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Par Mme Djaltou ABOUBAKER OSMAN
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TITRE DE LA THÈSE

Epidémiologie des mycobactéries en Polynésie Française

Pour obtenir le grade de DOCTORAT d'AIX-MARSEILLE UNIVERSITÉ

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Membres du Jury de la Thèse :
Rapporteur : Monsieur le Pr. Jean-Louis GAILLARD
Rapporteur : Monsieur le Pr. Jean-Louis KOECK
Examinateur : Monsieur le Pr. Pierre-Edouard FOURNIER
Directeur de thèse : Monsieur le Pr. Michel DRANCOURT

Unité de Recherche sur les Maladies Infectieuses et Tropicales Émergentes (URMITE)
Professeur Didier RAOULT, Directeur

SOMMAIRE

AVANT-PROPOS

Le format de présentation de cette thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille. Le candidat est amené à respecter des règles qui lui sont imposées et qui comportent un format de thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie exhaustive sur le domaine de cette thèse. Par ailleurs, la thèse est présentée sur article publié, accepté ou soumis associé d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

Professeur Didier RAOULT

RÉSUMÉ

La tuberculose est l'une des maladies les plus meurtrières au monde; elle se situe en seconde position juste après l'infection au virus de l'immunodéficience humaine (VIH) avec laquelle parfois elle est en co-infection. En 2013, les données de l'Organisation Mondiale de la Santé indiquent que neuf millions de personnes ont développé la tuberculose dont 1,5 million de patients décédés (OMS, 2014). La tuberculose est due à un groupe d'agents infectieux phylogénétiquement proches formant le complexe *Mycobacterium tuberculosis.* Ce complexe est actuellement formé de dix espèces, *M. tuberculosis* étant l'espèce la plus fréquemment responsable de la tuberculose humaine. *M. tuberculosis* est un bacille à croissance lente (le temps de doublement est de 24 heures) qui forme des colonies rugueuses. Il existe quelques cas de tuberculose zoonotique mais la transmission est essentiellement interhumaine et aéroportée. Son évolution clonale comporte une co-évolution du pathogène avec son hôte. Le complexe *M. tuberculosis* comporte également des bacilles tuberculeux formant des colonies lisses à croissance rapide.

Ces souches n'ont été isolées qu'à partir de prélèvements cliniques chez l'homme, il n'y a pas de telle souche d'origine animale. Ces souches lisses forment un groupe génétiquement hétérogène avec de génomes de grande taille (4.48 ± 0.05 Mb) et pourraient former un lien entre *M. tuberculosis* et des mycobactéries de l'environnement telles que *Mycobacterium kansasii*.

Dans une première partie de notre thèse, nous avons revu les articles sur les souches lisses de *M. tuberculosis* et nous avons montré que les trois premiers isolats, ont été obtenus chez des patients en France, à Madagascar et en Polynésie Française par Georges Canetti entre 1968 et 1970. Suite à l'isolement d'une souche lisse à partir d'un ganglion cervical chez un enfant Somali de 2 ans en 1997, ces bacilles tuberculeux ont été nommés "*Mycobacterium canettii*". Aujourd'hui, moins d'une centaine de ces souches ont été isolées à partir de patients exposés aux pays formant la Corne de l'Afrique, principalement la République de Djibouti, qui présente la plus forte prévalence. La raison de cette spécificité géographique et la rareté ne sont pas comprises. Dans une deuxième partie, nous avons cherché à savoir si les souches "*M. canettii*" persistaient en Polynésie Française après la première description par G. Canetti.

La Polynésie Française est une région pour laquelle il n'y a plus de données sur la tuberculose depuis 30 ans. Pour cela nous avons procédé à l'analyse génotypique de 34 isolats de *M. tuberculosis* et de 87 isolats de mycobactéries non-tuberculeuses en provenance de la Polynésie Française. Nous avons identifié ainsi quatre sous-groupes phylogénétiques appartenant au lineage Euro-américain, un génotype Beijing (lineage Est-asiatique) avec une dissémination mondiale associée à des formes résistantes aux antibiotiques et un génotype Manu (lineage Indo-océanique). Nous n'avons pas retrouvé de souches lisses. Nous avons également décrit des isolats représentatifs d'une nouvelle lignée de *M. tuberculosis* et identifié les mycobactéries non-tuberculeuses qui sont des agents infectieux opportunistes rencontrés en Polynésie Française dont une nouvelle espèce que nous avons nommée *Mycobacterium massilipolynesiensis*. Dans une dernière partie, nous avons envisagé une transmission par voie digestive de *"M. canettii"* sur la base des données de notre revue. En effet, la comparaison des données cliniques avec les quelques données expérimentales suggère l'eau potable et les aliments contaminés comme des sources potentielles avec une porte d'entrée orale.

Selon cette hypothèse, nous avons testé la résistance de "*M. canettii*" à la chaleur et nous avons observé la croissance de cette dernière entre 25°C et 45°C. En conclusion, l'étude des isolats circulant dans des régions isolées et peu étudiées nous a permis de décrire deux nouvelles lignées de *M. tuberculosis* et une nouvelle espèce de mycobactérie non-tuberculeuse. D'autre part, les données que nous avons obtenues ici pourraient former une base en vue d'élucider les réservoirs et les sources de souches lisses.

Mots clés: *Mycobacterium tuberculosis*, "*Mycobacterium canettii"*, souches lisses du bacille tuberculeux, Polynésie Française, infection, diagnostic, environnement, réservoir, amibes.

SUMMARY

Tuberculosis is one of the deadliest diseases in the world; in second position just after the human immunodeficiency virus (HIV) with which it is sometimes co-infection. In 2013, the World Health Organization (WHO) data show that nine million people developed tuberculosis including 1.5 million patients who died (WHO, 2014). Tuberculosis is caused by a group of phylogenetically close infectious agents forming the *Mycobacterium tuberculosis* complex. This complex is currently comprised of ten species; *M. tuberculosis* is the species most frequently responsible for human tuberculosis. *M. tuberculosis* is a slow growing bacterium (doubling time 24 hours) which forms rough colonies. There are few cases of zoonotic TB but transmission is essentially human to human and airborne. His clonal evolution has a co-evolution of the pathogen with its host. The complex also includes tubercle bacilli forming smooth colonies rapidly growing. These strains were isolated from clinical specimens than in humans, there is no such animal strain. These smooth strains form a genetically heterogeneous group with large genomes (4.48 ± 0.05 Mb) and could be a link between *M. tuberculosis* and environmental mycobacteria such as *Mycobacterium kansasii*.

In the first part of our thesis, we reviewed the articles on the smooth strains of *M. tuberculosis* and we showed that the first three isolates were obtained from patients in France, Madagascar and French Polynesia by Georges Canetti between 1968 and 1970. Following the isolation of a smooth isolate from a cervical ganglion in a Somali child from 2 years in 1997, the smooth tubercle bacilli were named "*Mycobacterium canettii*". Today, less than a hundred of these strains were isolated from patients exposed to countries forming the Horn of Africa, especially the Republic of Djibouti, which has the highest prevalence. The reason for this geographical specificity and rarity are not understood. In the second part, we looked at whether smooth tubercle bacilli "*M. canettii*" persisted in French Polynesia after the first description by G. Canetti. French Polynesia is a region for which there is no data on tuberculosis since 30 years. For this we performed the genotyping of 34 isolates of *M. tuberculosis* and 87 non-tuberculous mycobacteria isolates from French Polynesia.

We identified four phylogenetic subgroups belonging to the Euro-American lineage, a Beijing genotype (East Asian lineage) with a worldwide dissimilation associated with antibiotic resistant forms and Manu genotype (lineage Indo-Oceanic). We have not found smooth strains.

We also describe isolates representative of a new lineage of *M. tuberculosis* and identified non-tuberculous mycobacteria which are opportunistic infectious agents encountered in French Polynesia including a new species that we named *Mycobacterium massilipolynesiensis.* In the last part, we considered a digestive tract transmission of "*M. canettii*" on the basis of our review. Indeed, the comparison of clinical data with the few experimental data suggests drinking water and contaminated food as potential sources with oral route of entry. According to this hypothesis, we tested the resistance "*M. canettii*" to heat and we observed the growth of the latter between 25°C and 45°C. In conclusion, the study of isolates circulating in isolated and few studied regions allowed us to describe two new lineages of *M. tuberculosis* and one new species of non-tuberculous mycobacteria. On the other hand, the data we obtained here could form a basis to elucidate the reservoirs and sources of smooth strains.

Key-Works: *Mycobacterium tuberculosis*, "*Mycobacterium canettii"*, smooth tubercle bacilli, French Polynesia, infection, diagnostic, environment, reservoir, amoeba.

INTRODUCTION & OBJECTIFS

INTRODUCTION

Les dix espèces du complexe *Mycobacterium tuberculosis* (CMT) sont responsables de la tuberculose humaine et de la tuberculose animale décrites chez les mammifères (singes, bovins, ovins, chèvres, chiens chats) et de certains oiseaux (perroquets) (Alexander, 2010; Ingen, 2012; Parsons, 2013; Ghodbane, 2013). Chez l'homme, la tuberculose est causée principalement par l'espèce *M. tuberculosis,* la forme pulmonaire étant la plus contagieuse et la seule mortelle directement d'homme à homme. *M. tuberculosis stricto sensu* est un bacille acido-résistant à croissance lente qui forme des colonies rugueuses sur milieu Middlebrook ou Löwenstein-Jensen. Les bacilles sont colorés en rose avec la coloration de Ziehl-Neelsen et ont une disposition caractéristique en cordes. Ces caractéristiques sont la base de l'identification microscopique de *M. tuberculosis* notamment dans les pays à ressources limitées dans lesquels les méthodes de biologie moléculaire (exemple GenExpert) n'ont pas été implantées (Simeão, 2009). D'un point de vue génotypique, six lignées de *M. tuberculosis* ont été définies (1-Indo-océanique, 2-Est-asiatique, y compris Beijing, 3-Est-afro-indienne, 4-Euro-américaine, 5-Ouest-Africaine ou *Mycobacterium africanum* I, 6-Ouest-Africaine ou *M.*

africanum II), distinctes de *Mycobacterium bovis* (Gagneux, 2006). Récemment une septième lignée, intermédiaire entre la lignée 1 et les lignées 2- 4, a été décrite en Ethiopie et à Djibouti dans la Corne de l'Afrique (Blouin, 2012, Firdessa, 2013) ainsi qu'une mycobactérie proche de *Mycobacterium pinnipedii* dans des prélèvements humains vieux de 1000 ans au Pérou, datés de la période 750–1350 et explorés par whole genome sequencing (Bos, 2014). Au sein du complexe *M. tuberculosis*, il existe des souches qui forment des colonies lisses, isolées en 1969 par Georges Canetti de l'Institut Pasteur à partir d'un échantillon clinique d'un fermier français présentant les symptômes de tuberculose pulmonaire. En 1987, l'étude de la composition lipidique de la paroi de quatre souches lisses (CIPT 1400100-59, -60, -61 et -62) montre que la "souche Canetti" a des lipides proches de lipides présents dans *Mycobacterium bovis* et dans *Mycobacterium kansasii*, mais absents dans *M. tuberculosis*. (Daffé, 1987). En 1997, des chercheurs des Pays-Bas (van Soolingen, 1997) isolent d'une adénopathie une nouvelle souche «lisse» de *M. tuberculosis* présentant un temps de génération court (temps de doublement de 17 heures) chez un enfant Somali de deux ans et proposent de la nommer *M. tuberculosis* subsp. Canetti en référence à la mycobactérie isolée en 1969 par Georges Canetti.

