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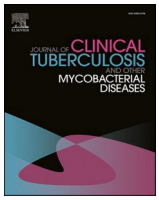

A cluster of six respiratory cultures positive for *Mycobacterium xenopi* –Clinical characteristics and genomic characterization

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ABSTRACT

Mycobacterium xenopi is a slow growing non-tuberculous mycobacterium (NTM) isolated from water systems and has been associated with pseudo-outbreaks and pulmonary infections in humans. We observed a cluster of six respiratory cultures positive for *M. xenopi* within a six-month period at our institution, approximately double our normal isolation rate of this organism. Only three of the six cases met clinical, radiographic, and microbiologic criteria for NTM infection. An investigation led by our hospital's Healthcare Epidemiology and Infection Program found no epidemiologic link between the six patients. Three isolates underwent whole-genome sequencing (WGS) and phylogenetic analysis confirmed they were non-clonal. *In vitro* susceptibility data found the isolates were sensitive to macrolides, moxifloxacin, and rifabutin. Our findings suggest that isolation of *M. xenopi* from pulmonary specimens may be increasing, further defines the genomic population structure of this potentially emerging infection, and establishes WGS as a useful tool for outbreak investigation strain typing.

1. Introduction

Mycobacterium xenopi is a slow growing non-tuberculous mycobacterium (NTM) that has been isolated from water systems and is associated with contamination and pulmonary disease in humans [1,2]. Prevalence is region specific and tends to be higher in Europe [3–6]. *M. xenopi* isolation has increased in recent years, although there have been improvements in diagnostic methods [7]. *M. xenopi* pulmonary infection can manifest in many forms, typically in older men with underlying lung disease, and some evidence suggests higher mortality rates compared with *Mycobacterium avium* complex [8–12].

Pseudo-outbreaks, defined as increased isolation of an organism without increase in cases meeting criteria for infection, have been linked to *M. xenopi*, typically from contaminated water or equipment [13–17]. However, reports have documented an upward trend in true infections due to *M. xenopi*, highlighting the importance of distinguishing pathogens from contaminants [7]. Multi-locus sequencing typing (MLST) methods have been established for *M. xenopi* [18]. Despite decades of literature on the organism, there have been no published reports of clinical outcomes data paired with whole genome sequencing and only six assembled genome sequences were publicly available in the National Center for Biotechnology Information (NCBI) sequence database as of January 2023 [19].

We report a cluster of six cases of pulmonary cultures positive for *M. xenopi* at our institution in a six-month period, documenting each case's clinical features, antimicrobial susceptibility testing (AST), treatment, outcomes, and infection control investigation. Whole genome sequencing and phylogenetic analysis was performed for three of the isolates.

2. Case reviews

2.1. Case report 1

A 78-year-old female with a history of smoking was found to have an incidental right upper lobe (RUL) lung nodule on computed tomography

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(CT) of the chest for which she underwent a RUL segmentectomy. Pathology of the lung was reported as adenocarcinoma and staining showed rare acid-fast bacilli (AFB). She received no additional therapy for her malignancy and was followed with serial imaging. CT imaging of the chest two years later showed a nodular opacity at the RUL surgical site (Fig. 1A), and she underwent a wedge resection of the lesion. AFB smear in the microbiology laboratory was negative, but the mycobacterial culture grew *M. xenopi* at three weeks.

Antibiotic therapy was not pursued as the patient was asymptomatic. A follow up CT of the chest one year later demonstrated sequelae of prior RUL resection but no other findings and the patient remains asymptomatic approximately fifteen months after diagnosis.

2.2. Case report 2

A 26-year-old female with a history of tetralogy of Fallot and low body mass index presented to an immediate care with acute inspiratory chest pain and night sweats for three months. A chest x-ray showed a RUL cavitary lung lesion and pneumomediastinum. CT of the chest showed RUL multiple cavitary lesions (Fig. 1B). AFB smear and culture of three sputum specimens were negative. She underwent a bronchoalveolar lavage (BAL). AFB smear in the microbiology laboratory was negative, but the mycobacterial culture grew *M. xenopi* at two weeks.

The patient started therapy with oral rifampin (RIF), ethambutol (EMB), azithromycin (AZM), and intravenous amikacin (AMK). After two weeks of therapy, she had a significant improvement in symptoms. Intravenous AMK was continued for 6 weeks. Three months into therapy, based on AST, the regimen was changed to rifabutin (Rfb), EMB, and AZM (Table 1). One year after diagnosis, a repeat CT chest showed persistent cavitary lesions for which she underwent a RUL partial lobectomy. The pathology results of the tissue showed granulomas and culture grew *M. xenopi*. She remains on EMB, Rfb, and AZM nearly two years after diagnosis.

