**Mycolicibacterium fortuitum** genomic epidemiology, resistome and virulome

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**BACKGROUND** Mycolicibacterium fortuitum is an opportunistic pathogen associated with human and animal infection worldwide. Studies concerning this species are mainly represented by case reports, some of them addressing drug susceptibility with a focus on a specific geographic region, so there is a gap in relation to the global epidemiological scenario.

**OBJECTIVES** We aimed to determine the global epidemiological scenario of *M. fortuitum* and analyse its traits associated with pathogenicity.

**METHODS** Based on publicly available genomes of *M. fortuitum* and a genome from Brazil (this study), we performed a genomic epidemiology analysis and *in silico* and *in vitro* characterisation of the resistome and virulome of this species.

**FINDINGS** Three main clusters were defined, one including isolates from the environment, human and animal infections recovered over nearly a century. An apparent intrinsic resistome comprises mechanisms associated with macrolides, beta-lactams, aminoglycosides and antituberular drugs such as rifampin. Besides, the virulome presented Type VII secretion systems (T7SS), including ESX-1, ESX-3, ESX-4 and ESX-4-bis, some of which play a role on the virulence of *Mycobacteriaceae* species.

**MAIN CONCLUSIONS** Here, *M. fortuitum* was revealed as a reservoir of an expressive intrinsic resistome, as well as a virulome that may contribute to its success as a global opportunistic pathogen.

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**Key words:** arr - rifampin - resistance - ESX - type VII secretion system - opportunist pathogen

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*Mycolicibacteriaceae* comprehends a wide spectrum of environmental and pathogenic bacteria that eventually arise in clinics affecting human and animal health. Recently, members of this family have been reclassified into new genera, and thus rapid-growing species, such as *Mycobacterium fortuitum*, now belong to the *Mycolicibacterium* genus.¹ Mycolicibacterium fortuitum is ubiquitous in the environment and its role as a pathogen is being recognised worldwide.² In fact, the burden of disease due to non-tuberculous mycobacteria (NTM) may be underestimated, as infections by these organisms are not on the reportable list.³ In addition, NTM infections are increasing globally in humans and animals, and *M. fortuitum* is among the most prevalent NTM species enrolled in this scenario.⁴,⁵,⁶ *M. fortuitum* infections have been reported with a high prevalence of resistance to several drugs, including macrolides, beta-lactams, aminoglycosides and tetracyclines, in addition to antituberular drugs (e.g., isoniazid, rifampin, ethambutol, clofazimine, ethionamide, and rifabutin).⁷,⁸,⁹ So far, studies of *M. fortuitum* are mainly represented by case reports in humans and animals, some of them addressing drug susceptibility with a focus on a specific geographic region, so there is a gap in relation to the global epidemiological scenario and systematic analysis of *M. fortuitum* traits associated with pathogenicity. The availability of *M. fortuitum* genomic information and metadata associated with these organisms (e.g., host, geographic location, year) is an opportunity to begin to fill the gap in the general biological characteristics of this zoonotic and widespread opportunistic bacteria.

**MATERIALS AND METHODS**

Genome sequences analysed - A total of 25 *M. fortuitum* genomes were retrieved from the NCBI site (https://www.ncbi.nlm.nih.gov/genome/browse/#/prokaryotes/14575/) in June 2021 (Table).

Genome sequencing and assembly - Genomic DNA of *M. fortuitum* 7G strain was extracted using NucleoSpin Microbial DNA (Macherey-Nagel) and sequenced with Nextera XT library kit on the Illumina HiSeq 2500 platform, generating 2 x 250 bp paired-end reads. The raw reads were submitted to quality control by NGS QC Toolkit v2.3.3⁰ and the genome was assembled using SPAdes v3.14.1.¹¹ The genome and the raw reads were deposited at NCBI under the accession numbers JAE-QRO000000000 and SRR15257947, respectively.

Drug susceptibility testing and cloning - *M. fortuitum* 7G strain was grown in trypticase soy agar (TSA) medium supplemented with 0.05% Tween-80 (Sigma-
Aldrich) at 22°C for 72 h. Drug susceptibility of the *M. fortuitum* 7G strain was evaluated by E-test method (bioMérieux) in Mueller-Hinton agar plates for various drugs: azithromycin, clarithromycin, streptomycin, tobramycin, meropenem, cefalotin, cefepime, and rifampicin. The *arr* and *rox* polymerase chain reaction (PCR) products were cloned into the pGEM T-Easy Cloning Vector System (Promega) and used to transform *Escherichia coli* DH5α lineage (rifampicin MIC of 4 µg/mL).