En 1998, Pfyffer et al. rapportent une autre souche isolée chez un patient Suisse âgé de 56 ans qui a vécu au Kenya (Pfyffer, 1997). Depuis lors, une centaine de souches lisses ont été isolées à partir de patients exposés aux pays tropicaux, principalement la République de Djibouti qui rapporte la plus forte prévalence et incidence (Miltegen, 2002, Fabre, 2004, Aboubaker Osman, 2015 soumis). La tuberculose causée par "*M. canettii"* est une maladie émergente dans la Corne de l'Afrique. L'autre spécificité frappante de ces souches lisses est qu'aucune transmission interhumaine n'a pu être identifiée, contrairement à *M. tuberculosis* (Koeck, 2011). Le réservoir naturel, la gamme d'hôtes et le mode de transmission de l'organisme sont à ce jour encore inconnus. L'étude phylogénétique des membres du CMT par Brosch et al. en incluant cinq souches lisses a montré que celles-ci possèdent des gènes codant pour la région de délétion 1 de *M.tuberculosis* (TbD1) et la région de différence RD9 (Brosch, 2002). Les analyses plus approfondies de Gutierrez et al. ont permis de mettre en évidence une structure de gènes mosaïques dans les gènes *gyrA* et *gyrB* montrant ainsi que les souches lisses présentent des recombinaisons intragéniques (Gutierrez, 2005). Pour cette équipe, l'hétérogénéité observée dans les souches lisses suggère que ces souches appartiennent à plusieurs génotypes (Gutierrez, 2005).

Blouin et al. ont identifié 18 polymorphismes nucléotidiques qui pourraient être liés à un seul événement de transfert horizontal de gènes entre les souches lisses et une mycobactérie environnementale inconnue (Blouin, 2014). Sur le plan expérimental, les travaux de Medie et al. ont montré que les organismes du CMT résistent à la digestion amibienne avec "*M. canettii"* s'échappant de l'amibe avant son enkystement. Les auteurs proposent que les cellulases des mycobactéries interviennent dans l'échappement à l'enkystement amibien (Medie, 2011). Dans notre laboratoire également, a été montrée la persistance de "*M. canettii"* dans le sol pendant au moins six mois (Ghodbane, 2014).

OBJECTIFS

Etant originaire de Djibouti, pays où la majorité des souches lisses a été isolée, nous nous sommes intéressés à ces microorganismes aux caractéristiques singulières au sein du complexe auxquels ils appartiennent. Dans ce travail de thèse, les objectifs étaient: (1) De faire une revue des données de la littérature sur l'état des connaissances concernant les souches lisses du complexe *M. tuberculosis,* dont *"M. canettii"* (2) De rechercher la présence de telles souches dans une collection de 34 isolats réalisée par le Dr. Didier Musso en Polynésie Française où une souche lisse avait été isolée, mais non publiée par Georges Canetti comme nous l'avons montré dans notre revue (3) De décrire l'épidémiologie des mycobactéries non-tuberculeuses en Polynésie Française (4) D'évaluer la résistance à la chaleur de souches lisses.

CHAPITRE 1-AVANT-PROPOS

L'année suivant la description d'une souche lisse de *M. tuberculosis* isolée chez un patient Somali de 2 ans (van Soolingen ,1997), une autre publication décrit une nouvelle souche lisse isolée chez un patient Suisse atteint de tuberculose mésentérique, qui avait vécu en Ouganda et au Kenya, avant de revenir en Suisse. Le patient co-infecté par le VIH, a développé les premiers signes de l'infection au Kenya (Pfyffer, 1998). De chercheurs français en collaboration avec le Service de Santé des Armées françaises à Djibouti identifient ensuite deux nouveaux cas d'infections causées par "*M. canettii*" chez deux soldats de la Légion étrangère française basée à Djibouti (République de Djibouti) souffrant de tuberculose pulmonaire (Miltegen, 2002). Les travaux ultérieurs indiquent que l'infection par le bacille tuberculeux lisse est corrélée avec un séjour dans la région de la Corne de l'Afrique (Fabre, 2004; Fabre, 2010; Koeck, 2011). D'un point de vue clinique, il est apparu que les patients infectés par "*M. canettii*" étaient significativement plus jeunes que ceux qui sont infectés par des souches rugueuses et que les expatriés français sont plus enclins à être infectés par des souches lisses par rapport aux Djiboutiens.

Les signes de l'infection de la forme pulmonaire de la tuberculose ne sont pas différents de celle causée par un membre du complexe *M. tuberculosis.* Aucune transmission interhumaine n'est rapportée (Koeck, 2011). Dans ce travail de revue, nous avons répertorié la liste de souches lisses publiées, étudié les spécificités cliniques, microbiologiques et génomiques pour dégager des pistes pour élucider le mode de transmission et le(s) réservoir(s) potentiel(s) de ces mycobactéries.

Bien que les souches lisses appartiennent au complexe *M. tuberculosis*, ces organismes forment un groupe remarquable par l'aspect lisse de ses colonies, l'absence de cord-factor et le temps de génération rapide. La forte prévalence des ganglions lymphatiques laisse suggérer une voie d'entrée orale et une contamination du tractus digestif.

Chapitre 1

REVUE

Smooth Tubercle Bacilli: Neglected Opportunistic Tropical Pathogens

Djaltou Aboubaker Osman, Feriel Bouzid, Stéphane Canaan, Michel Drancourt

Soumis à Frontiers in Public Health

Smooth Tubercle Bacilli: Neglected Opportunistic Tropical Pathogens

Running title: *Mycobacterium canettii*

Djaltou Aboubaker Osman [1, 2]‡, Feriel Bouzid [1, 3]‡, Stéphane Canaan [3], Michel Drancourt [1]*

1. Aix-Marseille Université, URMITE, UMR CNRS 7278, IRD 198, Inserm 1095, Marseille, France.

2. Institut de Recherche Médicinale (IRM), Centre d'Études et de Recherche de Djibouti (CERD).

3. Centre National de la Recherche Scientifique (CNRS) - Aix-Marseille Université - Enzymologie Interfaciale et Physiologie de la Lipolyse UMR7282. Marseille, France.

‡ DAO and FB contributed equally to this work.

*Corresponding author: Professor Michel DRANCOURT, Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, Faculté de Médecine, 27, Boulevard Jean Moulin, 13385 Marseille Cedex 5, France. Tel: +33 4 91 32 43 75; Fax: +33 4 91 38 77 72

Email: michel.drancourt@univ-amu.fr

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Abstract

Smooth tubercle bacilli (STB) including ‘‘*Mycobacterium canettii’’* are members of the *Mycobacterium tuberculosis* complex (MTBC) which cause non-contagious tuberculosis in human. This group comprises less than one hundred isolates characterized by smooth colonies and cordless organisms. Most STB isolates have been obtained from patients exposed to the Republic of Djibouti but seven isolates, including the three seminal ones obtained by Georges Canetti between 1968 and 1970, were recovered from patients in France, Madagascar, Sub-Sahara East Africa and French Polynesia. STB form a genetically heterogeneous group of MTBC organisms with large 4.48 ± 0.05 Mb genomes which may link *Mycobacterium kansasii* to MTBC organisms. Lack of inter-human transmission suggested a yet unknown environmental reservoir. Clinical data indicate a respiratory tract route of contamination and the digestive tract as an alternative route of contamination. Further epidemiological and clinical studies are warranted to elucidate areas of uncertainty regarding these unusual mycobacteria and the tuberculosis they cause.

Keywords: *Mycobacterium tuberculosis* complex, “*Mycobacterium canettii”, Canetti strain, Mycobacterium kansasii,* smooth tubercle bacilli, clinic, pathogenesis, evolution, Djibouti, Horn of Africa, experimental work, bioinformatics, lipids, lipolytic enzymes, amoebas, cellulases.

Introduction

In 2013, nine million people developed tuberculosis (TB) and 1.5 million people infected with TB died *(1)*. The vast majority of cases were caused by *Mycobacterium tuberculosis stricto sensu*, a cord-forming organism exhibiting rough colonies *(2-4)* while a few cordless isolates, referred as 'smooth tubercle bacilli' (STB) were reported to form smooth colonies *(5)*. The first three STB isolates made by Georges Canetti in 1968-1970 *(6)* were further named '*Mycobacterium canettii*' following the isolation of an additional STB isolate from a tuberculous lymph node in a Somali child *(7)*. Then, a total of 93 STB have been isolated from patients exposed to tropical countries, mainly the Republic of Djibouti, which reports the highest prevalence and incidence of STB *(5, 7-17)*. The reason for this geographical specificity is not really understood. Despite its rarity, STB deserve special attention due to their epidemiological, clinical and microbiological characteristics, which are unique among the *M. tuberculosis* complex (MTBC).

