extracted with custom script and stored in a Federal Acquisition STreamlining Act (FASTA) file for running with standalone Basic Local Alignment Search Tool (BLAST) program. The standalone blast is run against "nr" database to reconfirm the results obtained by Ion Reporter software (version 5.2).

RESULTS

The patient from Ahmedabad after removal of left complex ovarian cyst on November 2016 through Laparoscopy complained of swelling and discharge after 20 days (Fig.1) General examination was normal. The ultrasound (USG) local report on 26 Jan 2017 revealed a sinus track at incision site from subcutaneous to intra muscular regions with underline collection (Port site infection). The tissue biopsy after HPE showed cellular debris with epithelium having necrotic areas. But no evidence of granuloma. However, the pus was subjected to various microbiological methods including culture and molecular techniques. The clinical specimen underwent KOH examination; Gram stain and ZNCF stain which were negative. Pyogenic and fungus culture were also negative. The clinical specimen was run on Mycobacteria Growth Indicator Tube (MGIT 960) system, an automated liquid media system for AFB culture, which was positive for Nontuberculous Mycobacteria/ Mycobacterium other than tuberculosis (NTM/MOTT) and gene expert was negative. The pure growth of NTM/MOTT was subjected to LPANTM CM, a Line probe assay for identification of common NTM/MOTT. The results showed good control bands but did not show any relevant conclusive identification bands. Further the sample was subjected to MALDI TOF where the sample was identified with score of 2.04 as M. farcinogenes (Fig. 2). Further the other molecular techniques were adopted for genome analysis of these bacteria. The analysis of 16srDNA using Sanger sequencing and Next generation Sequencing revealed the two pictures that were observed in both techniques and found to be similar (Fig.3,4a and 4b) for M. farcinogenes (DSM43637).

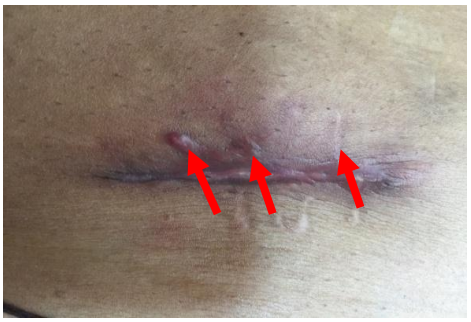

Fig.1 Before treatment with swelling wound.

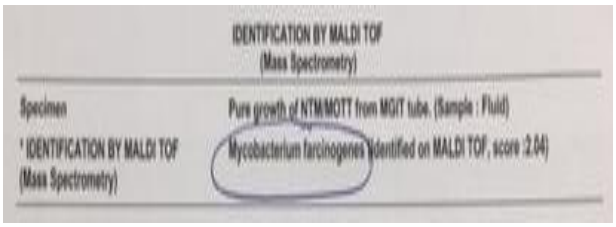

Fig.2 Report of M. farcinogenes score from MALDI-TOF

Fig.3 NCBI blast result of Sanger sequencing of strain DSM 43637.

Mycobacterium farcinogenes partial 16S rRNA gene, strain DSM 43637

Sequence ID: LT718447.1 Length: 1496 Number of Matches: 1

Range 1: 884 to 1132 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
433 bits(234)	1e-117	246/251(98%)	3/251(1%)	Plus/Minus

```
Query  1     CCGAGTTGACCCCGGCAGTCTCTCACGAGTCCCCACCATAACGTGCTGGCAACATGAGAC  60
Sbjct  1132  CCGAGTTGACCCCGGCAGTCTCTCACGAGTCCCCACCATAACGTGCTGGCAACATGAGAC  1073
Query  61    AAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCC  120
Sbjct  1072  AAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCC  1013
Query  121   ATGTACCAC-TGCACACAGGCCACAAGGGAACCGACATCTCTGCCGGCGTCCTGTGCACG  179
Sbjct  1012  ATGCACCACCTGCACACAGGCCACAAGGGAACCGACATCTCTGCCGGCGTCCTGTGCATG  953
Query  180   TCAAACCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAATCCACATGCCTCCGCCGCTTG  239
Sbjct  952   TCAAACCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAATCCACATGC-TCCGCCGCTTG  894
Query  240   TGCCGGGCCCC  250
Sbjct  893   TGC-GGGCCCC  884
```

Fig.4a NCBI Blast from NGS showing sequence of Partial sequence of 16S rRNA gene (strain DSM 43637).