2.3. Case report 3

A 48-year-old male with a history of an eating disorder in remission, chronic low BMI, and previous smoker presented to the emergency department with chest pain for two days. A CT of the chest showed bilateral upper lobe cavities. Nine months later, a CT of the chest showed increase in the size of a left upper lobe (LUL) cavitary nodule (Fig. 1C). A LUL wedge resection was performed (Fig. 2A) and surgical pathology revealed granulomas (Fig. 3A) with a positive AFB stain (Fig. 3B). AFB smear in the microbiology laboratory was negative, but mycobacterial culture grew *M. xenopi* at 2 weeks.

The patient started RIF, EMB, and AZM. A follow up CT of the chest two months later showed a residual cavitary nodule near the resection margin in the left lower lobe (LLL). Three months into treatment, based on AST, moxifloxacin (MXF) was added and RIF was changed to Rfb. One month later, the patient developed elevated transaminases and Rfb was discontinued. CT scan of the chest at 12 months revealed an increase in the cavity size. Fifteen months after initial culture, the patient underwent a LUL wedge resection, and pathology showed granulomas. The AFB smear from the resected tissue was negative and culture has remained negative to date. The patient's symptoms have resolved and he continues on EMB, AZM, and MXF fourteen months after diagnosis.

2.4. Case report 4

A 66-year-old male with a history of hypertension was diagnosed with new atrial fibrillation by his primary care physician. A CT of the chest performed for workup of the atrial fibrillation revealed a RUL nodule (Fig. 1D). He underwent a RUL wedge resection of the lesion (Fig. 2B) and surgical pathology showed granulomas but AFB stain was negative (Fig. 3C). AFB smear in the microbiology laboratory was negative, but the mycobacterial culture grew *M. xenopi* at five weeks.

The patient was not treated with antibiotics given his lack of symptoms and repeat CT of the chest 18 months later showed sequelae of the RUL resection only. The patient remains asymptomatic fourteen months after diagnosis.

2.5. Case report 5

A 39-year-old male with a history of hypertension presented with shortness of breath and night sweats. Eight months into symptoms, CT of the chest showed ground glass pulmonary opacities and cavities in both lungs (Fig. 1E). BAL culture was negative for growth. Four months later, the patient underwent a right lung wedge resection (Fig. 2C). Surgical pathology of the lung tissue revealed granulomas and AFB staining was