Particularities of the STB infection

No environmental or animal STB isolates have been identified, contrary to that of *M. tuberculosis (18)*. Indeed, the three seminal STB isolates were not reported by Canetti himself, but were rather identified through two indirect sources *(6, 19)*. Accordingly, the precise history of these seminal isolates is poorly known, although it began prior to 1969, as deduced from a study on *M. tuberculosis* var. *hominis*, Canetti strain mycolic acids submitted for publication in 1968 *(20)*. This first isolate was obtained from a 20-year-old French farmer suffering from pulmonary TB although he had apparently never left France *(6,19)*. Canetti obtained a second isolate from a 54-year-old farmer also suffering from pulmonary TB in Madagascar, then a third isolate from a man suffering tuberculous adenitis in Papeete, Tahiti *(6, 19)*. Surprisingly, the first ever reported STB isolates were therefore from three patients

with no reported contact with the Horn of Africa, where the vast majority of cases had been reported. In 1997, a fourth STB isolate (So93 strain) was reported as *'M. canettii' (7)*. The more general term 'STB' used here was quoted in a report on *M. tuberculosis* smooth variants in Djibouti *(5)*. Since 1997, a survey of the literature found a total of 93 STB isolates, mainly obtained from patients exposed to tropical countries (Supplementary Table 1). Indeed, 82/93 (88%) isolates were obtained from patients exposed to the Republic of Djibouti, 2/93 (2%) from patients exposed to Uganda including one also exposed to Kenya, 2/93 (2%) from patients exposed to Somalia, three other patients exposed to France, French Polynesia and Madagascar, and four cases with unknown geographical exposure (Figure 1A). With the notable exception of the three seminal isolates, all isolates were obtained between the 23° 26' 16" N and 23° 26' 16" S parallels in tropical countries with a coastline (Figure 1A). Following the description of the first cases in 1969 and 1970, few cases were reported between 1991 and 1997, although 29/93 cases were described from 1998 to 2000. A second peak in case reporting was observed between 2002 and 2003, with 17 cases being described, and a third peak took place eight years later with 10 new cases (Supplementary Figure 1). It should be noted that the number of published cases significantly correlates to the number of STB papers published over the same time period (P=1.208e-13, Pearson's correlation), suggesting a positive bias in reporting cases (Supplementary Figure 1). Interest in STB isolates gained ground around the 2000s, suggesting that efforts were concentrated where the main strains were collected, mainly in the Horn of Africa. Furthermore, the unusual macroscopic phenotype of the STB strains may delay their diagnosis and may even result in them being under-reported. Clinical data available for 85/93 patients *(5, 7, 8, 10, 11, 13-17)* indicate 44/85 (52%) had the pulmonary form and 41/85 (48%) had the extra-pulmonary form, including lymph node involvement in 32% of cases (Figure 2). In Djibouti, no significant difference was found in the prevalence of the pulmonary form between STB

[17/30 (56.6%)] and *M. tuberculosis stricto sensu* [2,188/3,772; (58%), P=0, 88>0, 1, X^2 test] (*Plan National de Lutte Anti Tuberculeuse*' 1997). However, the prevalence of enlarged lymph nodes in STB (12/30; 40%) was significantly higher than in *M. tuberculosis stricto sensu* (717/3,772; 19%) (P= 0.038, X^2 test). In Djibouti, a recent epidemiological investigation found that all STB lymph nodes were diagnosed in children and that all STB children had lymph nodes which were infected *(8)*. Indeed, the So93 strain was also obtained from lymphadenitis in a two-year-old Somali child *(7)*. Of note, the age of children with STB lymph nodes in the Horn of Africa shows a bimodal distribution with 7/14 children ≤ 4 years. This is the median age reported for *Mycobacterium avium hominissuis* lymph nodes *(21)*. This observation suggests that young children are infected by suction of contaminated fomites. These clinical observations suggest an oropharyngeal portal of entry for STB. Moreover, reports of STB-infected mesenteric lymph nodes *(15)*, as well one case of STB ascites *(19)* suggest a digestive tract route of infection in addition to the respiratory tract route. Interestingly, in contrast to classical TB infection, there is no evidence of human-to-human transmission of STB infection, suggesting the existence of an as yet unknown environmental reservoir *(5)*. Accordingly, '*M. canettii*' (CIPT140010059) was shown to survive in experimentally infected soil for a minimum of 12 months (*22*). Taken together, these observations suggest that soil may be a direct or indirect source of STB through drinking water and food, entering and replicating at the oropharyngeal portal of entry and spreading into the respiratory and digestive tracts (Figure 2).

Particularities of the STB organisms

The generation time of STB is two-three times shorter than that of *M. tuberculosis* strains in both liquid media and solid media at 30°C and 37°C (three and eight days for STB and *M. tuberculosis* H37Rv, respectively, at 37°C as measured by BACTEC 460 System in numerical growth units), a feature also of *Mycobacterium microti* (*9*). By definition, STB

present smooth colonies which are white to pale beige and glossy (Figure 1B) (5), correlating with the presence of a large amount of triglycosyl glycolipids *(7,23,24)*. Through electron microscopy scanning, colonies were observed to vary from small, singular, flat and cone-shaped to larger compound colonies formed by a homogeneous distribution of bacilli in singlets or aggregated in small clumps instead of the cord-like aggregates usually seen with rough MTBC strains *(7)* (Figure 1B). Specific biochemical traits, including antibiotic susceptibility patterns, are reported in Supplementary Table 2. Matrix-Assisted Laser Desorption Ionisation-Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) fingerprinting *(25)* yields a distinctive peptide spectrum for '*M. canettii*' (Figure 1C).

Five available whole STB genomes indicate a 4.4202 Mb -4.52595-Mb chromosome larger than that of the other MTBC members. No phages have been observed, but a controversial 55-kb prophage was identified in STB-I *(17, 26)*, nine spacers matching the *Mycobacterium marinum* strain M prophage and two spacers matching the Thibault or Redi *Mycobacterium* phages. Three additional prophages, phiBN42_1, phiBN44_1, phiMCAN_1, have been described respectively as '*M. canettii*' CIPT 140070010, '*M. canettii'* CIPT 140060008 and '*M. canettii'* CIPT 140010059 *(26)*. These prophages may play a major role in the evolution of STB, as previously reported for *M.* abscessus *(27)*. Further study found that some STB isolates lacked the insertion element *IS*1081, while a new *ISMycA1* (GenBank accession number AJ619854) was discovered in the '*M. canettii*' CIPT140010059 genome *(12)*. IS*MycA1* encodes a transposase which, surprisingly, shares 48% amino-acid sequence identity with IS-encoded transposases of the *Mycobacterium ulcerans* plasmid *(28)*. IS*MycA1* is a distinctive characteristic of STB in comparison with the other MTBC members *(12)*. Indeed, the original ''*M. canettii''* strain (CIPT 140010059) and So93 are indistinguishable from the other MTBC members as a result of sequencing of16S rRNA and housekeeping genes (*rpo*B, *kat*G, *rps*L and *gyr*A*) (7)*. Nevertheless, further analysis of six housekeeping

genes yielded 14 (A-N) STB clonal groups *(12, 17)*. The multiple locus variable number of tandem repeats analysis (MLVA) *(10, 11)* highlighted that ETR-A (allele 10), ETR-C (alleles 6 and 10), MIRU-02 (allele 3), MIRU-40 (allele 8), and Mtub29 (allele 5) were unique to STB strains *(10)*. Compared to *M. tuberculosis,* H37Rv investigations showed the presence of an intact region of deletion RD9 and the *M. tuberculosis* specific deletion (TbD1) *(11,29,30)*. Genomic analysis revealed that the precorrin gene *cob*F, preserved in many environmental mycobacteria, including *M. kansasii (31)*, is also present in all STB but is absent in all other MTBC members *(8, 17)*. In STB, repetitive sequences of the PE-PGRS families are highly diverse; in particular, PE_PGRS62 is polymorphic and positively selected in STB, while it is highly preserved in MTBC *(31)*. Indeed, STB strains show unprecedented high genetic heterogeneity with traces of intra-species horizontal gene transfer (HGT) compared to the worldwide population of MTBC strains, which represent one of the most extreme examples of a genetically homogeneous group *(8, 12, 17)*. Recently, distributive conjugal transfer was found to be a predominant mechanism for lateral gene transfer among STB, supporting the high heterogeneity observed in this group *(32, 33)*. These observations led to a new evolutionary scenario for the emergence of pathogenic *M. tuberculosis* from an environmental organism, such as *Mycobacterium kansasii*, through transitional ‘‘smooth’’ tubercle bacilli *(34-36)*.

STB infection models

Only amoebas have been used as a cell model for ‘*M. canettii’* infection *(37)*. In this model, 89% of ‘*M. canettii’* organisms which were co-cultured with free-living *Acanthamoeba polyphaga* amoeba were ingested by trophozoites , a ratio which is significantly higher than for *M. tuberculosis*, *Mycobacterium bovis* and *Mycobacterium avium (37).* This difference correlates with a 2.56 µm larger size for ‘*M. canettii*’ and smoothness reflecting the specific

169 presence of glycolipid containing triglycosyl. In a *Mycobacterium marinum-Acanthamoeba*
170 coculture model, it was shown that lipooligosaccharide modulates the phagocytosis of
171 mycobacteria in *Acanthamoeba (38)*. In contrast to *M. tuberculosis* and *M. bovis,* '*M.*
172 *canettii*' survives into cytoplasmic vacuoles and escapes from encystment *(37)*. This specific
173 behaviour could be related to the activation of cellulases Cel6, Cel12 and CBD2 to lyse the
174 cellulose cell wall of the amoebal exocyst *(39, 40)*. In the absence of any known reservoir *(5)*,
175 further studies presenting animal models with contradictory results may not be relevant to
176 natural human infection. A first model of guinea pigs which were inoculated subcutaneously
177 and intramuscularly with 1 mL 10^3 or 10^5 colony-forming units (CFU) of So93 or *M.*
178 *tuberculosis* H37Rv did not show signs of clinical disease for eight weeks *(7)*. However,
179 necropsy found overwhelming disseminated tuberculous lesions and severe loss of body fat
180 deposits in guinea pigs inoculated with So93, in contrast to animals inoculated with *M.*
181 *tuberculosis* H37Rv. In all animals, it has been found that the liver, spleen as well as the
182 lungs were infected. Virulence, measured by microscopic and bacteriological examination
183 and average root index of virulence calculation, was lower for *M. tuberculosis* H37Rv than
184 for So93 *(7)*. In a further study, BALB/c mice were infected by 2 x 10^5 viable cells of '*M.*
185 *canettii'* (strains CIPT 140010059 and So93) or *M. tuberculosis* H37Rv *(41)*. Two and three
186 weeks after infection, '*M. canettii'* induced larger perivascular infiltrates and significantly
187 smaller areas of granuloma in the lung than *M. tuberculosis* H37Rv. Also, *'M. canettii'* CIPT
188 140010059 induced sustained TNF-α and iNOS expression in lungs combined with delayed
189 and moderate IFN-γ expression. Four weeks post-infection, '*M. canettii'* strains yielded
190 almost 100% survivals significantly higher than 40-50% survivals in *M. tuberculosis*-infected
191 animals. In addition, lung replication of *'M. canettii'* strains was significantly lower than that
192 of *M. tuberculosis* H37Rv at all time points. At the final time point, pneumonic areas induced
193 by the *'M. canettii'* CIPT 140010059 were significantly smaller than those produced by *M.*

tuberculosis H37Rv *(41)*. In a further model, BALB/c mice were infected with 2.5 x 10^5 viable cells of '*M. canettii'* CIPT 140010059 or ten major genotypes of *M. tuberculosis* (H37Rv, Africa, Amesterdam, Beijing, Erdman, Haarlem, IS-in-Ori, Less-trans, Somalia, Zerocopy) *(42)*. *'M. canettii'* and *M. tuberculosis* H37Rv did not induce lung pathology for three weeks, and *'M. canettii'* caused limited pneumonia with mild peribronchiolitis, perivasculitis and alveolitis in the absence of granuloma formation at day 56 post-infection; at day 120 post-infection, '*M. canettii'* and *M. tuberculosis* H37Rv yielded a similar 10% death rate *(42)*. Additional animal models were conducted by infecting BALB/c and C57BL/6 mice with 10^3 CFUs of STB-D, STB-L, STB-K or STB-J, *M. tuberculosis* TbD1 positive or *M. tuberculosis* TbD1 negative by intranasal aerosol *(17)*. The STB strains effectively multiplied in the lungs and disseminated to the spleen three weeks after inoculation, but consistently persisted for less time during the chronic infection phase (30 weeks), compared to both *M. tuberculosis* strains. Furthermore, 128 days after inoculation, histopathological analyses revealed less severe lung lesions and inflammation in STB-infected mice than in *M. tuberculosis* infected mice *(17)*. The lower virulence and persistence of STB strains correlated to differences in both innate and adaptive immune responses *(17)*. In infected SCID mice, recruitment of activate innate cells was observed in the lung parenchyma three weeks post-infection with STB to a lower extent compared to *M. tuberculosis* infection. In addition, 13-week post-infection lung recruitment of activated $CD4^+$ and $CD8^+$ lymphocytes was quantitatively lower in STB-infected mice compared to *M. tuberculosis*-infected mice *(17)*.

