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Short Communication

Prolongation of incubation time improves clinical diagnosis of *Mycobacterium xenopi* infection and allows susceptibility testing of mycobacterial strains against multiple antibiotics

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ABSTRACT

Objectives: Mycobacterium xenopi is a nontuberculous mycobacterium (NTM) whose clinical diagnosis and drug susceptibility studies are frequently hampered by poor in vitro growth. Extending the culture incubation time from 42 days (common-standard) to 56 days could improve the likelihood of diagnosis and provide strains for phenotypic drug susceptibility profiling of this poorly studied but clinically relevant mycobacterium.

Methods: Time-to-positivity of mycobacterial cultures incubated for 56 days were analysed and compared. Clinical mycobacteriosis was defined by ATS/IDSA criteria. In vitro susceptibility of *M. xenopi* isolates was tested by broth microdilution.

Results: Of 3852 mycobacteria-positive cultures (26 different mycobacterial species),*M. xenopi* required by far the longest growth time in culture, exceeding the 42 days commonly used in routine diagnostics in 41.2% of cases versus 4.7% for other NTM and 2.0% for *Mycobacterium tuberculosis* complex ($P < 0.001$). Prolonging the incubation time to 56 days had a great impact on *M. xenopi* diagnosis, as 56.3% (27/48) of patients would have not fulfilled the ATS/IDSA criteria at an incubation limited to 42 days. All 40 *M. xenopi* isolates from patients with clinical mycobacteriosis were fully susceptibility to macrolides and rifamycins in vitro and to moxifloxacin, amikacin and linezolid.

Conclusion: These results indicate that a significant percentage (56.3%) of positive culture for*M. xenopi* would have incorrectly been reported as negative to clinicians without prolonging the incubation time to 56 days. Moreover, 56.3% of patients with *M. xenopi* disease would have missed the diagnosis along with an appropriate germ-based antimycobacterial treatment, otherwise fully effective.

1. Introduction

In the past years, the frequency of pulmonary disease caused by nontuberculous mycobacteria (NTM) has been observed to be steadily increasing [1,2]. Despite improvements in radiological and microbiological techniques, the diagnosis of NTM disease, and consequent establishment of an effective antibiotic treatment, still remains a puzzling task.

Among the NTM, *Mycobacterium xenopi* is a slowly-growing mycobacterium characterised by particularly challenging laboratory isolation [3]. Its slow in vitro replication kinetics, coupled with a non-optimised incubation time for this mycobacterium according to current guidelines [4], significantly limit *M. xenopi* recovery from biological specimens, potentially justifying the heterogeneous epidemiology observed in Europe and the USA [5]. As *M. xenopi* is an environmental NTM [6,7], insufficient *M. xenopi* recovery from clinical specimens has a critical impact on the definition of its clinical significance in positive patients and in the identification of patients who require antibiotic treatment [4,5].

To date, the optimal combined antimycobacterial treatment for *M. xenopi* has not been established and the difficulty of in vitro

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susceptibility testing is the main reason why poor data are available on effective antibiotic treatments [8–10].

The present study reports the optimisation of *M. xenopi* diagnosis, identification of patients with clinical disease and in vitro drug susceptibility testing by modification of standard culture procedures for mycobacteria in a large Italian reference centre over a 7-year period.

2. Materials and methods

This retrospective, non-interventional study included all respiratory, biopsy or other biological (non-environmental) specimens processed for cultural analysis of mycobacteria between January 2012 and December 2018 at ASST Grande Ospedale Metropolitano Niguarda (Milan, Italy). Cultures were grown on solid egg Löwenstein–Jensen (LJ) medium and in Middlebrook 7H9 broth in an automated bacteriological BACTEC MGIT 960 system at a temperature of 35 °C for a maximum of 56 days. Both cultures were constantly monitored for mycobacterial growth during the entire culture period. Time-to-positivity (TTP) of the first positive culture (either liquid or solid), expressed in days, was registered after microscopic confirmation by Kinyoun staining. Mycobacterial species identification was performed using GenoType Mycobacterium CM, Mycobacterium AS, NTM-DR, MTBC or MTBDRplus (Hain Lifescience GmbH, Nehren, Germany) according to manufacturer's instructions.