**Phylogenetic analysis** - The *M. fortuitum* genomes were annotated using Prokka v1.14.6(12) and submitted to Roary v3.13.0(13) to determine the core genome. The single-nucleotide polymorphism (SNP) sites of the concatenated core genes (180,276 bp) were obtained using snp-sites v2.5.1(14). A phylogenetic neighbor joining tree was generated using PhyML v3.1 in Seaview v4(15) with 1000 bootstrap replicates.

**Resistome and virulome analysis - *M. fortuitum* genomes** were surveyed for antibiotic resistance and virulence genes through ABRicate (https://github.com/tsee-mann/abricate) based on The Comprehensive Antibiotic Resistance Database(16) and Virulence Factor Database. (17) In addition, T7SS was searched based on the identification of the T7SS core proteins(18) using HMMer package v3.1b2. (19)

### RESULTS AND DISCUSSION

Here, based on 25 *M. fortuitum* genomes (drafts and complete) available in the NCBI database and a sequenced genome from Brazil (this study; accession number JAEQR0000000000), we performed a genomic epidemiology analysis and *in silico* and *in vitro* characterisation of the resistome(20) of this species. This is a diverse set of *M. fortuitum* genomes considering the occurrence of the strains in terms of space, time, and origin, since they were isolated from environments, animals, and humans in several countries for almost a century (1923-2020). A core genome SNP analysis generated a neighbor-joining tree that revealed three main clusters (Figure). Based on the available metadata, it was possible to associate clusters I and III with isolates from different countries, while cluster II is composed only of isolates from South Africa. All isolates from cluster I (India, Mozambique, and Cambodia; 2008-2012) and II (South Africa; 2011-2012) were from human infections. Cluster III is quite diverse with isolates from human and animal infections, as well as from the environment, being recovered over almost a century (1923 to 2020) (Table).

The *in silico* inference of the resistome was performed using CARD, resulting in the identification, in all genomes of clusters I, II and III, of the followed genes: *aph* (aminoglycoside O-phosphotransferase), *aac* (aminoglycoside acetyltransferase), *arr* (NAD+ rifampin ADP-ribosyltransferase), *blaF* (beta-lactamase), *erm* (23S ribosomal RNA methyltransferase), *rhpA* (RNA polymerase-binding protein), *rox* (rifampin monooxygenase) and *tap* (multidrug efflux pump). Each of these genes was found in the same chromosomal location, with no evidence of association with mobile platforms, suggesting the constitutive nature of this resistome in this species. In addition to this *in silico* analysis and to gain some insights into the functionality of these antibiotic resistance genes (ARGs), we performed *in vitro* analyses with *M. fortuitum* 7G strain, defining the minimum inhibitory concentration (MIC) for the antibiotic classes represented in the resistome. The *M. fortuitum* 7G strain showed high resistance rates to macrolides (azithromycin > 256 µg/mL and clarithromycin > 32 µg/mL), aminoglycosides (streptomycin > 32 µg/mL and tobramycin > 32 µg/mL), carbapenem (meropenem > 32 µg/mL), cephalosporins (cefalotin > 256 µg/mL and cefepime > 256 µg/mL) and rifampicin > 32 µg/mL. In fact, Nash et al.(21) observed that *M. fortuitum* strains were naturally resistant to macrolides and that this resistance would be associated with the *erm* gene. Erm belongs to a diverse family of proteins encoded by a heterogeneity of alleles, some of them (*erm*37-41) intrinsically associated with *Mycobacteriaceae* species. Here, *erm*39 was identified in all *M. fortuitum* genomes and its functionality was demonstrated in *M. fortuitum* 7G, as it had already been shown in the CT6 strain.(21) The *aph*(3’)-Ic gene was identified for the first time in an environmental *M. fortuitum* strain, being involved in molecular mechanisms of streptomycin resistance in some *Mycobacteriaceae* and *Streptomyces*. (22) It is worth noting the identification in all genomes of a set of determinants, related to different resistance mechanisms, associated with the rifampin class of antibiotics. The *arr*, *rhpA*, *rox* and *tap* genes may impact resistance to rifampicin, (23,24) a first-line drug that has been used to treat *Mycobacterium tuberculosis* infections for more than half a century. We then experimentally determined the activity of some of these genes (*arr* and *rox*) carried by the *M. fortuitum* 7G strain. Based on *arr* and *rox* genes cloning and transformation in heterologous system, it was demonstrated that this *arr* allele was associated with a high rate of resistance (32 µg/mL), while *rox* did not improve the *E. coli* rifampicin MIC, indicating that this Arr offers resistance to rifampin for *M. fortuitum* 7G strain. Besides the conservative *M. fortuitum* resistome, a class 1 integron carrying a qac/sul1 gene cassette was identified in a genome (E3337) from cluster III (Table) and, therefore, this would be the first evidence of this genetic element of resistance in *Mycolicibacterium*. Class I integron is a genomic platform in which antibiotic resistance genes are acquired and expressed, contributing to the emergence of resistance in a one-step fashion, (25) therefore, it represents a possibility to increase the resistance spectrum in one generation. In general, in bacteria, plasmids are another genetic element strongly associated with the bacterial resistome, but particularly for *M. fortuitum*, its resistome was entirely associated with the chromosomal genomic context. In fact, a previous survey of *Mycolicibacterium* mobilome showed an 8 kb non-mobilisable plasmid, without association to any ARG, shared by two *M. fortuitum* metagenomes (SCH6189132/cluster II/South Africa and MTB7/cluster III/Morocco). (18)