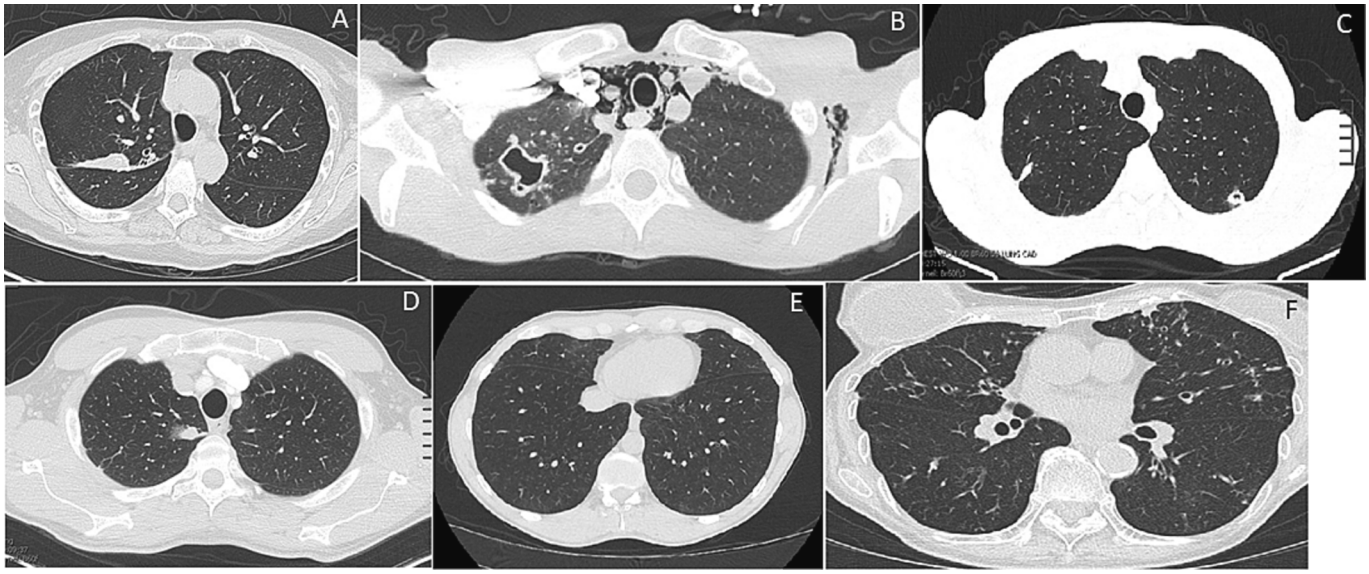

Fig. 1. Computed tomography (CT) of the chest images. Fig. 1A: Case 1. Right upper lobe band-like opacity. Fig. 1B: Case 2. Large right upper lobe cavitary lesions. Fig. 1C: Case 3. Upper lobe cavitary nodules. Fig. 1D: Case 4. Right upper lobe nodule. Fig. 1E: Case 5. Left and right upper lobe cavitary nodules. Fig. 1F: Case 6. Multifocal bronchiectasis and tree-in-bud opacities.

Table 1
Susceptibility reports of five *Mycobacterium xenopi* isolates.

	In vitro susceptibility testing results of *Mycobacterium xenopi* (MIC in micrograms/milliliter)										
Case Number	Amikacin	Ciprofloxacin	Clarithromycin	Clofazimine	Doxycycline	Linezolid	Minocycline	Moxifloxacin	Rifabutin	Rifampin	Streptomycin	Trimethoprim-Sulfamethoxazole
1	4	2	< 0.06	0.06	8	4	4	0.12	< 0.12	0.5	8	1/19
2	8	> 8	< 0.06	0.06	> 8	4	8	1	< 0.12	2	16	> 4/76
3	8	4	0.12	0.06	> 8	4	> 8	0.5	< 0.12	4	16	> 4/76
4	32	2	0.25	0.12	> 8	2	> 8	1	2	> 4	> 32	> 4/76
5	2	0.5	< 0.06	< 0.015	2	2	2	0.25	< 0.12	0.12	4	0.3/4.8

positive (Fig. 3D). AFB smear of the tissue in the microbiology laboratory was also positive and mycobacterial culture grew *M. xenopi* at six weeks.

The patient was started on RIF, EMB, AZM, and MXF. Two months later, he reported improvement in symptoms. MXF was discontinued at three months based on AST. Eight months into therapy, repeat CT of the chest showed stable findings and no new lesions. The patient remains on RIF, EMB, and AZM one year after diagnosis.

2.6. Case report 6

An 88-year-old-female with a history of non-small-cell lung carcinoma of the RUL three years prior presented with cough for one year. A CT of the chest showed multifocal bronchiectasis with tree-in-bud opacities (Fig. 1F). Sputum AFB smear in the microbiology laboratory was negative, but the mycobacterial culture grew *M. xenopi* at four weeks. AST was not performed.

Given the patient's advanced age and limited symptoms, the patient was not treated. A repeat CT of the chest six months later showed stable radiographic findings and the patient's symptoms remain unchanged.

3. Methods

This study was approved by the Institutional Review Board (IRB) at Northwestern University (STU00217076).

3.1. Mycobacterial culture

Mycobacterial cultures were processed with 4% sodium hydroxide. AFB smear was performed via fluorescent auramine-rhodamine stain with a confirmatory Kinyoun stain. The specimens were inoculated in broth via the BACTEC Mycobacteria Growth Indicator Tube System (BACTEC MGIT 960 System, Becton-Dickinson, Franklin Lakes, NJ). Positive broth cultures were sub-cultured to Middlebrook 7H11 agar (Thermo Fisher Scientific, Waltham, MA). All *M. xenopi* isolates grew between 3 and 6 weeks with yellow pigment on 7H11 media. Colonies underwent Sanger sequencing of the *rpoB* gene, and all sequences aligned (greater than 99% sequence identity) to the *M. xenopi* gene sequences available on GenBank (accessions KF910160.1, AP0222314.1, JN881349.1, HQ141571.1, and EU597594.1). AST was performed at the National Jewish Health Advanced Diagnostic Laboratories via broth microdilution.