Clinical mycobacteriosis was defined as the isolation of *M. xenopi* in (i) at least one sample of bronchoaspirate/bronchoalveolar lavage, (ii) at least two samples of sputum or (iii) at least one biopsy sample (mainly lymph nodes) according to American Thoracic Society/Infectious Diseases Society of America (ATS/IDSA) guidelines [5].

Mycobacterial strains collected from the first respiratory or biopsy specimens of patients with a diagnosis of clinical mycobacteriosis by *M. xenopi* were tested in vitro against a panel of 13 antibiotics by the broth microdilution method using Sensititre™ Myco SLOMYCO AST plates (Thermo Fisher Scientific, UK). Microdilution plates were incubated at 42 °C and the results were evaluated after 5–15 days. Clinical breakpoints for minimum inhibitory concentrations (MICs) established for *Mycobacterium kansasii* were used [11,12].

3. Results

3.1. Time-to-positivity of cultural analysis for Mycobacterium tuberculosis complex and nontuberculous mycobacteria

Between January 2012 and December 2018, a total of 44 375 biological specimens from patients receiving treatment or diagnostic procedures at our centre and affiliates were processed for cultural analysis of mycobacteria, of which 3852 were positive.

Fig. 1A depicts the median and interquartile range (IQR) TTP values for different mycobacterial classes. The shortest TTP was observed for rapidly-growing NTM (median 4 days, IQR 3–8 days), followed by slowly-growing NTM (median 8 days, IQR 5–15 days) and *M. tuberculosis* complex (median 9 days, IQR 5–15 days). *M. xenopi* showed the longest TTP, with cultures requiring a median of 36 days (IQR 18–51 days) to became positive compared with 7 days (IQR 4–14 days) for other NTM and 9 days (IQR 5–15 days) for *M. tuberculosis* ($P < 0.001$ by Mann–Whitney test for all comparisons).

M. xenopi was the slowly-growing NTM species requiring the most prolonged time of growth in culture ($P < 0.001$, Mann–Whitney test) (Fig. 1B); the TTP was >42 days in 41.2% of cases

