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## Short Communication

## Extended spectrum of antibiotic susceptibility for tuberculosis, Djibouti

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## ABSTRACT

In the Horn of Africa, there is a high prevalence of tuberculosis that is reported to be partly driven by multidrug-resistant (MDR) *Mycobacterium tuberculosis strictu sensu* strains. We conducted a prospective study to investigate *M. tuberculosis* complex species causing tuberculosis in Djibouti, and their in vitro susceptibility to standard anti-tuberculous antibiotics in addition to clofazimine, minocycline, chloramphenicol and sulfadiazine. Among the 118 mycobacteria isolates from 118 successive patients with suspected pulmonary tuberculosis, 111 strains of *M. tuberculosis*, five *Mycobacterium canettii*, one '*Mycobacterium simulans*' and one *Mycobacterium kansasii* were identified. Drug-susceptibility tests performed on the first 78 isolates yielded nine MDR *M. tuberculosis* isolates. All isolates were fully susceptible to clofazimine, minocycline and chloramphenicol, and 75 of 78 isolates were susceptible to sulfadiazine. In the Horn of Africa, patients with confirmed pulmonary tuberculosis caused by an in vitro susceptible strain may benefit from anti-leprosy drugs, sulfamides and phenicol antibiotics.

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## 1. Introduction

Tuberculosis remains a major deadly infectious disease worldwide, with 10.4 million new cases and 1.4 million deaths from the disease in 2015 [1]. However, the burden of tuberculosis varies greatly from one country to another, as illustrated by the ten-fold variation in incidence from 27/100 000 inhabitants in wealthy regions of America to an average rate of 275/100 000 inhabitants in Africa [1]. The Horn of Africa, comprising Djibouti, Ethiopia, Somalia and Eritrea, is a hotspot for tuberculosis, with a high incidence of 378/100 000 inhabitants in Djibouti and 192/100 000 inhabitants in Ethiopia in 2015 [1]. In Djibouti, the high prevalence of tuberculosis has been reported to be partly driven by so-called multidrug-resistant (MDR) *Mycobacterium tuberculosis* isolates [2,3], defined as being resistant to at least isoniazid and rifampicin [4]. Moreover, in Djibouti, tuberculosis is caused not only by *M. tuberculosis* isolates, but also by *Mycobacterium canettii* in 3% and up to 6% of cases [3,5]. Notably, *M. canettii* organisms are naturally resistant to pyrazinamide and streptomycin [6].

With a view to updating information on the microbiology of pulmonary tuberculosis in Djibouti, we prospectively identified mycobacterial isolates from patients presenting with clinically-suspected pulmonary tuberculosis, and extended in vitro drug susceptibility tests for classic anti-tuberculous antibiotics to include clofazimine [7], minocycline [8], chloramphenicol [9] and sulfadiazine [10], all of which were shown to inhibit mycobacteria.

## 2. Materials and methods

## 2.1. Patients and sample processing

This study was approved by the IHU Méditerranée Infection, Ethics Committee Approval n°2016-025, Marseille, France. The study was conducted in collaboration with the Hôpital Pneumo-phtisiologie Chakib Saad Omar in Djibouti as part of their routine activity. All patients with suspected pulmonary tuberculosis who attended this hospital between May and December 2016 were included in the study. Informed and consenting patients answered an anonymized questionnaire containing personal information (age, sex, nationality, address, job and travel out of Djibouti), human immunodeficiency virus (HIV) status, medical history and reported tuberculosis cases in their family. Collected sputa were systematically examined by microscopy observation after Ziehl-Neelsen staining. Samples were

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stored at 4 °C before shipment. Every two weeks, samples that tested positive for microscopic detection of acid-fast bacilli were shipped to the biology laboratory in the Hôpital d'Instruction des Armées Alphonse Laveran, Marseille, France, with an average transport time of eight days. Samples were then decontaminated using the BD BBL™ MycoPrep™ Mycobacterial System Digestion/Decontamination Kit (Becton Dickinson, Le Pont-de-Claix, France), and cultured on Coletsos medium (Bio-Rad, Marnes-la-Coquette, France) and on egg-based Lowenstein-Jensen medium (Bio-Rad, Marnes-la-Coquette, France), at 37 °C for 60 days. Colonies of positive cultures were stained with fluorescent auramine-O staining (Sigma, Saint-Quentin Fallavier, France). The presence of mycobacteria was confirmed by detecting stained bacilli after fluorescence microscopy examination.