3.2. Whole genome sequencing and phylogeny

DNA was extracted from mycobacterial cultures with the QIamp BiOstic Bacteremia DNA Kit (Qiagen, Hilden, Germany). Multiplexed sequencing libraries were prepared using the plexWell Plus 24 kit (seqWell, Beverly MA, USA) and sequenced on the Illumina MiSeq using a 600-cycle V3 kit to yield paired-end 300 bp reads. Reads were quality trimmed and adapter sequences removed using fastp [20]. Genome sequences were *de novo* assembled using SPAdes v3.9.1 [21]. Residual adapter contamination was identified in the assembly contigs using NCBI FCS-adaptor v0.2.3 (https://github.com/ncbi/fcs]) and removed. Assembly contigs shorter than 200 bp or those with an average read coverage of less than 5 were removed. Genomes assemblies were deposited in GenBank under BioProject accession number PRJNA918685. One complete genome assembly of strain JCM 15561 (NCBI RefSeq accession NZ AP022314.1) and five draft genome assembly sequences of DSM 43995 (NCBI assembly accession GCA 002102015.1) NCTC10042 (GCA 900453395.1), RIVM700367 (GCA 000257745.1), 3993 (GCA 000523715.1), and 4042 (GCA 000523695.1) were available from NCBI at the time of analysis (January 8, 2023) and were downloaded (Table 2). Of note, three of the available records (JCM 15561, DSM 43995, and NCTC10042) are separate assemblies of the same type strain, ATCC 19250, and one sequence was

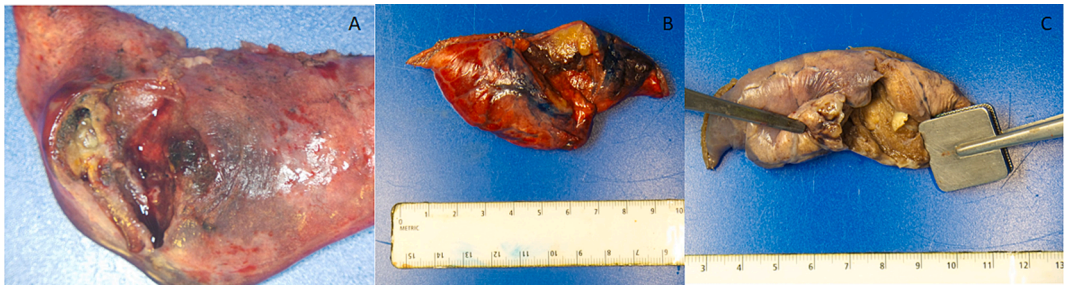

Fig. 2. Gross images of resected lung tissue. Fig. 2A: Case 3. Gross image of left upper lobe pulmonary wedge resection. A 15.0 g, 7.0 × 4.0 × 2.0 cm lung wedge specimen was serially sectioned to reveal a 2.0 × 1.5 × 1.5 cm well-circumscribed gray-white lesion. Additional sectioning at during intraoperative consult revealed an additional 0.3 × 0.2 × 0.2 cm gray-tan nodule. Fig. 2B: Case 4. Gross image of right upper lobe pulmonary wedge resection. Multiple fragments of red to yellow-tan tissue measuring 0.7 × 0.5 × 0.3 cm in aggregate and a 9.9 × 4.9 × 2.6 cm lung wedge were sectioned to reveal a 3.0 × 1.6 × 1.5 cm yellow firm homogeneous nodule. Fig. 2C: Case 5. Gross image of right upper lobe pulmonary wedge resection. A 22.5 g, 11.5 × 8.5 × 1.8 cm lung wedge specimen. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