Conclusions

With less than one hundred reported cases, STB infection remains a neglected infectious disease in tropical countries in East Africa. Indeed, their unique morphological features,

which are unusual among the MTBC, with smooth, shiny luxuriant and rapidly growing colonies, may lower their presumptive identification as MTBC members. Their cordless appearance observed after Ziehl-Neelsen staining further complicates first-line identification in endemic countries. The reservoirs and mode of transmission remain unknown but comparing clinical data with scarce experimental data suggests contaminated drinking water and food as potential sources, with local replication in the oropharynx and cervical lymph nodes and further dissemination in the respiratory and digestive tracts. In terms of this hypothesis, looking for STB in the stools of patients would be of interest, as it has been observed in patients with *M. tuberculosis* pulmonary tuberculosis *(43, 44)*. Likewise, genetic and genomic data including large genome size and the abundance of phage sequences, suggest that STB form a heterogeneous group of tuberculosis organisms with intermediate features in between mammal-adapted *M. tuberculosis* organisms and environmental organisms such as *M. kansasii (36)*. By means of conclusion, the data reviewed here could form the foundation of efforts towards elucidating the reservoirs and sources of STB, along with the development of laboratory tests aimed at a point-of-care diagnosis of STB infection *(45)*.

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Conflict of interest

The authors declare no conflict of interest.

Figure legends:

Figure 1: A) Geographical sources for STB infection in 93 patients. B) Aspects of STB and *M. tuberculosis* H37Rv colonies on 7H10 solid Middlebrook medium and Ziehl-Neelsen staining of mycobacteria. STB present smooth colonies and distribution of bacilli in singlets or aggregated small clumps instead of the cord-like aggregates usually seen with the rough H37Rv strains. C) MALDI-TOF spectrum for "*M. canettii*" (a) and *M. tuberculosis* H37Rv (b).

Figure 2: STB tuberculosis anatomical sites of infection and potential environmental sources and routes of contamination. Number of STB cases per site is indicated in brackets. Blue is for digestive tract, red for other anatomical sites. Question marks indicate hypothetical routes of contamination.

Supplementary material legends:

Table S1: Information on reported smooth tubercle bacilli strains.

Table S2: Classification of STB in comparison with *M. tuberculosis* H37Rv.

Figure S1: Cumulative number of cases and number of publications based on the year of isolation.

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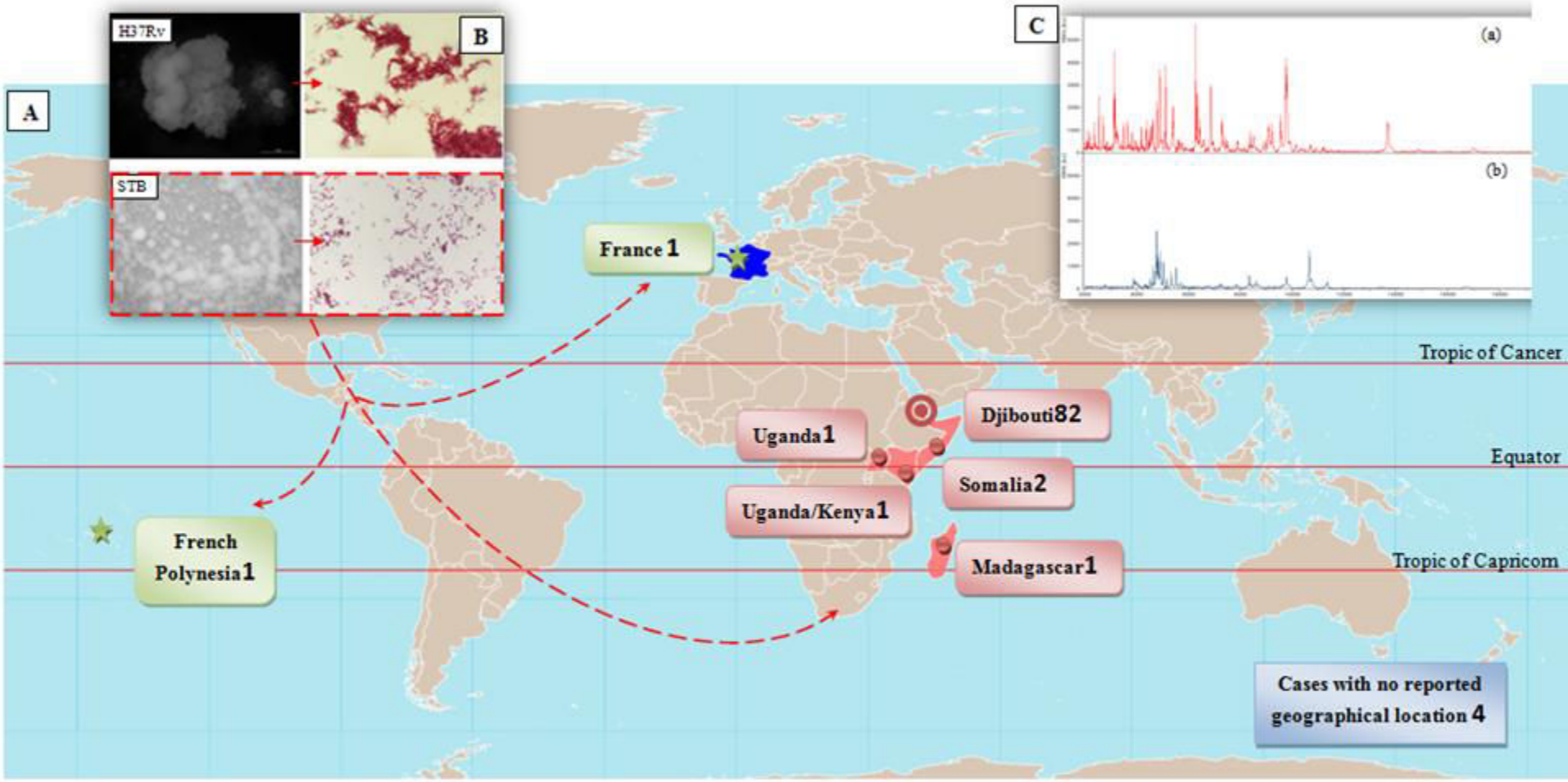
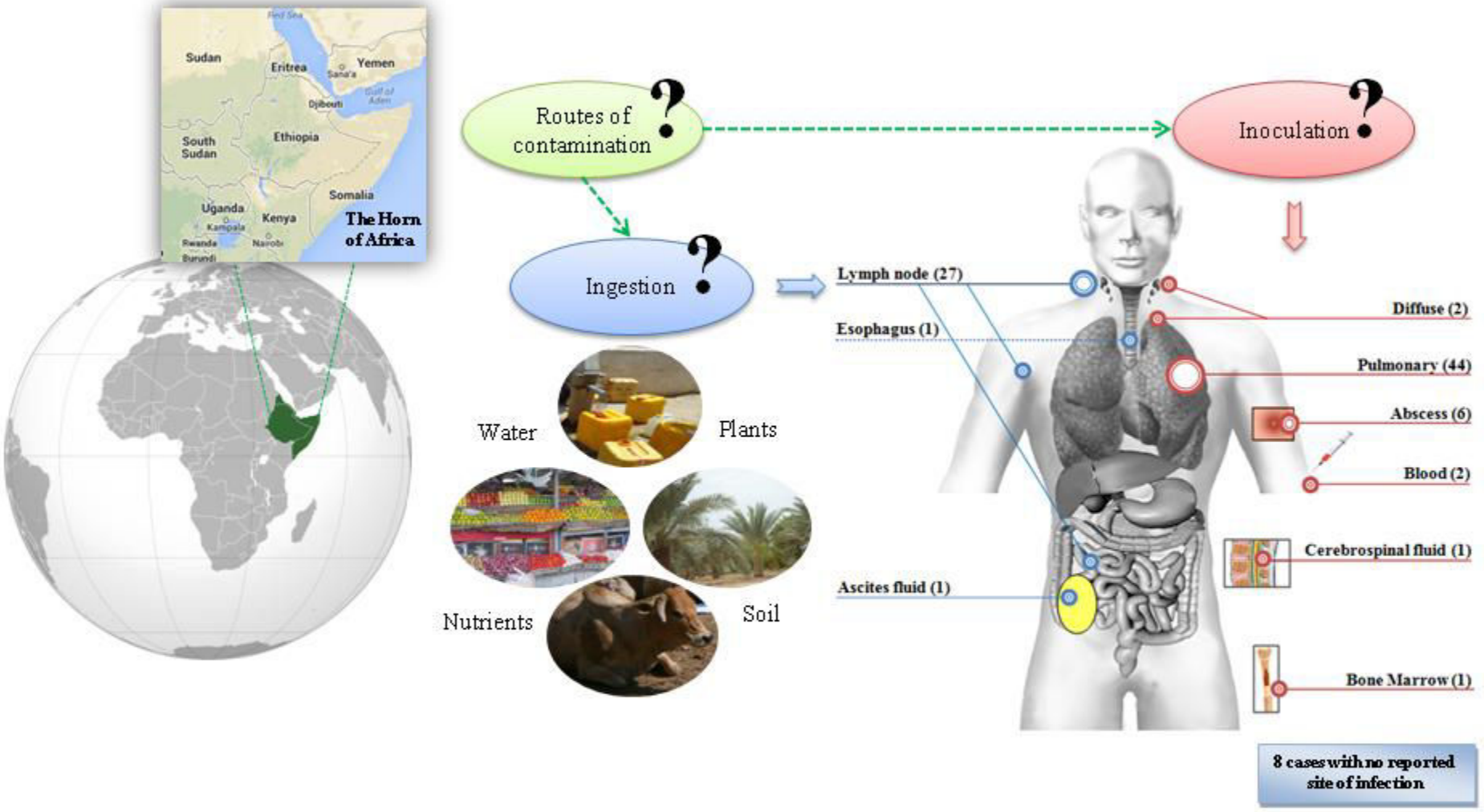