Primer	Phylum	Class	Order	Family	Genus	Species	% ID	Count	DB counters	F:R %	% of total reads	% of valid reads	% of mapped reads	% of mapped reads per primer
V3								476	476 : 0		0.05	49.53	81.23	100
	Actinobacteria							476	476 : 0		0.05	49.53	81.23	100
		Actinobacteria						476	476 : 0		0.05	49.53	81.23	100
			Actinomycetales					476	476 : 0		0.05	49.53	81.23	100
				Mycobacteriaceae				476	476 : 0		0.05	49.53	81.23	100
					Mycobacterium			476	476 : 0		0.05	49.53	81.23	100
						Mycobacterium alvei / Mycobacterium boenickei / Mycobacterium conceptionense / Mycobacterium farcinogenes / Mycobacterium fortuitum / Mycobacterium houstonense / Mycobacterium neworleansense / Mycobacterium peregrinum / Mycobacterium porcinum / Mycobacterium septicum	99.37 - 100	476	476 : 0	45.59 : 54.41	0.05	49.53	81.23	100

Fig.4b IR suite showing species level identification of bacteria (Strain DSM 43637 from fig. 3 and 4a).

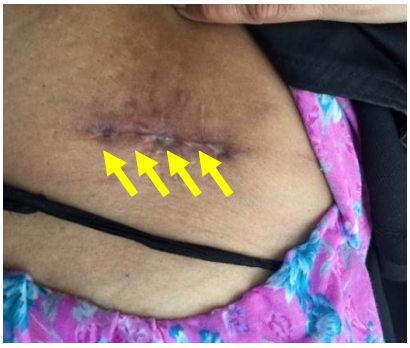

Fig.5 After treatment with normal.