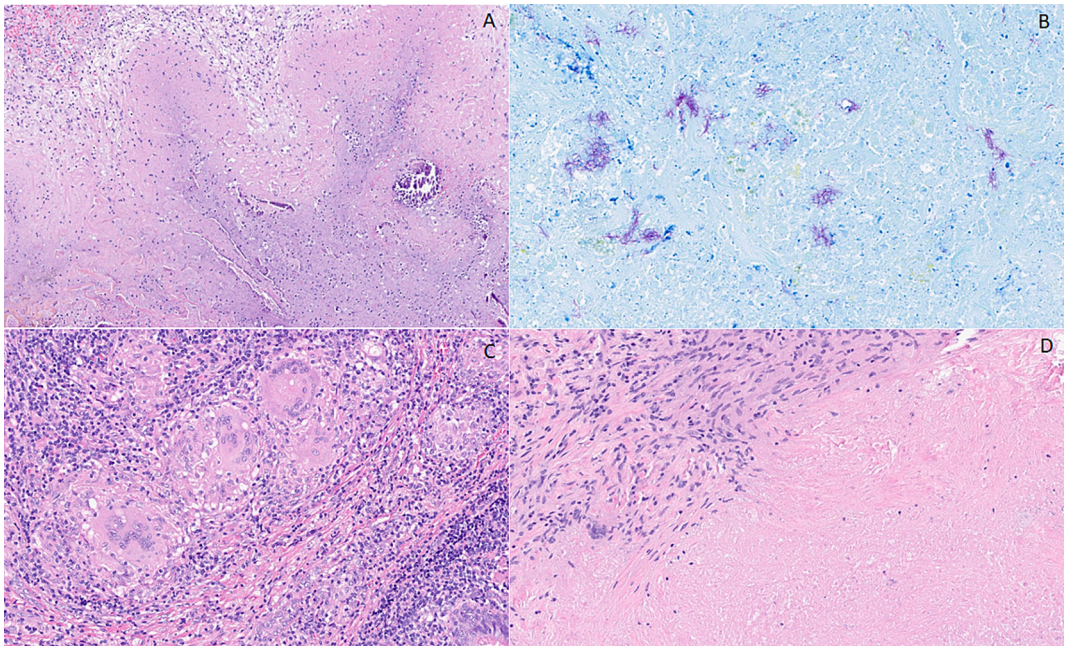

Fig. 3. Surgical pathology slide images of lung tissue. Fig. 3A: Case 3. H&E of left upper lobe lung tissue demonstrating rare granulomas. Fig. 3B: Case 3. AFB stain demonstrating numerous acid-fast bacilli. Fig. 3C: Case 4. H&E of right upper lobe lung tissue demonstrating necrotizing granulomas. AFB staining was negative. Fig. 3D: Case 5. H&E of right upper lobe lung tissue demonstrating non-caseating granulomas.

generated from a type strain of unknown provenance (RIVM700367). The species of all nine genome sequences in this analysis were confirmed to be *M. xenopi* from 16S rRNA gene sequences. For each of the five draft assembled sequences from NCBI, pseudoread with a length of 250 bp and a depth of 20 reads across each contig were generated. Reads from the three newly sequenced isolates and pseudoreads generated from the five draft genome sequences from NBCI were aligned to the complete genome sequence of JCM 15561 using bwa v. 0.7.15 [22]. Pseudoreads were generated from genome sequences using the pseudoreads.pl script (https://gist.github.com/egonozer/d2f5b17a0ae62e76fbe9857bb81c1dd0) that is based on functionality embedded in Snippy v4.6.0 (https://github.com/tseemann/snippy/releases/tag/v4.6.0). Single nucleotide variants relative to the reference were identified using bcftools v1.9 using a haploid model and filtering read alignments base quality lower than 25 or alignment quality less than 30. Variants were further filtered as previously described [23] using the bcftools filter software (https://github.com/egonozer/bcftools_filter) to remove variants with single nucleotide variant (SNV) quality score less than 200, read consensus less than 75%, read depths less than five, less than one read in both directions, or located within repetitive regions (as defined by blast alignment of the reference genome sequence against itself). For the total set of all consensus sequences, variant positions with base calls in less than 100% of isolate sequences (i.e. non-core positions) were masked with N's using ksnp matrix filter.pl [24]. Maximum likelihood phylogenetic trees were generated from genome alignments with IQ-TREE v2.0.7 [25,26].

4. Results

The number of *M. xenopi* isolates per year at our institution since 2009 ranges between 2 and 6, approximately half the rate we observed in this study (Fig. 4). Specimens from Case 1 and 2 were processed the same day, raising concern for a possible pseudo-outbreak and prompting an investigation by the Healthcare Epidemiology and Infection Program, which evaluated hospital locations, procedure rooms, and shared equipment. Because the investigation found no epidemiologic links between the six cases, environmental cultures were not performed. Of note, only Case 2 had more than one positive culture for *M. xenopi*,

Table 2
Isolate and sequence assembly characteristics. Abbreviations: BAL = bronchoalveolar lavage, RUL = right upper lobe, N = novel allele, U = unknown, ND = not defined.

ID	Assembly Accession	BioSample Accession	Year Isolated	Location isolated	Host	Specimen Source	Contigs	Assembly Size (nt)	GC%	6-gene MLST						
										atpD	fusA1	glnA1	pheT	secA1	topA	ST
Case1	GCA_027854195.1	SAMN32603692	2021	USA	*Homo sapiens*	Lung tissue	181	4,889,376	65.91	1	1	1	1	2	1	2
Case2	GCA_027854205.1	SAMN32603693	2021	USA	*Homo sapiens*	BAL	208	4,889,461	65.91	1	1	1	1	2	1	2
Case3	GCA_027854175.1	SAMN32603694	2021	USA	*Homo sapiens*	Lung tissue	162	4,879,895	65.91	1	1	1	1	2	1	2
3993	GCA_000523715.1	SAMN02641613	2012	USA	*Homo sapiens*	Sputum	37	5,287,678	65.84	1	1	1	1	2	1	2
4042	GCA_000523715.1	SAMN02641614	2012	USA	*Homo sapiens*	RUL biopsy	93	5,477,373	65.99	1	N	1	1	2	1	U
DSM_43995	GCA_002102015.1	SAMN04216958	1959	UK	*Xenopus laevis*	Skin lesion	146	4,933,400	65.90	1	1	1	1	1	1	1
JCM_15661T	NZ_AP022314.1	SAMD00197529	1959	UK	*Xenopus laevis*	Skin lesion	1	4,917,655	65.91	1	1	1	1	1	1	1
NCTC10042	GCA_900453395.1	SAMEA101170168	1959	UK	*Xenopus laevis*	Skin lesion	21	5,251,936	65.90	1	1	1	1	1	1	1
RIVM700367	GCA_000257745.1	SAMN02470128	unknown	unknown	unknown	unknown	117	4,434,836	66.11	1	1	2	1	2	3	ND

though only Case 2 and Case 3 had repeat specimens sent for culture.