A
H37Rv
B
STB
C
(a)
(b)
France 1
Uganda 1
Djibouti 82
Somalia 2
Uganda/Kenya 1
Madagascar 1
French Polynesia 1
Tropic of Cancer
Equator
Tropic of Capricom
Cases with no reported geographical location 4


Sudan
Eritrea
Yemen
Sana'a
Djibouti
Gulf of Aden
South Sudan
Ethiopia
Somalia
Uganda
Kampala
Kenya
Nairobi
Rwanda
Burundi
The Horn of Africa
Routes of contamination
Inoculation
Ingestion
Water
Plants
Nutrients
Soil
Lymph node (27)
Esophagus (1)
Ascites fluid (1)
Diffuse (2)
Pulmonary (44)
Abscess (6)
Blood (2)
Cerebrospinal fluid (1)
Bone Marrow (1)
8 cases with no reported site of infection

Supplumentary table 1: Informations on reported smooth tubercle bacilli strains

First Id	Alias	Collection Strain Code	TB site	Year isolation	Country of isolation	Patient nationality	Geographical exposure	Sexe	Age	Genetic pattern	Principal reference
HB3177		CIPT140010059	Pulm	1968	FR	FR	FR	M	20	STB-A	[6]
HB3178		CIPT140010060	Pulm	1969	FR	FR	Madagascar	M	54	STB-A	[6]
HB3253		CIPT140010061	LN	1970	FR polynesia	FR	Papeete	M	NR	STB-A	[6]
CIPT140010062		CIPT140010062	NR	NR	NR	NR	NR	NR	NR	NR	[23]
910563			NR	1991	FR	NR	NR	NR	NR	NR	[6]
So93		So93	LN	1993	NLA	SOM	SOM	M	2	NR	[7]
NZM 217/94		NZM 217/94	LN	1993	Sw	Sw	Uganda and Kenya	M	56	STB-C	[17]
NR	19990160		Pulm	1998	FR	FR	DJ	M	36	STB-C	[16]
NR	19991574		Pulm	1999	FR	FR	DJ	M	55	STB-D	[16]
NR			Pulm	2002	FR	FR	DJ	M	21	NR	[15]
Percy3	19990516	CIPT140060008 Percy3	Pulm	1998	DJ	DJ	DJ	F	8	STB-D	[14]
Percy6	19990515	Percy6	Pulm	1998	DJ	FR	DJ	F	35	STB-D	[14]
Percy8	19990161	Percy8	LN	1998	DJ	FR	DJ	F	4	STB-C	[14]
Percy21a	19990160	Percy21a	Pulm	2000	DJ	FR	DJ	M	36	STB-C	[14]
Percy21b	20000342	Percy21b	NR	2000	DJ	DJ	DJ	F	NR	STB-D	[14]
Percy22		Percy22	Abs	2003	DJ	FR	DJ	NR	NR		[14]
Percy26	19991708	Percy26	LN	1999	DJ	DJ	DJ	F	18	STB-D	[14]
Percy29a	19990589	Percy29a	Pulm	1999	DJ	ETH	DJ	F	34	STB-D	[14]
Percy30		Percy30	NR	2003	DJ	DJ	DJ	NR	NR	NR	[14]
Percy31b		Percy31b	Blo	2003	DJ	ETH	DJ	NR	NR	NR	[14]
Percy50		Percy50	Pulm	1983	DJ	FR	DJ	NR	NR	NR	[14]
Percy58b		Percy58b	Pulm	2002	DJ	ETH	DJ	NR	NR	NR	[14]
Percy74	20010389	Percy74	Pulm	2001	DJ	DJ	DJ	M	NR	STB-D	[14]
Percy94b		Percy94b	Pulm	2002	DJ	DJ	DJ	NR	NR	NR	[14]
Percy99c	19991704	Percy99c	Pulm	1999	DJ	DJ	DJ	M	22	STB-D	[14]
Percy101		Percy101	Abs	2002	DJ	DJ	DJ	NR	NR	NR	[14]
Percy103		Percy103	BM	2002	DJ	DJ	DJ	NR	NR	NR	[14]
Percy106		Percy106	Pulm	2002	DJ	ETH	DJ	NR	NR	NR	[14]
Percy144		Percy144	LN	2002	DJ	FR	DJ	NR	NR	NR	[14]
Percy150	20001049	Percy150	Pulm	2000	DJ	DJ	DJ	M	NR	STB-D	[14]
Percy156	20010933	Percy156	LN	2001	DJ	DJ	DJ	M	27	STB-D	[14]
Percy189b	20001248	Percy189b	Pulm	2000	DJ	DJ	DJ	M	NR	STB-D	[14]
Percy197b		Percy197b	Pulm	2002	DJ	FR	DJ	NR	NR	NR	[14]
Percy199b	20001247	Percy199b	AF	2000	DJ	DJ	DJ	F	NR	STB-D	[14]
Percy205	20001246	Percy205	Pulm	2000	DJ	DJ	DJ	M	63	STB-D	[14]
Percy206	20001245	Percy206	Pulm	2000	DJ	DJ	DJ	M	NR	STB-D	[14]
Percy212	19981514	Percy212	LN	1998	DJ	DJ	DJ	M	32	STB-C	[14]
Percy229	19990645	Percy229	LN	1997	DJ	NR	DJ	NR	NR	STB-L	[14]
Percy245b	20010188	Percy245b	LN	2000	DJ	DJ	DJ	F	NR	STB-D	[14]
Percy246	20010390	Percy246	LN	2001	DJ	DJ	DJ	M	NR	STB-D	[14]
Percy257	19980862	Percy257	LN	1998	DJ	DJ	DJ	F	40	STB-D	[14]
Percy32	19990711	Percy32	LN	1999	DJ	DJ	DJ	M	13	STB-B	[14]
Percy79	20010391	Percy79	Pulm	2001	DJ	DJ	DJ	M	NR	STB-F	[14]
Percy26b		Percy26b	NR	2003	DJ	DJ	DJ	NR	NR	NR	[14]
Percy94	19991705	Percy94	Pulm	1999	DJ	DJ	DJ	M	42	STB-H	[14]
Percy213	19980864	Percy213	Pulm	1998	DJ	DJ	DJ	M	20	STB-H	[14]
Percy214	19980863	CIPT140070013 Percy214	Pulm	1998	DJ	DJ	DJ	F	27	STB-H	[14]
Percy258	19980865	Percy258	Pulm	1998	DJ	DJ	DJ	M	40	STB-H	[14]
Percy25	19991709	CIPT140070002 Percy25	LN	2000	DJ	DJ	DJ	M	7	STB-E	[14]
Percy65		CIPT140070017 Percy65	LN	1999	DJ	DJ	DJ	F	4	STB-J	[14]
Percy89	20000587	CIPT100070005 Percy89	LN	2000	DJ	ETH	DJ	F	NR	STB-G	[14]
Percy99b	20000473	CIPT140070007 Percy99b	Pulm	2000	DJ	DJ	DJ	M	NR	STB-I	[14]
Percy157	19991669	Percy157	Pulm	1999	DJ	DJ	DJ	M	NR	STB-N	[9, 11]
	19990263		Pulm	1997	FR	DJ	NR	NR	NR	STB-F	[13]
			CF	2005	USA	SUD	Uganda	F	30	NR	[12]
Percy300		Percy300	Blo	2003	DJ	DJ	DJ	NR	NR	NR	[11]
Percy329	20050642	Percy329	Pulm	2005	DJ	FR	DJ	NR	NR	STB-CD	[9, 11]
Percy358		Percy358	NR	2003	DJ	FR	DJ	NR	NR	NR	[11]
Percy516		Percy516	LN	2006	DJ	FR	DJ	NR	NR	NR	[11]
Percy673		Percy673	Abs	2007	DJ	DJ	DJ	NR	NR	NR	[11]
Percy756		Percy756	Pulm	2009	DJ	DJ	DJ	NR	NR	NR	[11]
NLA000200937		NLA000200937	Pulm	2002	NLA	ERI	DJ	NR	NR	NR	[11]
NLA000201000		NLA000201000	Pulm	2002	NLA	SOM	DJ	NR	NR	NR	[11]
NLA000400617		NLA000400617	Abs	2004	NLA	SOM	DJ	NR	NR	NR	[11]
Percy301		Percy301	LN	2003	DJ	DJ	DJ	NR	NR	NR	[11]
Percy525		Percy525	Pulm	2006	DJ	DJ	DJ	NR	NR	NR	[11]
Percy302		CIPT140070010 Percy302	Abs	2004	DJ	DJ	DJ	NR	NR	STB-K	[11]
Percy327		CIPT140070008 Percy327	Abs	1997	DJ	DJ	DJ	NR	NR	STB-L	[11]
NLA000701671		NLA000701671	Pulm	2007	NLA	SOM	DJ	NR	NR	NR	[11]
MTB_K116		MTB_K116	NR	NR	NR	NR	SOM	NR	NR	NR	[10]
3151/08			NR	NR	NR	GER*	NR	NR	NR	NR	[47]
NR	19990264		Pulm	1998	FR	NR	DJ	NR	NR	STB-F	[9]
NR	20040352		LN	2004	DJ	NR	DJ	NR	NR	STB-M	[9]
Percy976		Percy976	Pulm	2010	DJ	DJ	DJ	M	18	STB-A	[8]
Percy977		Percy977	Pulm	2010	DJ	DJ	DJ	F	22	STB-A	[8]
Percy979		Percy979	Pulm	2010	DJ	DJ	DJ	F	39	STB-A	[8]
Percy1004		Percy1004	LN	2010	DJ	DJ	DJ	M	14	NR	[8]
Percy1049		Percy1049	Pulm	2011	DJ	ETH	DJ	F	36	STB-A	[8]
Percy1060		Percy1060	Diffuse	2011	DJ	DJ	DJ	M	33	STB-A	[8]
Percy1062		Percy1062	Pulm	2011	DJ	FR	DJ	M	40	STB-C	[8]
Percy1064		Percy1064	Pulm	2011	DJ	DJ	DJ	M	55	STB-C	[8]
Percy1077		Percy1077	Esophagus	2011	DJ	FR	DJ	M	48	STB-A	[8]
Percy1078		Percy1078	LN	2011	DJ	FR	DJ	F	3	STB-A	[8]
Percy1079		Percy1079	LN	2011	DJ	FR	DJ	M	1	STB-A	[8]
Percy1084		Percy1084	LN	2011	DJ	FR	DJ	M	4	STB-A	[8]
Percy1085		Percy1085	LN	2011	DJ	FR	DJ	F	8	STB-A	[8]
Percy1086		Percy1086	Diffuse	2012	DJ	DJ	DJ	M	51	STB-A	[8]
Percy1101		Percy1101	Pulm	2011	DJ	DJ	DJ	F	26	STB-C	[8]
Percy1105		Percy1105	Pulm	2012	DJ	FR	DJ	M	44	STB-A	[8]
Percy1115		Percy1115	LN	2012	DJ	FR	DJ	M	3	STB-A	[8]
Percy1116		Percy1116	LN	2012	DJ	FR	DJ	M	12	STB-A	[8]
Percy1129		Percy1129	LN	2013	DJ	FR	DJ	F	11	STB-A	[8]
Percy1130		Percy1130	Pulm	2013	DJ	DJ	DJ	M	35	STB-A	[8]