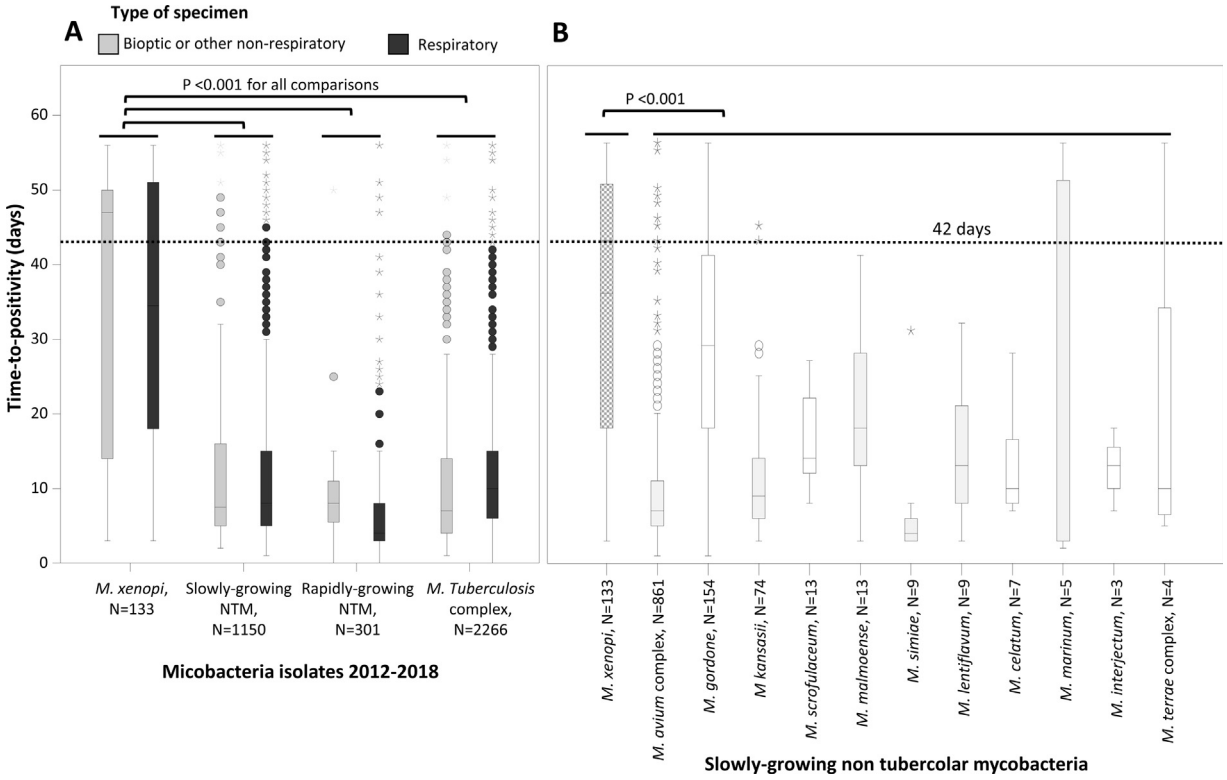

Fig. 1. Comparison of time-to-positivity (TTP) for mycobacteria cultures over a 7-year period. Box plots for median and interquartile range of TTP values are reported for (A) different mycobacterial classes and specimen types and (B) specific species of slowly-growing nontuberculous mycobacteria (NTM). All positive liquid cultures between January 2012 and December 2018 were analysed. The horizontal dotted line represents the current standard for incubation time (42 days). *P*-values were calculated by Mann–Whitney test.

compared with only 4.7% of cases for other NTM and 2.0% for *M. tuberculosis* complex ($P < 0.001$, Fisher's exact test).

3.2. Impact of prolonged incubation time on clinical diagnosis of Mycobacterium xenopi disease

Of the total 133 *M. xenopi* strains analysed, 126 (94.7%) were from respiratory samples, 5 (3.8%) were from lymph node or tissue biopsies and 2 (1.5%) were from urine samples. Sputum was the most frequently collected specimen in our daily practice, accounting for 75.2% (100/133) of *M. xenopi*-positive samples. TTP values for sputum samples were <42 days in 65.0% of cases compared with 37.5% in bronchoalveolar lavage and 30% in bronchoaspiration samples ($P = 0.026$, Kruskal–Wallis test).

For the determination of TTP, the results both of liquid MGIT and solid LJ media were taken into consideration. However, in 125 (94.0%) of 133 samples the first to turn out positive was liquid MGIT culture.

When ATS/IDSA criteria for the definition of *M. xenopi* disease were applied, it was found that 27/48 patients (56.3%) received this