Only isolates for Case 1, Case 2, and Case 3 yielded sufficiently high-quality genomic DNA for WGS. Despite multiple extraction techniques, the other isolates yielded DNA of low quantity, possibly due to the hardy cell walls of mycobacteria. MLST from the assembly sequences based on the previously described 6-gene typing schema [18] revealed that all three isolates belonged to ST-2. Despite this, the core genome phylogenetic tree generated from the three isolates in this study with six previously deposited genomes in GenBank (Fig. 5) revealed that while the case isolates shared a common branch and ST, they were not clonal. The core genome alignment of the three newly sequenced isolates differed between 2126 and 4213 pairwise single nucleotide variants (SNV's), verifying that they were distinct isolates and not part of either an outbreak or a pseudo-outbreak.

In vitro AST was performed on five of the isolates (Case 1–5). There were disparate AST results between the isolates, further supporting different sources of origin for the isolates (Table 1). All isolates showed susceptibility to clarithromycin (CLR), linezolid (Lzd), MXF, and Rfb. Four out of five were susceptible to AMK. Only 2 out of five were susceptible to RIF and trimethoprim-sulfamethoxazole (TMP-SMX), and only 1 was susceptible to ciprofloxacin (CIP). None of the isolates were susceptible to doxycycline or minocycline. An examination of mycobacterial antimicrobial resistance genes found no homologs of resistance-associated mutations in *ermB*, *gyrA*, *inhA*, *katG*, *pncA*, *rpoB*, *rpsL*, or *rsmG / gidB* in any of the three case isolates (Table 3) [27,28]. No homologs resistance-associated genes *erm* (*M. tuberculosis* H37Rv locus ID Rv1988), associated with macrolide resistance [29], *stp* (locus ID Rv2333c, associated with tetracycline resistance [30], or *lfrA* (*M. smegmatis* FDAARGOS 679 locus ID FOB87 RS25035, efflux pump conferring resistance to fluoroquinolones, rifamycins, and isoniazid (INH) [31] were found in any of the case isolates.

5. Discussion

M. xenopi is a slow growing mycobacterium that has been known to cause NTM infection in humans for decades, yet little is known about optimal treatment, correlation of *in vitro* susceptibility testing and outcomes, resistance mechanisms, and strain typing with whole genome sequencing [1–16].

We observed a time–space cluster of six cases of pulmonary cultures positive for *M. xenopi* within a six-month period, approximately double our normal rate. Of note, three of the six cases do not meet IDSA diagnostic criteria for pulmonary NTM infection. Case 1 and Case 4 did not meet criteria due to a lack of respiratory symptoms and Case 6 had only one positive sputum culture for *M. xenopi* [32]. An investigation by our hospital's Healthcare Epidemiology and Infection Program did not identify any common environmental source to suggest a pseudo-outbreak, and whole genome sequencing and phylogenetic analysis demonstrated that the isolates were not clonal and thus not likely to have stemmed from an isogenic source such as a colonized water system.

Isolates in this study showed highest *in vitro* susceptibility to CLR, Lzd, MXF, and Rfb, and low susceptibility to RIF, tetracyclines, CIP, and TMP-SMX. Examination of the sequences of common mycobacterial resistance genes in the *M. xenopi* genomes did not reveal any mechanisms for the high *in vitro* minimum inhibitory concentrations to RIF and tetracyclines observed. The genome analysis also could not explain the fluoroquinolone resistance observed in the isolate from Case 2 relative to other isolates more sensitive to MXF. This suggest there may be other mechanisms, potentially efflux pumps or modifying enzymes, driving lowered susceptibility to these antibiotics. Further study is needed to better elucidate antimicrobial resistance mechanisms in *M. xenopi*.