LEGEND

Pulm, pulmonary
LN, lymph node
AF, ascites fluid
BM, bone marrow
Abs, abscess
Blo, blood
Esophagus
Diffuse
CF, cerebrospinal fluid
NR, No Reported

DJ, Djibouti
FR, France
NLA, Netherlands
Sw, Switzerland
ETH, Ethiopia
SOM, Somalia
ERI, Eritrea
SUD, Sudanese
GER, Germany

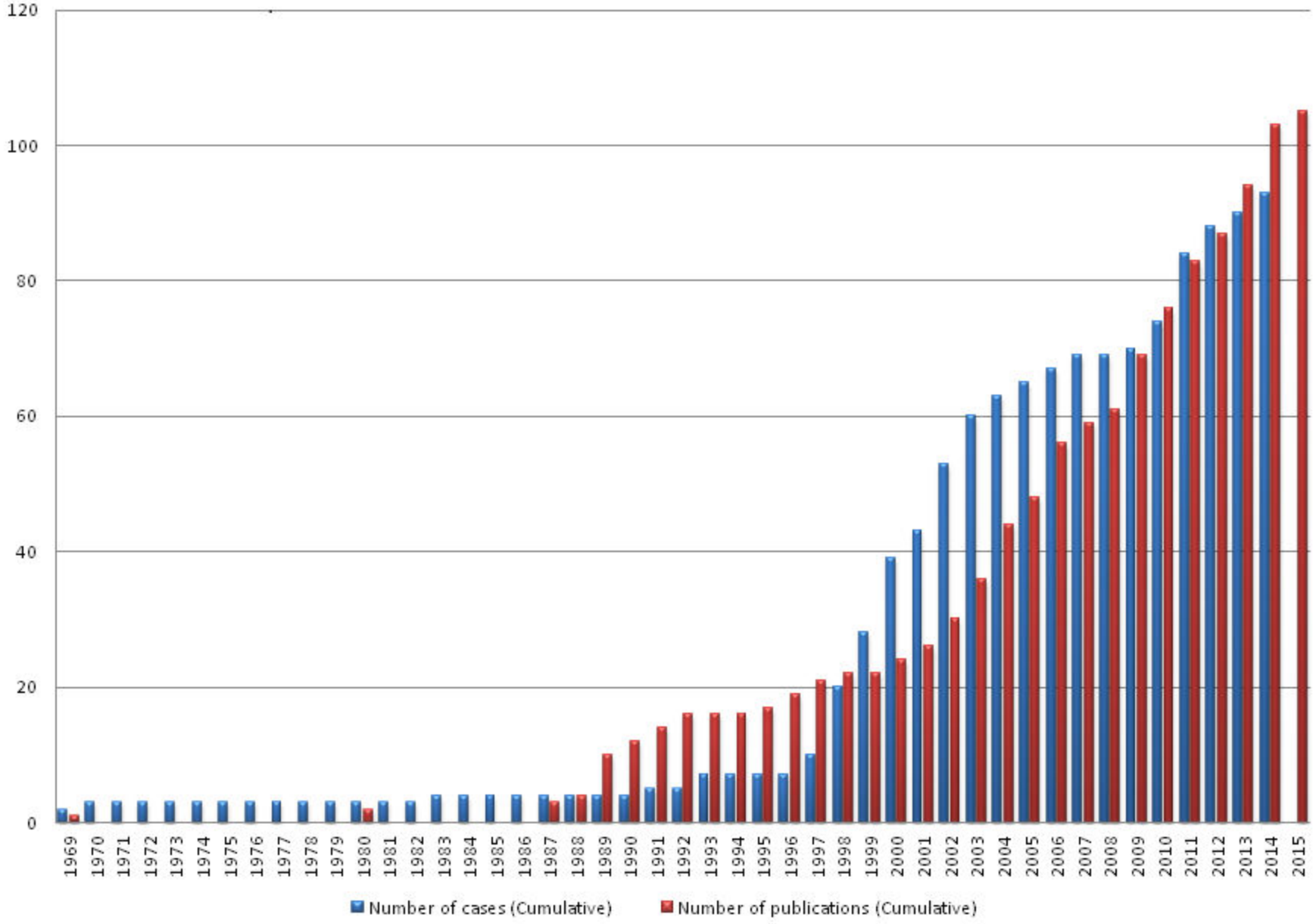
F: Female
M: Male

Yellow color: hallmarking of diagnosed cases out of Djibouti
Red color: alias and genetic pattern assigned by deduction
*Information obtained by correspondence with the author

Supplementary Table 2: Classification of STB in comparison with *M. tuberculosis* H37Rv.

Characteristics	**STB**	***M. tuberculosis* H37Rv**	**References**
Morphological			
Colony appearance	Eugonic smooth	Eugonic rough	*(5)*
Depth of growth	Aerophilic	Micro-aerophilic	*(22)*
Doubling time in liquid Tween Albumin medium	17 hours	25 hours	*(7)*
Generation time - LJ medium - BACTEC 460 System	 22 (16–25 days) 3 days	 23 (18–25 days) 8 days	*(5) (17)*
Growth on minimum solid media (Trypticase-soy agar)	Positive	Negative	*(5)*
Biochemical			
Nitrate reductase	Present	Present	*(5)*
Niacin production	Absent	Present	*(5)*
Drug susceptibility			
Pyrazinamide **(PZA) 100 mg/L**	Resistant	Sensitive	*(47)*
***Thiophen*-2-carboxylic acid hydrazine (TCH) 2 mg/L**	Resistant	Resistant	*(15)*

Streptomycin **(SM)**	2 – 10 µg/mL	0.25–1.0 µg/mL	*(16)* *(46)*
Isoniazid **(INH)**	0.2–1 µg/mL	0.016–0.06 µg/mL	
Rifampin **(RIF)**	0.2 µg/mL	0.06–0.25 µg/mL	
Ethambutol **(EMB)**	2.5 – 7.5 µg/mL	0.06–0.25 µg/mL	
Molecular			
Genome size (**Mb**)	4.29797 – 4.52595	4.4115	*(17)*
GC%	65.40 – 65.60	65.50	*(17)*
TbD1 region	Present	Absent	*(29)*


120
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Number of cases (Cumulative)
Number of publications (Cumulative)

CHAPITRE 2-AVANT-PROPOS

Nous avons rapporté dans notre revue que les trois souches séminales de "*M.canettii*" ont été isolées hors de la Corne de l'Afrique: la première a été isolée en France par Georges Canettii à partir d'un fermier de 20 ans vivant dans le Calvados et souffrant d'une tuberculose pulmonaire et n'ayant apparemment jamais quitté la France (Goh, 2001; Didier, 2005). G. Canetti obtient un second isolat chez un fermier âgé de 54 ans souffrant de tuberculose pulmonaire à Madagascar, puis un troisième isolat chez un patient souffrant d'une adénite tuberculeuse à Papeete, Tahiti (Polynésie Française) (Goh, 2001; Didier, 2005). Il n'existe aucune description de ces trois souches par G. Canetti. Ces trois souches sont décrites indirectement à travers des travaux respectivement sur le lien entre la composition lipidique et l'aspect des colonies au sein du complexe *Mycobacterium tuberculosis* (Daffé, 1987; Lemassu, 1992) et la mise en œuvre d'outils de la biologie moléculaire pour une rapide différenciation entre les membres de ce même complexe (Goh, 2001) puis une thèse de médecine (Bruno, 2005). Nous ne disposons d'aucune donnée pour expliquer le lien entre ces premières souches isolées hors d'Afrique et la restriction géographique des suivantes à la Corne de l'Afrique.

Nous avons procédé à l'analyse génotypique d'une collection de 34 isolats du complexe *M. tuberculosis* isolées en Polynésie Française par Dr. Didier Musso. Nous n'avons pas identifié de telles souches lisses décrites dans la littérature mais deux isolats représentatifs de deux nouvelles lignées de *M. tuberculosis*. Plus d'enquêtes épidémiologiques seront nécessaires pour mieux cerner la répartition géographique des souches lisses.

Article 2

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ORIGINAL ARTICLE

Diversity of *Mycobacterium tuberculosis* lineages in French Polynesia

Djaltou Aboubaker Osman [a,b], Michael Phelippeau [a], Michel Drancourt [a,], Didier Musso [c,*]**

[a] *Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes (URMITE), UMR CNRS 7278, IRD 198, INSERM 1095, Faculté de Médecine, Marseille, France*
[b] *Institut de Recherche Médicinale (IRM), Centre d'études et de Recherche de Djibouti (CERD), Djibouti*
[c] *Pôle de recherche et de veille sur les maladies infectieuses émergente, Institut Louis Malardé, Tahiti, French Polynesia*

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French Polynesia;
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Mycobacterial interspersed repetitive unit;
Mycobacterium tuberculosis;
Spoligotyping;
tuberculosis

Abstract *Background/Purpose:* French Polynesia is an overseas territory located in the South Pacific. The incidence of tuberculosis in French Polynesia has been stable since 2000 with an average of 20 cases/y/100,000 inhabitants. Molecular epidemiology of *Mycobacterium tuberculosis* in French Polynesia is unknown because *M. tuberculosis* isolates have not been routinely genotyped.
Methods: From 2009 to 2012, 34 isolates collected from 32 French Polynesian patients were identified as *M. tuberculosis* by probe hybridization. These isolates were genotyped using spoligotyping and 24-loci mycobacterial interspersed repetitive units (MIRUs)-variable number of tandem repeat (VNTR). Spoligotype patterns obtained using commercial kits were compared with the online international database SITVIT. MIRU-VNTR genotyping was performed using an in-house protocol based on capillary electrophoresis sizing for 24-loci MIRU-VNTR genotyping.
Results: The results of the spoligotyping method revealed that 25 isolates grouped into six previously described spoligotypes [H1, H3, U likely (S), T1, Manu, and Beijing] and nine isolates grouped into six new spoligotypes. Comparison with the international database MIRU-VNTRplus distributed 30 isolates into five lineages (Haarlem, Latin American Mediterranean, S, X, and Beijing) and four as unassigned isolates.
Conclusion: Genotyping identified four phylogenetic lineages belonging to the modern Euro–American subgroup, one Beijing genotype responsible for worldwide pandemics, including

* Corresponding author. Pôle de recherche et de veille sur les maladies infectieuses émergente, Institut Louis Malardé, PO Box 30, 98713 Papeete, Tahiti, French Polynesia.
** Corresponding author. Unité de Recherche sur les Maladies Infectieuses et Tropicales Émergentes, CNRS, UMR 6236 – IRD 198, Faculté de médecine, Aix-Marseille Université, 27 Boulevard Jean Moulin, 13385 Marseille cedex 05, France. Tel.: +33 491 385 517; fax: +33 491 387 772.
E-mail addresses: michel.drancourt@univ-amu.fr (M. Drancourt), dmusso@ilm.pf (D. Musso).