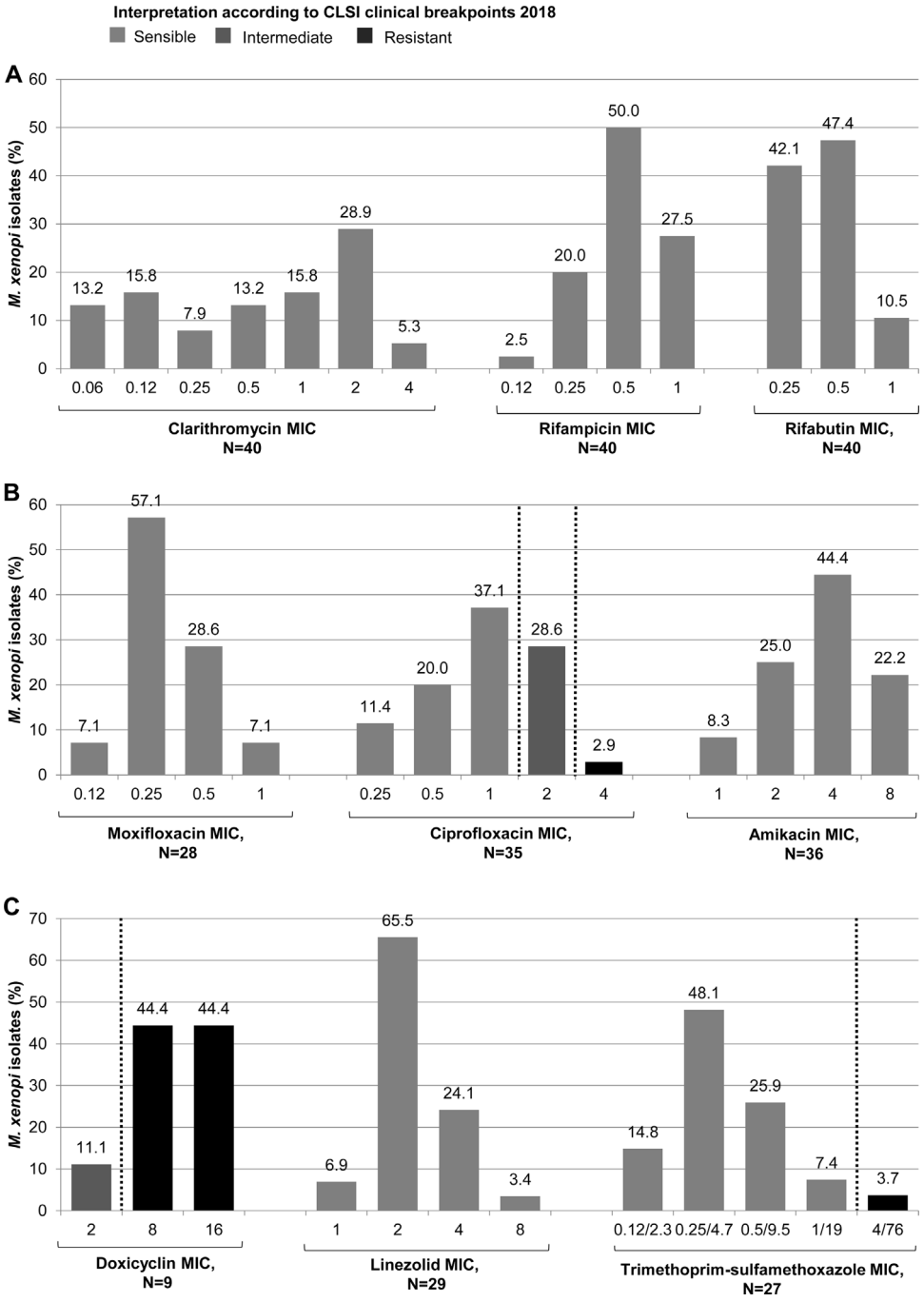

Fig. 2. Minimum inhibitory concentrations (MICs) of *Mycobacterium xenopi* isolates for (A) suggested and (B) alternative antibiotics used in the clinic against nontuberculous mycobacteria. MICs were determined by broth microdilution using Sensititre™ Myco SLOMYCO AST plates for *M. xenopi* isolates from patients with a diagnosis of *M. xenopi* disease according to ATS/IDSA criteria. Only the first isolate for each patient was analysed. Percentages of isolates at each MIC are reported in the histograms. The total number of strains analysed for each compound is reported in brackets. Vertical dotted lines represent the CLSI clinical breakpoints for resistance established for *Mycobacterium kansasii* [12]. ATS, American Thoracic Society; IDSA, Infectious Diseases Society of America; CLSI, Clinical and Laboratory Standards Institute.

diagnosis only thanks to the prolongation of incubation time from 42 days to 56 days for both culture media. Indeed, criteria were fulfilled based on specimens characterised by a median TTP of 53 days (IQR 45–55 days).

3.3. *In vitro susceptibility profile of* Mycobacterium xenopi *strains responsible for clinical disease*

Fig. 2 shows the results of determination of *M. xenopi* susceptibility to a panel of nine drugs used for combined antimycobacterial therapy and for which clinical breakpoints for susceptibility evaluation are available.

According to Clinical and Laboratory Standards Institute (CLSI) criteria, all 40 *M. xenopi* isolates tested from patients with clinical disease showed in vitro MICs within the susceptible range for macrolides (clarithromycin), rifampicin and rifabutin (Fig. 2A) as well as for moxifloxacin, amikacin and linezolid (Fig. 2B).

Strains of *M. xenopi* resistant to ciprofloxacin, doxycycline or trimethoprim/sulfamethoxazole accounted for 2.9%, 88.9% and 3.7% of those isolated, respectively (Fig. 2B); 28.6% of isolates had an intermediate MIC for ciprofloxacin (Fig. 2B).

4. Discussion

To improve *M. xenopi* recovery and to allow antimicrobial susceptibility testing for a higher number of patients with clinical disease, in the last 7 years our third-level centre has processed more than 44 000 biological specimens for cultural analysis for mycobacteria using an incubation time both for liquid MGIT and solid LJ of 56 days instead of the 42 recommended by the CLSI [4].