An obstacle to *M. xenopi* infection management is the lack of correlation between *in vitro* AST and clinical response. Data suggests *in vitro* resistance to RIF and EMB, despite better outcomes with this regimen [33]. A randomized controlled trial (RCT) found a lower mortality rate in patients treated with RIF plus EMB compared to RIF, EMB, and INH,

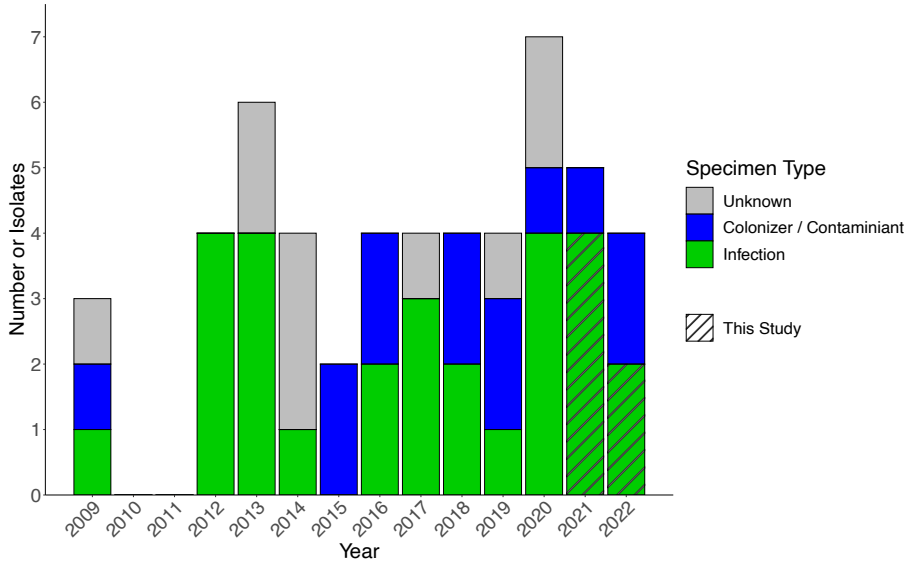

Fig. 4. Epidemic curve of *Mycobacterium xenopi*. True infections were determined by clinician's documentation that the organism was contributing to clinical disease, whereas colonizers/contaminants were felt not to contribute to clinical disease. If it was unclear based on chart review, it was listed as unknown significance. All six isolates in this study were felt to have clinical significance and are listed separately.

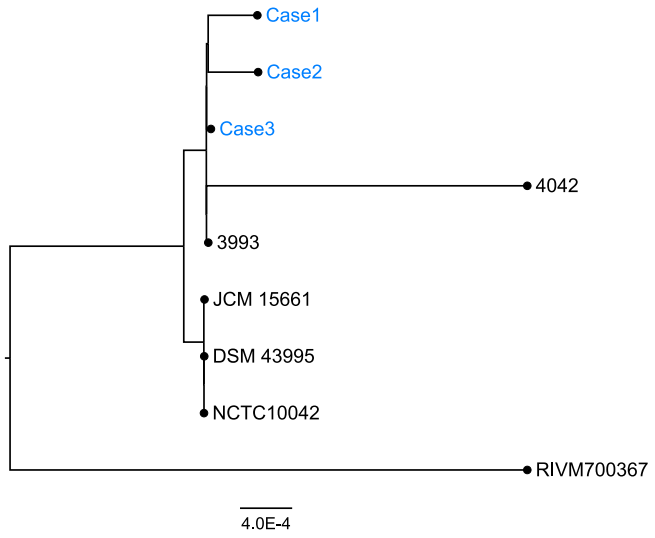

Fig. 5. Core genome maximum likelihood phylogenetic tree of *M. xenopi* isolates (Case 1, Case 2, and Case 3 from this study in blue, others were previously deposited to NCBI GenBank). Tree is midpoint rooted. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

but also found no correlation of *in vitro* AST and clinical response [34]. In contrast, a case series found no difference in clinical outcomes between patients treated with RIF plus EMB versus RIF, EMB, and INH [35]. A review found no benefit of INH or aminoglycosides on outcomes, whereas fluoroquinolone-based regimens had lower rates of short-term relapse [36]. A prospective RCT found no clinical difference between CLR versus MXF containing regimens [11]. The Infectious Diseases Society of America (IDSA), therefore, recommends a 3-drug regimen including a macrolide or fluoroquinolone in addition to RIF and EMB, with consideration of intravenous medications if cavities or severe bronchiectasis [32]. Although ours was a small study, the AST data supports use of AZM, MXF, and Rfb as potential empiric treatment options in *M. xenopi* infections, whereas tetracyclines, CIP, and TMP-SMX may be less effective.

Our cases show the diversity of pulmonary infections of *M. xenopi* ranging from pulmonary nodules, cavitary lesions, to multifocal tree-in-bud opacities. Two out of six cases were asymptomatic and the patients are doing well without treatment (Case 1 and 4). Two out of three cases with cavitary lesions required wedge lung resections due to persistent imaging findings despite prolonged therapy (Cases 2 and 3). Case 2 had a persistent positive AFB culture after one year of therapy, demonstrating difficulty in eradication.