## 2.2. Identification tests

Mycobacteria were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) after direct deposit of the colonies [11]. Briefly, colonies were spotted on a MALDI-TOF target plate and then covered with 1 µL matrix solution. After drying, the target plate was introduced into the Microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) for analysis. Generated spectra were compared with the Bruker peptide profile database and identification was performed using the pattern-matching process as previously described [11]. 16S rRNA and *rpoB* gene sequencing [12,13] were then performed to identify non-tuberculous mycobacteria. *M. tuberculosis* complex isolates were identified at the species level by specific multiplex polymerase chain reaction (PCR) amplification of regions of difference (RD) RD4, RD9 and RD12 [14] using *M. tuberculosis* H37Rv, *Mycobacterium bovis* BCG Pasteur and *M. canettii* CIP 140010059<sup>T</sup> as positive controls and *Mycobacterium smegmatis* mc<sup>2</sup> 155 as a negative control. *M. canettii* identification was confirmed by partial *hsp65* gene sequencing [6] and quantitative real time PCR (qPCR) targeting the *cobF* gene using *cobF*\_Forward\_5'-GCGACTGCTCGTTTCAGG-3', *cobF*\_Reverse\_5'-GATGCGTGCCCGACCTC-3' and *cobF*\_Probe\_5'-6FAM-CCGGACACCCGAATCTGGTGG-3'. For all tuberculous isolates, real-time PCR targeting *M. tuberculosis*-specific deletion 1 (TbD1) was performed using TbD1\_Foward 5'-CAAA GGAACCGCGAAAGTTA-3', TbD1\_Reverse 5'-ACCGTGATAA GCACCAGGAC-3' and TbD1\_Probe\_5'-6FAM-TCGCGGTGATGTT GCTCTTCG-3'. All qPCR experiments were conducted using the CFX96® qPCR, incorporating qPCR reagents (Takyon, Eurogentec, Liège, Belgium). The qPCR cycle was 50 °C for two minutes, 95 °C for five minutes followed by 40 cycles of 95 °C for one second and 60 °C for 35 seconds and finally 45 °C for 30 seconds.

## 2.3. Drug susceptibility testing

Drug susceptibility tests for streptomycin, isoniazid, rifampicin, ethambutol and pyrazinamide were performed in liquid medium by an automated BACTEC™ MGIT™ 960 method using the BACTEC™ MGIT™ 960 SIRE kit and the BACTEC™ MGIT™ 960 PZA kit (Becton Dickinson). Final concentrations in MGIT tubes were 1 µg/mL for

streptomycin, 0.10 µg/mL for isoniazid, 1 µg/mL for rifampicin, 5 µg/mL for ethambutol and 100 µg/mL pyrazinamide according to the manufacturer's recommendations (Becton Dickinson). *M. tuberculosis* H37Rv was used as a control strain. Further susceptibility tests were performed by molecular hybridization using the GenoType MTBDRplus kit (Hain LifeScience) to detect rifampicin and isoniazid resistance. For the clofazimine, minocycline, chloramphenicol and sulfadiazine (CMCS) susceptibility test, mycobacterial liquid cultures were calibrated at a final concentration of 10<sup>5</sup> colony-forming units/mL according to McFarland standards. Drug stock solutions in dimethyl sulfoxide (Euromedex, Souffelweyersheim, France) were stored at -20 °C, except for sulfadiazine, which was freshly prepared. The applied drug concentrations were 1.5 µg/mL for clofazimine, 4 µg/mL for minocycline, 5 µg/mL for chloramphenicol and 20 µg/mL for sulfadiazine, based on previous studies [7–10]. The CMCS susceptibility test was performed by adapting the automated MGIT960 method. For each isolate tested, one drug-free growth control tube and four additional drug-containing tubes were inoculated with 0.5 mL mycobacterial suspension. An antimicrobial susceptibility testing set comprising the five tubes was incubated in the Bactec MGIT 960 instrument (Becton Dickinson). Results were analysed by the Bactec MGIT 960 system within seven to ten days and interpreted as previously described [15].

## 3. Results

Over an eight-month prospective study, 200 samples and questionnaires from 200 patients were sent to the laboratory. Seventy samples did not grow and 12 were contaminated. Of the 118 mycobacteria isolated from 118 sputa, MALDI-TOF-MS identified 116 *M. tuberculosis* complex isolates and one *M. kansasii* isolate, and there was one unidentified isolate. Of 116 *M. tuberculosis* complex isolates, the analysis of restriction digests (RDs) by PCR multiplex yielded 111 *M. tuberculosis* and five *M. canettii* isolates. Molecular characterization showed that 51/111 (46%) *M. tuberculosis* isolates were TbD1-positive. The five *M. canettii* isolates were firmly identified by *cobF* gene-positivity, the presence of TbD1 region and a C-to-T transition in the *hsp65* gene. One '*M. simulans*' and one *M. kansasii* isolates were firmly identified based on a 16S rRNA and *rpoB* gene sequences with 99% sequence similarity with the references. For the 110 *M. tuberculosis*-infected patients whose sex was known, 79 (71.8%) were men and 31 (28.1%) were women, and the average age was 33 (range 13–73) years. Of the 108 patients whose geographical origin was known, 92 patients (85%) were Djiboutian, 13 (12%) were Ethiopian, two were Somalian and one was Yemenite. Only eight patients (7.2%) reported a previous history of treatment for tuberculosis. Information on HIV status was known for 88 of the *M. tuberculosis*-infected patients, of whom three (3.4%) were HIV-positive. The five patients infected by *M. canettii* were all Djiboutian (three men and two women, average age: 34 years) who originated from five different neighborhoods (Table 1). These patients reported no tuberculosis history and were HIV-negative. Three of these patients reported a stay in Somaliland and one reported a stay in Ethiopia (Table 1). Travel to Somaliland in 3/5 (60%) of *M. canettii*-infected patients was