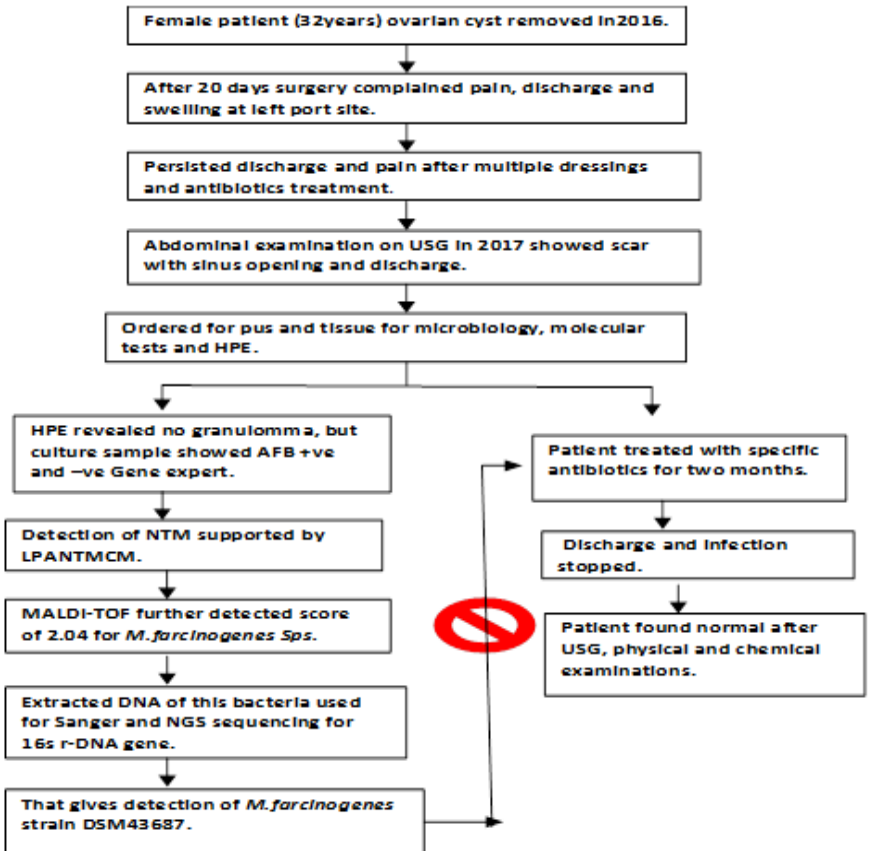

Fig.6 Flow chart for detection of bactetial species, strain and treatment of patient.

DISCUSSION

The female patient suffered from left port infection which was expressed 20 days later the procedure. It should be noted here thet the patient was on multiple higher antibiotics and still did not respond. This warrants for a definite request for AFB smear and culture by the clinician in such clinician scenarios. After 5 months she visited an ID specialist, where pus was drain. The ZNCF stained smear was positive for AFB and culture by MGIT 960 grew NTM/MOTT. Specimen by LPANTMCM was not conclusive as the bands on this method are few, related to common Mycobateria. For this reason, MALDI-TOF was used. It should also be noted here that of LPANTMCM does not give conclusive results, the strain should be sent to a reference laboratory for correct identification, which would also mean correct therapy and cure for the patient. Though the score of MALDI-TOF was good, the strain was further subjected to gene sequencing. Identification today is of great impotence for therapy, case and epidemiology.

The strain is DSM 43637 in addition to others like M.forcinogenes M15, M16 and M39.[10,12] The result suggested the correct identification of the bacterium. The species was well differentiated by 16S r-DNA and 16S-23S internal transcribed spacer DNA from M.senegalense earlier by other workers from clinical sources.[10-12] Later on its whole genome sequencing NCTC 10955 (DSM 43637) was done by Croce et al.[18] It is a non tuberculosis species responsible for bovine farcy. This strain consists of 6,139,893 bps with G+C content of 63.73% and composed of 5,816 protien-coding genes and 76 RNA genes. M.forcinogenes has also been isolated now from soil [21] and rarely implicated as a human pathogen.[14] Our results also support the document of later workers strongly, revealing its pathogenecity in human.

Further our clinical specimen is subjected to gene sequencing for confirmation using latest methods using DNA extracted from cultured Micobacteria. This DNA was used for NGS and Sanger sequencing analysis following ion torrent suite software for data analysis. The results obtained by MALDI-TOF/mass spectrophotometer was correlated with the data of NGS and Sanger sequencing technologies to confirm the species and strain present (DSM43637) in our patient sample and corroborated with the results of Croce et al.[18]

TREATMENT

After confirmation of the NTM, M.farcinogenes, finally from port site infection, IV Amikacin 1gm daily with combination of tablets Clarithromycin 500mg BD (2 times daily) and Linezolid 600mg OD (once a day) for 9 months were planned to be administered. Since the patient developed toxicity for Amikacin, it was stopped after 2 months and both oral medications were continued according to sensitivity. Finally, patient improved and the discharge stopped totally after 45 days. After 2 months, no discharge was noted and also no collection was available to process a repeat culture. Sonography

physical and clinical examinations showed total recovery from collection in the residual track. Treatment was discontinued after 9 months. (Fig.5).

CONCLUSION

(Fig.6) M. farcinogenes strain (DSM43637) was isolated from pus, which was the **first report of its kind** in a clinical sample from our laboratory Ahmedabad Gujarat India. This involved latest microbiological and molecular tools to identify the species with high sensitivity and specificity from clinical samples.

CONFLICT OF INTEREST

There are no conflicts of interest among authors.

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