The *M. fortuitum* virulome was accessed using ABRicate based on Virulence Factor Database. Four genes were identified in all genomes: *icl*, *ideR*, *phoP*, and *relA* (except *relA* in GA-0871). These genes were associated with
# TABLE
Metadata and resistome of the *Mycobacterium fortuitum* genomes

| Genome/strain | ap(3'')-lc | arr-1 | blaF | erm39 | aac(2')-Ib | rhpA | tap |rox| sul1 | T7SS | Country | Year | Source | Cluster | Accession number |
|---------------|------------|-------|------|-------|-----------|------|-----|---|------|------|---------|------|--------|---------|------------------|
| E2981         | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | Cambodia | 2011 | ND     | I       | LZKN0000000000 |
| E3377         | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | Cambodia | 2011 | ND     | I       | LZS0000000000 |
| GiA-0871      | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | India    | 2008 | human  | I       | MBER0000000000 |
| isolate_4     | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | China    | 2013 | human  | I       | JAAZW00000000000 |
| E2378         | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | Cambodia | 2010 | ND     | I       | LZKM0000000000 |
| 1165541.1     | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | Mozambique | 2012 | human  | I       | LZLP0000000000 |
| NS-7455       | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | India    | 2010 | human  | I       | MBEK0000000000 |
| SCH5383401    | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | S. Africa | 2012 | human  | II      | LMIH000000000 |
| SCH5661144-a  | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | S. Africa | 2012 | human  | II      | LZSR0000000000 |
| SCH5661144-b  | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | S. Africa | 2011 | human  | II      | LZSS0000000000 |
| SCH6189132-b  | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | S. Africa | 2012 | human  | II      | LZIP0000000000 |
| SCH5646012    | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | S. Africa | 2012 | human  | II      | LZSN0000000000 |
| MTB7          | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | S. Africa | 2016 | human  | III     | VHZP000000000 |
| E1336         | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | Cambodia | 2010 | ND     | III     | LZLZ0000000000 |
| 1242461       | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | Mozambique | 2012 | human  | III     | LZLZ0000000000 |
| CT6           | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | USA      | 2014 | soil   | III     | NZ_CP011269.1   |
| E190          | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | Cambodia | 2010 | ND     | III     | LZIW0000000000 |
| NCTC1542      | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | ND       | 1923 | fish   | III     | UGQY0000000000 |
| E1141         | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | Cambodia | 2010 | ND     | III     | LZIV0000000000 |
| isolate_1     | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | China    | 2013 | human  | III     | JAAZW00000000000 |
| JCM6368       | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | Japan     | 2015 | ND     | III     | BCSZ0000000000 |
| isolate_2     | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | China    | 2013 | human  | III     | JAAZWM0000000000 |
| E3337         | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | Cambodia | 2011 | ND     | III     | LZK0000000000 |
| 1089381.4     | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | Mozambique | 2012 | human  | III     | LZLO0000000000 |
| 7G            | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | Brazil   | 2020 | cat    | III     | JAEQR0000000000 |
| ATCC 6841      | X          | X     | X    | X     | X         | X    | X   | X | X    | X    | USA      | 1938 | human  | III     | CP014258.1      |
stress response, persistence, and iron uptake, indirectly impacting the virulence. In addition, we also searched for the T7SS, which is the only specialised secretion system of these organisms. T7SS is encoded by six paralogous chromosomal loci (ESX-1, -2, -3, -4, -4 bis, and -5) and has been associated with several functions, including virulence.\(^{(26,27)}\) All \textit{M. fortuitum} genomes had ESX-1, ESX-3, ESX-4 and ESX-4-bis Type VII secretion systems, some of which play an essential role in \textit{Mycobacterium} virulence, nutrient uptake and conjugation.\(^{(26,27)}\)

Here, the ubiquitous \textit{M. fortuitum} bacterium proved to be a reservoir of an expressive intrinsic resistome and virulome, despite the spatiotemporal diversity of the strains, which indicates a constitutive trait of the species that may contribute to its success as a global opportunist pathogen.

**AUTHORS’ CONTRIBUTION**

SMM, ELF and ACPV - Conception, design, data analysis and manuscript writing; FFJ and NVR experimental work. The authors declare no conflict of interest.

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