**Table 1**  
Synopsis of five patients diagnosed with *M. canettii* pulmonary tuberculosis, Djibouti.

Isolate	Sex	Age	Nationality	Job	Address in Djibouti	Travel outside Djibouti	HIV status
DJ 480	M	30	DJ	No occupation	Quartier 3	Somaliland 2015	No
DJ 514	F	41	DJ	No occupation	Quartier 7 bis	Somaliland 2014–2015	No
DJ 517	M	27	DJ	No occupation	Cité Poudrière	No	No
DJ 613	F	27	DJ	No occupation	Quartier 2	Somaliland 2012	No
DJ 734	M	47	DJ	Cooking	Quartier 7	Ethiopia 2015	No

M: men, F: women, DJ: Djiboutian, HIV: human immunodeficiency virus.

**Table 2**  
*In vitro* drug susceptibility testing of mycobacteria isolated from patients with suspected pulmonary tuberculosis, Djibouti.

	Drugs					n/N Isolates
	STR (1 µg/mL)	INH (0.1 µg/mL)	RIF (1 µg/mL)	ETH (5 µg/mL)	PZA (100 µg/mL)	
Drug susceptibility profiles	S	S	S	S	S	55/73 <i>M. tuberculosis</i>
	S	S	S	S	R	1/73 <i>M. tuberculosis</i>
	S	R	S	S	R	1/73 <i>M. tuberculosis</i>
	R	S	S	S	S	7/73 <i>M. tuberculosis</i>
	R	S	S	S	R	3/3 <i>M. canettii</i>
	S	R	R	S	R	1/1 " <i>M. simulans</i> "
	R	R	R	S	R	4/73 <i>M. tuberculosis</i>
	R	R	R	R	R	5/73 <i>M. tuberculosis</i> 1/1 <i>M. kansasii</i>

STR: streptomycin, INH: isoniazid, RIF: rifampicin, ETH: ethambutol, PZA: pyrazinamide, green square, S: susceptible; red square, R: resistant, n/N: number of obtained profiles/total number of isolates.

significantly higher than the 4/111 (3.6%) of *M. tuberculosis*-infected patients ( $P=0.001$ , Fisher's exact test), but this was not true for travel to Ethiopia ( $P=1$ , Fisher's exact test). One '*M. simulans*' isolate with uncertain pathogenicity was documented in a 40-year-old Djiboutian man living in Balbala, a suburb of Djibouti-town. This HIV-negative patient had been initially diagnosed with cavitary pulmonary disease and had been unsuccessfully treated for non-documented MDR tuberculosis. One *M. kansasii*-infected patient was a 46-year-old Djiboutian man working as a painter and living in Quartier 7 of Djibouti. He reported a stay of one year in Somaliland in 2012. The patient reported no tuberculosis history, was HIV-negative and had been previously diagnosed with non-documented MDR tuberculosis.

Drug susceptibility testing was performed for 78/118 (66%) isolates including 73 *M. tuberculosis*, three *M. canettii*, one '*M. simulans*' and one *M. kansasii* isolates. As for SIRE and PZA susceptibility tests, 55/78 (70%) isolates were susceptible to all anti-tuberculosis drugs tested (Table 2). The three *M. canettii* isolates were resistant to streptomycin and pyrazinamide. The '*M. simulans*' isolate was resistant to rifampicin, isoniazid and pyrazinamide. The *M. kansasii* isolate was resistant to the standard concentrations applied for the anti-tuberculous antibiotics. Nine MDR among the 73 *M. tuberculosis* isolates were obtained by the BACTEC method and confirmed by the molecular hybridization technique. HIV status was reported for seven MDR-tuberculosis patients, all of whom were HIV-negative. Past medical history of tuberculosis was indicated in four of the nine cases of MDR *M. tuberculosis*. For the CMCS susceptibility test, all 78 tested mycobacteria, including the nine MDR *M. tuberculosis* isolates, were inhibited by 1.5 µg/mL clofazimine, 4 µg/mL minocycline, 5 µg/mL chloramphenicol and 20 µg/mL sulfadiazine, except for three *M. tuberculosis* isolates, including two MDR that were resistant to sulfadiazine.