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remote islands in the South Pacific, and one *Manu* genotype of the ancestral lineage of *M. tuberculosis*.

Introduction

Mycobacterium tuberculosis complex infections are one of the major global public health problems, especially in the Pacific region. The Pacific is a very large area and the situation of tuberculosis (TB) differs from one region to another. The situation is alarming in the Western Pacific region (21% of the global TB burden), where recent estimates indicate that there are approximately 1.9 million incident TB cases and 260,000 deaths annually, with the situation exacerbated by TB—human immunodeficiency virus (HIV) co-infection and the emergence and spread of multidrug-resistant TB.[1] Regarding TB surveillance, the Pacific TB Laboratory Initiative was created in 2004 in collaboration with the Western Pacific Regional Office of the World Health Organization (WHO), the Secretariat of the Pacific Community, the U.S. Centers for Diseases Control and Prevention, and reference laboratories located in Australia and New Zealand, with the main objective of improving the quality of sputum microscopy and surveillance for TB drug resistance.[2] In the Western Pacific region, the prevalence of TB decreased by 46% and the mortality by 42% from 2000 to 2008.[2] The epidemiology of TB is different in the Pacific Island Countries and Territories (PICTs) compared with the Western Pacific region (South East Asia). The PICTs comprise 22 countries and territories and three subregions, namely, Micronesia, Melanesia, and Polynesia. From 2000 to 2008, the notification rate of TB cases in the PICTs ranged from 159 to 207 cases/100,000 inhabitants in Melanesia, from 91 to 147 cases/100,000 inhabitants in Micronesia, and from 15 to 22 cases/100,000 inhabitants in Polynesia; the highest TB case notification rate was reported in Kiribati (Micronesia), 399 cases/100.000 inhabitants in 2006.[3] Six PICTs were identified as high-burden TB countries (TB cases $\geq$ 70/100,000 inhabitants).[3] In the PICTs, only 45 multidrug-resistant *M. tuberculosis* isolates were reported between 2004 and 2009, with the highest prevalence being reported among migrant workers in Micronesia.[3] Although rates of drug resistance and multidrug-resistant *M. tuberculosis* were low in the PICTs up to 2009 (except in the Federated States of Micronesia), formal drug-resistance surveillance has not been widely implemented in the region.

French Polynesia is an overseas French territory located in the South East Pacific (274,000 inhabitants; 118 islands distributed in 5 archipelagos, 67 islands are inhabited). In French Polynesia, the TB control program is coordinated by the Director of Health/Ministry of Health. Based on mandatory clinical declarations, the TB incidence has been stable since 2000 with an average of 20 cases/100,000 inhabitants,[3,4] and 80% of the cases being pulmonary TB. From 2000 to 2012, no case of TB multidrug resistance and no *M. tuberculosis*—HIV co-infections were reported in French Polynesia.[4]

Concerning the genetic diversity of *M. tuberculosis* in the Pacific region, data are principally available for the Western Pacific region.[5,6] Because there are no laboratory tools, very little is known about the molecular epidemiology of *M. tuberculosis* in the PICTs, except for studies conducted in Kiribati (Micronesia)[7] and Papua New Guinea (Melanesia).[8]

Molecular tools have been proven to be useful in obtaining a better understanding of TB epidemiology.[9] An initial study on *M. tuberculosis* molecular epidemiology was conducted in French Polynesia in 1991/1992 using the IS6110 restriction fragment length polymorphism,[10] however, *M. tuberculosis* isolates have not been routinely genotyped using new molecular tools.

To describe the genetic diversity among clinical isolates of *M. tuberculosis* in French Polynesia, we genotyped 34 *M. tuberculosis* complex strains isolated over a 4-year period using spoligotyping and 24-loci mycobacterial interspersed repetitive units (MIRUs)-variable number of tandem repeat (VNTR).

Methods

Ethics statement

This study was approved by the Ethics Committee of French Polynesia under reference Number 61/CEPF. The study was a noninterventional study conducted on samples collected for TB laboratory diagnosis and under medical prescription by a physician.

Sample collection

The study was conducted on *M. tuberculosis* strains isolated from 2009 to 2012 at the Institut Louis Malardé, Tahiti, a French Polynesian health and research institute in charge of infectious diseases control and surveillance, which receives samples from the whole of French Polynesia. Specimens were received with a confidential medical questionnaire form.

Clinical isolate processing and drug-susceptibility testing

Specimen processing for TB diagnosis was performed at the Institut Louis Malardé, Tahiti. All specimens received for tuberculosis diagnosis were routinely investigated using direct microscopic examination after Ziehl—Neelsen staining and cultured on egg-based Lowenstein—Jensen medium

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(bioMérieux, La Balme-les-Grottes, France). First-line identification of colonies was made using Ziehl—Neelsen staining before hybridization with *M. tuberculosis* complex probes (AccuProbe *M. tuberculosis* complex, bioMérieux, La Balme-les-Grottes, France). For all strains identified as *M. tuberculosis*, antibiotic susceptibility was performed using a broth microdilution method for rifampin, isoniazid, streptomycin, and ethambutol.

Genotyping methods

Genotyping was performed at the Reference Laboratory for Mycobacteria of the Institut Hospitalo-Universitaire Méditerranée Infection, Marseille, France. Genotyping was performed using Beijing typing by polymerase chain reaction (PCR), spoligotyping, and 24-loci MIRUs-VNTR.

Beijing typing

To screen Beijing isolates, real-time PCR was performed with primers targeting an IS6110 insertion in *Rv2820* that is unique to the *Beijing* lineage.[11] The real-time PCR mix consisted of 10 μL of MasterMix (Eurogentec, Angers, France), forward (5′-CTCGGCAGCTTCCTCGAT-3′) and reverse (5′-CGAACTCGAGGCTGCCTACTAC-3′) primers (0.5 μL each of 20 pM working stocks), 0.5 μL of 0.1 nmol/μL working stock of the probe (6VIC-AACGCCAGA-GACCAGCCGCCGGCT), 3.5 μL nuclease-free water, and 5 μL DNA. The reactions were set up in a 96-well PCR plate and processed on a CFX96 real-time PCR system (Bio-Rad, Marnes-la-Coquette, France). PCR cycling parameters were as follows: 50°C for 2 minutes followed by 40 cycles of 95°C for 5 minutes, 95°C for 1 second, and 60°C for 35 seconds and 45°C for 30 seconds.

Spoligotyping

Spoligotype patterns,[12] obtained using a commercial kit according to manufacturer guidelines (Gentaur, Paris, France), were compared using the online international *M. tuberculosis* molecular markers spoligotype database

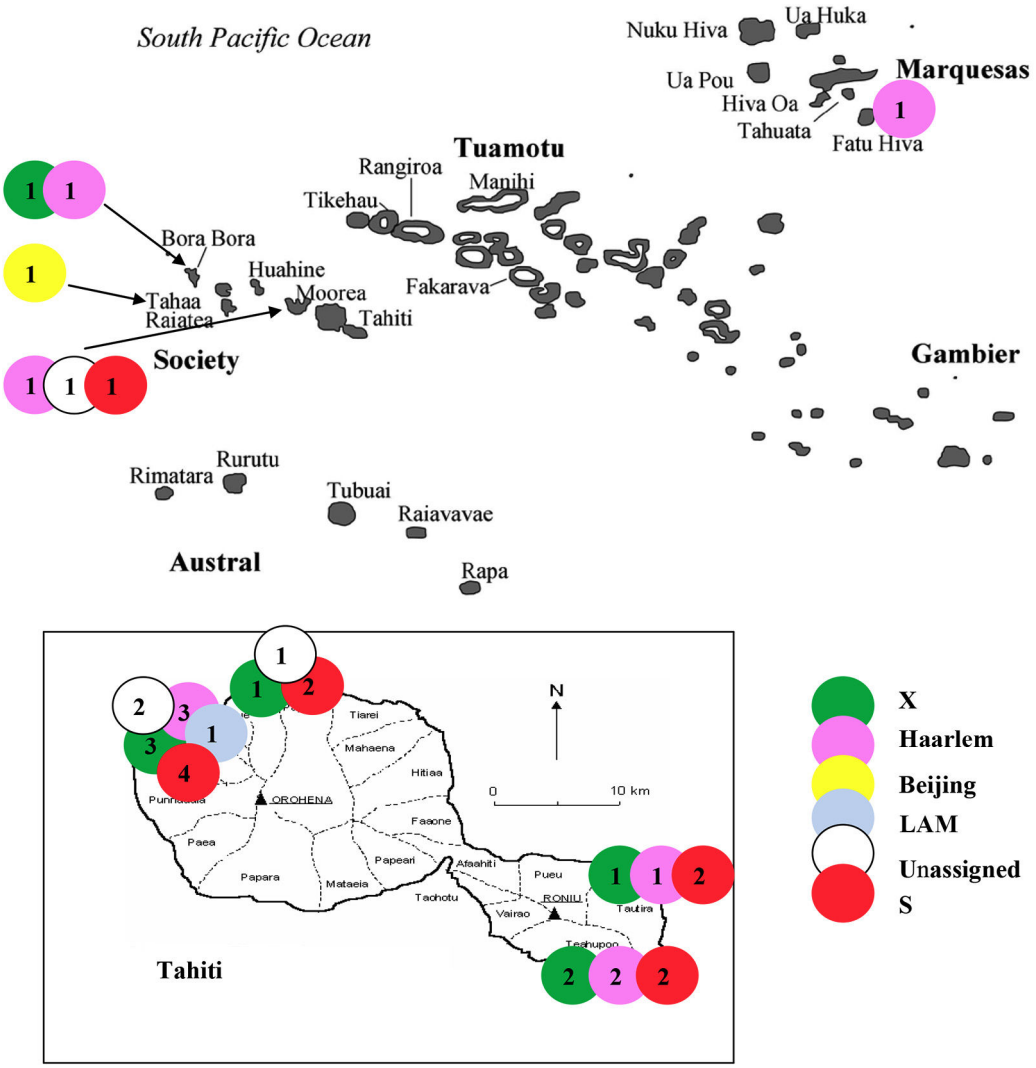

Figure 1. Geographic distribution of the 34 *Mycobacterium tuberculosis* isolates in French Polynesia with a focus on Tahiti Island. Each lineage is represented by a different color. LAM = Latin American Mediterranean.