The CLSI suggest the possibility to prolong the standard incubation time when specific species of NTM are suspected, but not in the case of *M. xenopi* [4]. However, in our 7 years' experience, *M. xenopi* has proved to be the mycobacterial species that requires, by far, the most prolonged time of growth both in liquid MGIT and solid LJ cultures. By using a relatively short incubation time of 42 days, 41.2% of positive specimens for *M. xenopi* would have been reported as negative to clinicians, a significantly higher proportion compared with other NTM (4.7%) or *M. tuberculosis* complex (2.0%). The requirement of *M. xenopi* for a high ambient temperature for optimal growth (42 °C) probably contributes to this finding, as a lower temperature (35 °C), corresponding to the growth optimum for *M. tuberculosis*, is commonly used in clinical settings [5,7,13].

Mycobacterium xenopi is an environmental NTM and contact with humans is not infrequent [6,7]. Therefore, the diagnosis of *M. xenopi* disease is not straightforward, leading to a difficult identification of the proper treatment protocol [8–10,14] and often to severe disease outcomes [15,16]. According to ATS/IDSA criteria, a single positive isolation from a non-sterile respiratory site (i.e. sputum) is not sufficient to ascribe clinical significance and to exclude colonisation [4,5]. To define clinical mycobacteriosis it is thus necessary to isolate *M. xenopi* from multiple non-sterile specimens during a period of time, or from biological sites with usually lower mycobacterial load (i.e. biopsies or bronchoalveolar lavage). For this reason, optimisation of laboratory procedures to improve *M. xenopi* recovery is a critical issue. Our modification of the standard diagnostic protocol had a great impact on *M. xenopi* disease diagnosis, as 56.3% (27/48) of our patients would have not fulfilled the ATS/IDSA criteria for clinical significance [4,5] if the incubation time was limited to 42 days. This supports the hypothesis that the correct diagnosis of *M. xenopi* disease may frequently be missed under non-optimised culture conditions, along with the correct diagnostic follow-up and antibiotic treatment.

Unlike pulmonary tuberculosis, the treatment decision for NTM is highly complex as it depends on the type of NTM isolated, contextual radiographic findings and potential individual benefit. Unfortunately, data on treatment, outcomes and mortality of *M. xenopi* disease are extremely scarce [16,17] and the high variability of drug combinations used clearly indicates the lack of consensus [18].

To date, few studies have been published on the susceptibility profile of *M. xenopi* in vitro, and current CLSI 2018 indications still do not report clinical MIC breakpoints for this mycobacterium. By applying the new interpretation criteria for *M. kansasii* [11,12], all of the *M. xenopi* isolates from patients with clinical disease in the current study were susceptible to macrolides (clarithromycin) and rifamycins (rifampicin and rifabutin), usually constituting the cornerstones of *M. xenopi* combination therapy [14,19]. In a recent study including 74 *M. xenopi* isolates from patients with *M. xenopi* colonisation/disease, 13.6% presented an intermediate/resistant clarithromycin MIC, whilst 41.9% and 14.9% were resistant to rifampicin and rifabutin, respectively [20]. As the drug susceptibility profile of the first *M. xenopi* strain isolated was analysed in the current study, either contextual to or anticipating clinical disease diagnosis and treatment, the lack of previous exposure to such antibiotics could account for this result. However, the limited number of subjects involved in both studies and the variability of clinical conditions does not allow firm conclusions.

Although in vitro results may not be predictive of general treatment success with that particular antibiotic in actual patients [9,10], the current results show the possibility of effective *M. xenopi* disease treatment, providing that a proper identification and diagnosis is made.

In conclusion, as the diagnosis of NTM disease still relies on culture, its appropriate request and execution is mandatory. To achieve this purpose, good communication between microbiologists and clinicians is critical, as the sensitivity of culture and laboratory diagnosis of NTM disease could still be optimised. For *M. xenopi*, prolongation of the incubation time to 56 days both for solid LJ and liquid MGIT cultures appears to be a clinically relevant adjustment. This adjustment was able to significantly increase the sensitivity of culture assay for the diagnosis of respiratory disease by this poorly studied mycobacterium and to demonstrate the maintenance of a favourable susceptibility profile of isolated strains to the first-line drugs most commonly used. Whether supported by further studies, current international guidelines should consider implementing this indication.

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Ethical approval

Not required.

Conflict of interests

None.

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