This study suffers from limitations. First, AST was performed on five of six isolates and WGS was performed on three of six isolates due to difficulties in extracting high-quality DNA. Optimization of automated

Table 3
Non-synonymous variants in antibiotic resistance-related genes relative to JCM 15661. *: embB reference gene sequence MYXE_RS01175 corrected to remove two frameshift mutations at poly-G sites likely the result of nanopore read errors. **: no gene mutations relative to reference identified. Mutations shown are nucleotide changes in the upstream promoter region. ***: Antibiotic abbreviations: AG = aminoglycoside, EMB = ethambutol, FQ = fluoroquinolone, INH = isoniazid, PZA = pyrazinamide, RIF = rifampin. ****: Corresponding amino acid positions from alignment of *M. xenopi* protein sequence to *M. tuberculosis* H37Rv protein sequence.

Gene	JCM15661Locus ID	Antibiotic ***	Case1	Case2	Case3	MX –>MTB****
embB*	MYXE_RS01175	EMB	–	N736S	P783A	736 –>760,783 –>809
gyrA	MYXE_RS00030	FQ	–	–	V754A	754 –>555
gyrB	MYXE_RS00025	FQ	–	–	–	
inhA**	MYXE_RS13650	INH	–	–	–	
katG	MYXE_RS11785	INH	–	S108N,N183H,Q410E	S108N,N183H,Q410E	108 –>140,183 –>215,410 –>444
pncA**	MYXE_RS11445	PZA	–	G-20A	–	
rpoB	MYXE_RS19350	RIF	I961T	–	–	961 –>965
rpsL	MYXE_RS19290	AG	–	–	–	
rsmG / gidB	MYXE_RS23550	AG	A61V,P206S	A61V	A61V,P206S	61 –>53,206 –>198

methods for DNA extraction may be necessary to expand WGS studies in *M. xenopi*. Nevertheless, this investigation is the first to pair clinical characteristics of pulmonary cultures positive for *M. xenopi* with whole-genome sequencing. It also adds to the public databases whole genome read and assembly data for three *M. xenopi* isolates, considerably increasing the amount of diverse genomic data available to better understand this relatively under-sequenced pathogen. Among the sequenced isolates that could definitely be identified as having been isolated from human sources, all shared a common branch on the core genome phylogenetic tree and four belonged to sequence type 2. Prior studies of *M. xenopi* sequence types also noted a predominance of ST-2 isolates among human infections in the Midwest and Northeast regions of the United States with ST-5 infections predominating in Southeast Canada [18,37]. The apparent abundance of ST-2 in human infections in this study and others underlines the inadequate discriminatory power of MLST approaches and further supports the importance of WGS as necessary to define strain relatedness in the context of outbreak or pseudo-outbreak investigations. This also demonstrates the need for future WGS studies in *M. xenopi* to better characterize the genomic diversity of isolates associated with human infection, potential geographic variations in predominating lineages, and whether there are distinctions from populations found in the environment or those associated with laboratory contamination.

Our study reinforces that *M. xenopi* is an emerging cause of NTM pulmonary infection in the United States, even among younger individuals with no underlying lung disease. We also demonstrated that that WGS is an essential tool for investigating pseudo-outbreaks and potentially has higher discriminatory power than MLST. Further study in this field should include development of *in vitro* AST testing methods that correlate with *in vivo* response, environmental surveillance studies for *M. xenopi* in tap water and heating systems, and discovery of new antimicrobials effective at treating non-tuberculous mycobacterial infections. Such investigations may also be better supported by broader and larger-scale WGS studies in this species.

Ethical statement

This study was approved by the Institutional Review Board (IRB) at Northwestern University (STU00217076).

Declaration of competing interests and funding sources

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CRediT authorship contribution statement

Kendall Kling: Conceptualization, Data curation, Formal analysis, Methodology, Supervision. **Rebecca Osborn:** Conceptualization. **Adil Menon:** Conceptualization. **Janna Williams:** Conceptualization. **Ryan Cardew:** Conceptualization. **Omar Al-Heeti:** Conceptualization. **Phillip Santoiemma:** Conceptualization. **Michael Angarone:** Conceptualization. **Samuel Gatesy:** Methodology. **Travis Kochan:** Methodology. **Teresa Zembower:** Conceptualization, Formal analysis, Supervision. **Karen Krueger:** Conceptualization, Methodology, Supervision. **Egon A. Ozer:** Conceptualization, Data curation, Formal analysis, Methodology, Supervision. **Chao Qi:** Conceptualization, Data curation, Formal analysis, Methodology, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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