#### 4. Discussion

In Djibouti, pulmonary tuberculosis is routinely diagnosed based on clinical data and positive microscopic observation of a sputum smear following Ziehl-Neelsen staining [3]. These criteria do not provide information on the mycobacterial species causing the tuberculosis and leave scope for the possibility that some patients who are clinically diagnosed with pulmonary tuberculosis are, in fact, infected by other mycobacteria. Accordingly, our prospective study revealed *M. canettii* in some patients in Djibouti, in line with observations previously reported in Djibouti [3,5]. In addition, we recovered one strain of *M. kansasii* and one strain of '*M. simulans*', which had not previously been reported in Djibouti. However, the interpretation of these two isolates remains uncertain given the absence of repeated sampling from these patients, and co-infection with tuberculous mycobacteria cannot be excluded for the same reason [16]. Indeed, it is the second isolate of '*M. simulans*' infection worldwide and, notably, the index case reported a few years ago was a Somali patient diagnosed with cavitary pulmonary disease mimicking MDR tuberculosis [17]. The patient reported in this paper had a similar medical history, yet denied having had any contact with Somalia. A few studies identified non-tuberculous mycobacteria as being responsible for pulmonary infection in Djibouti, including *Mycobacterium chelonae*, *Mycobacterium fortuitum* and *Mycobacterium peregrinum* [3].

Our *in vitro* susceptibility data yielded a 9/73 (12.3%) frequency of MDR among *M. tuberculosis* isolates, which is significantly lower than 72% (23/32) ( $P=1.10^{-9}$ ,  $\chi^2$  test) and 57% (51/88) ( $P=2.10^{-9}$ ,  $\chi^2$  test) previously reported in Djibouti [2,3]. The fact that previous retrospective studies incorporated data from 2007–2011 [2,3], and that tested samples had been collected from hospitalized patients, may account for differences in estimations of the actual MDR rate [2]. Moreover, because the present strain series is limited in terms of

the number of strains and the length of the collection, it may not be representative of the overall current epidemiology of tuberculosis in Djibouti. Only larger and longer-term studies will give a reasonable overview of the situation. We observed that one '*M. simulans*' isolate was resistant to isoniazid and rifampicin, as previously reported [17], and was susceptible to streptomycin and resistant to pyrazinamide, contrary to that reported for the first strain [17]. Whether this observation indicates a natural heterogeneity of antibiotic susceptibility pattern in '*M. simulans*' will depend on the observation of further isolates. *M. kansasii* is naturally resistant to pyrazinamide [18], but higher inhibitory concentrations of streptomycin, isoniazid, rifampicin and ethambutol than those applied in our study are required [19].

In the absence of any standardized published protocol to test the in vitro susceptibility of *M. tuberculosis* complex mycobacteria to the anti-leprosy antibiotics, sulfadiazine and chloramphenicol, we tested a single concentration in the upper limit of the inhibitory concentrations previously reported [7–10]. Bearing this limit in mind, we observed that all *M. tuberculosis* complex isolates, including nine MDR isolates, were susceptible to clofazimine, minocycline and chloramphenicol, and 94% were susceptible to sulfadiazine. In this paper, we report the antimicrobial activity of clofazimine, minocycline, chloramphenicol and sulfadiazine against '*M. simulans*' and *M. canettii*, thereby confirming previous observations [10,17]. Moreover, the inhibition of *M. kansasii* by chloramphenicol is herein demonstrated for the first time.

## 5. Conclusions

This study illustrates the impact of accurate identification of mycobacteria recovered from respiratory tract specimens to confirm and refine the diagnosis of tuberculosis in countries with a high incidence of tuberculosis, where several *M. tuberculosis* complex species are circulating. In Djibouti, mycobacteria responsible for suspected pulmonary tuberculosis were susceptible in vitro to at least three different antibiotics from an extended spectrum of anti-tuberculous, anti-leprosy, sulfamide and phenicol antibiotics. We propose that such strains could be qualified as 'multidrug-susceptible', opposing the definition of multidrug-resistant strains.

Anti-leprosy, sulfamide and phenicol antibiotics are all orally administered, may be included in Directly Observed Treatment Short course strategy, and their potential toxicity is well established, given the 30–50 years of prescription worldwide [20]. Data reported here indicate that these antibiotics could be considered during the empirical treatment of patients presenting with confirmed tuberculosis in the Horn of Africa.

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**Competing interests:** None declared.

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