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SITVIT (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/).[13–15]

MIRU-VNTR genotyping

We developed an in-house protocol based on capillary electrophoresis sizing for performing MIRU-VNTR genotyping. Prior to the amplification, the concentration of DNA extracts was determined using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNA was then diluted to a final concentration of 1 ng/μL and stored at −20°C. PCRs for the MIRU-VNTR manual typing were conducted using HotStartTaq polymerase kit (QIAGEN, Marnes-la-Coquette, France) including the Q solution (20 μL) containing 2 μL of the DNA at 1 ng/μL and 18 μL of premix for the 24 loci as described by Supply.[16] In this study, to determine the amplicon fragment sizes, we used the LabChip GXII system (PerkinElmer, Hopkinton, MA, USA) consisting of an array of 12 short capillaries covering the size range from 50 bp to 5 kb and prefilled with gel polymers. The samples were analyzed in a 96-well plate in a single experiment without intervention. Because the sipper is rinsed between samples, cross-contamination was avoided. PCR products were diluted 10-fold to make up a final volume of 20 μL with distilled water and analyzed using the HT DNA high sensitivity LabChip Kit (PerkinElmer). The assay was carried out according to the manufacturer's instructions. PCR products were loaded in a systematic manner in which the *M. tuberculosis* H37Rv reference strain was added as a control for size assignments. The resulting gel images were stored as a .tiff file. Size for each band was determined using both a ladder and internal markers. Copy numbers were obtained by comparing these band sizes with an allele naming table for each tandem repeat locus made available elsewhere and were saved in Microsoft Excel (Microsoft, Redmond, WA, USA) format. To distribute the MIRU-VNTR patterns into lineages, tools available on the MIRU-VNTRplus website (http://www.miru-vntrplus.org) were applied.

Clustering analysis

The Hunter and Gaston Discriminatory Index (HGDI) was used to calculate the discriminatory power of both methods.[17] HGDI is calculated using the following formula: $1 - \left[\frac{1}{N(N-1)}\sum_{J=1}^{s} nj(nj-1)\right]$.

Table 1 Clinical description of 32 patients and the 34 *Mycobacterium tuberculosis* isolates in French Polynesia, 2009–2012

Groups[a]	All	Haarlem	LAM	S	Beijing	X group	Unassigned
Patients characteristics[b]							
Age (y)							
<20	20	22	0	45.5	0	0	0
20–60	65	67	100	45.5	100	87	100
>60	15	11	0	9	0	13	0
Sex[a]							
Female	20	56	0	9	0	0	50
Male	80	44	100	91	100	100	50
Clinical presentation[b]							
Cough	74	78	100	54	NA	87	100
Asthenia	59	56	100	64	NA	38	100
Weight loss	50	44	100	27	NA	75	75
Hemoptysis	21	11	0	18	NA	38	25
Fever	50	33	100	45.5	NA	63	75
Sweats	21	33	100	9	NA	13	25
Home living conditions							
Number of persons at home	5.5	5	1	6.6	15	4	6.3
Number of rooms at home	3.5	4	NA	3.2	1	3	4
Positive AFB[b]	41	22	100	9	100	38	100
Organ involvement[b]							
Pulmonary involvement	65	89	100	100	100	87	100
Extrapulmonary involvement	32	33	0	55	0	13	25
Outcome[b]							
Treatment completed	91	100	100	73	100	100	100
Lost to follow up	6	0	0	18	0	0	0
Death	3	0	0	9	0	0	0

[a] Based on MIRU-VNTR.
[b] Results expressed in percentages.
AFB = acid fast bacilli; LAM = Latin American Mediterranean; MIRU = mycobacterial interspersed repetitive unit; NA = Not available; VNTR = variable number of tandem repeat.

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Results

Sample stains, patient characteristics, and drug-susceptibility testing

From 2009 to 2012, 34 *M. tuberculosis* complex clinical isolates (MT1—MT34) collected from 32 French Polynesian patients were genotyped. *M. tuberculosis* strains were isolated from respiratory tract specimens, pleural effusion samples, and biopsy specimens (lung scan-guided and lymph node biopsies). Patients lived in 19 different cities on five different islands of three French Polynesian archipelagos; the location of the 34 strains isolated from the 32 patients is reported in Figure 1. Patient demographic characteristics and available clinical data for all the patients are reported in Table 1. All patients were new confirmed cases of tuberculosis according to the WHO classification for TB cases,[18] with the exception of one patient with positive *M. tuberculosis* isolates (MT33 and MT34) cultured in 2011, who was previously treated in 2009 for pulmonary tuberculosis and was considered lost to follow up. The MT33 and MT34 isolates obtained from this patient displayed the same genotype suggesting a relapse. Two young patients (sputum isolates MT11 and MT15) were investigated as contacts of a nearby confirmed case of pulmonary tuberculosis whereas all other individuals were suspected of tuberculosis prior to specimen collection on the basis of clinical and/or radiological signs. A 75-year-old man suffering from lymphoma and treated with chemotherapy died during treatment. For one patient, the MT24 isolate from a cervical lymph node and the MT29 isolate from sputum yielded the same spoligotype and clustered in the same MIRU-VNTR group identified. All strains were susceptible to the four tested antibiotics except the isolate MT31, isolated from a patient from the Marquesas archipelago, which was resistant to streptomycin.

All patients were treated with a first-line antibiotic regimen for tuberculosis (2 rifampin, isoniazid, pyrazinamide, and ethambutol + 4RH (rifampicin + isoniazid) regimen) as recommended by the WHO[18] and French Infectious Diseases Society. None of the patients was co-infected with HIV.

Beijing typing

First-line screening of the isolates by real-time PCR Beijing lineage yielded only one positive isolate (MT22).

Spoligotyping

The 24 isolates were scattered into 12 spoligotypes. Twenty-five strains were scattered into six previously described spoligotypes: H1 (6 strains), H3 (3 strains), U likely S (6 strains), T1 (8 strains), Manu2 (1 strain), and one Beijing strain isolated from an incarcerated patient (MT22). SIT62 (H1 variant), SIT50, and SIT75 (H3 variants) were the dominant clusters. Nine strains exhibited patterns not previously reported in the list of all spoligotype international types, consisting of three isolates—MT8, MT15, and MT21 (spoligotype "A")—two isolates—MT5 and MT17 (spoligotype "B")—and one isolate each—MT9, MT23, MT26, and MT27 (spoligotypes "C—F").

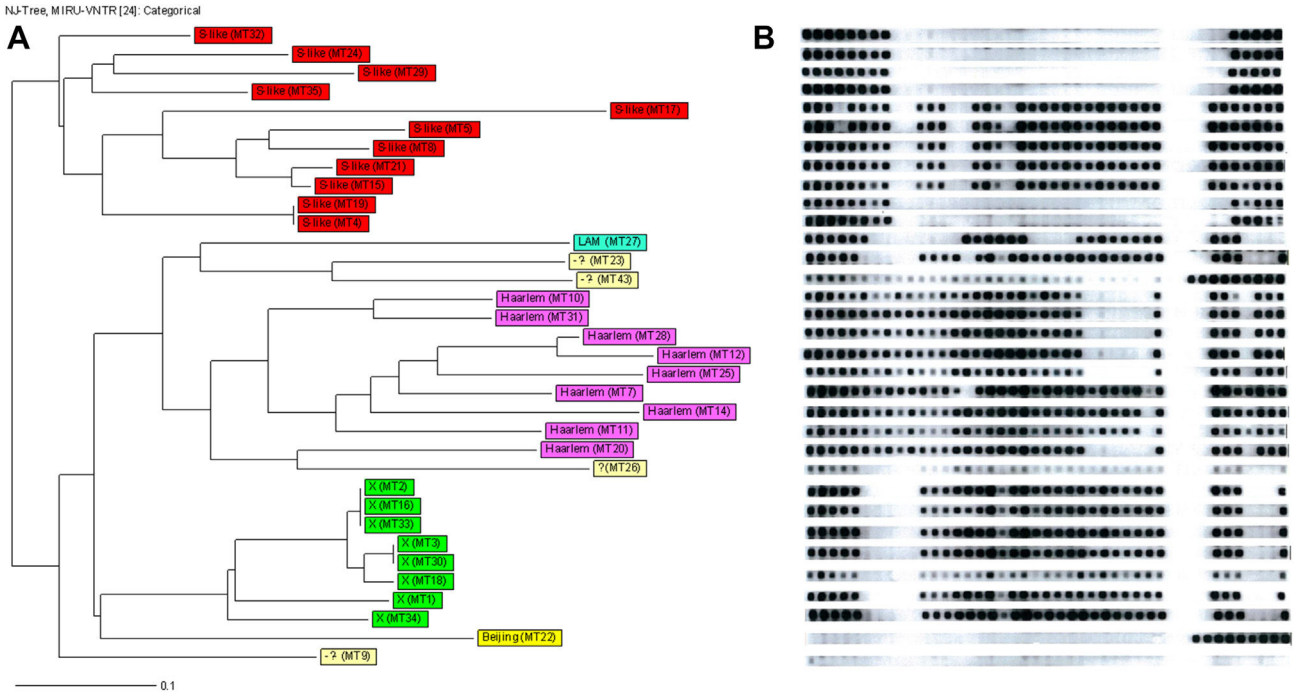

Figure 2. Neighbor-joining (NJ) tree combining of (A) 24-loci MIRU-VNTR and (B) spoligotyping of 34 *Mycobacterium tuberculosis* strains investigated in French Polynesia. Six lineages were identified and are represented by the same colors as in Figure 1. MIRU = mycobacterial interspersed repetitive unit; VNTR = variable number of tandem repeat.

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MIRU-VNTR

The MIRU-VNTR loci of the 34 isolates are reported in Figures 2 and 3.[19] Twenty-nine isolates (85.3%) belonged to the modern Euro–American lineage: 11 isolates were identified as members of the S lineage, one of the Latin American Mediterranean (LAM) lineage, nine isolates of the Haarlem lineage, and eight of the X lineage.

The similarity option of the MIRU-VNTRplus website (http://www.miru-vntrplus.org) confirmed isolate MT22 as a Beijing group B (East Asian lineage).[7]

Tree-based analysis was adopted to enable the assignment of the other isolates to lineages.[19] Eleven isolates were identified as members of the S lineage, one of the LAM lineage, nine of the Haarlem lineage, and eight of the X lineage. Four isolates exhibited a unique profile different from the ones available in the database (February 2015).

Combined spoligotyping and MIRU-VNTR

Overall, 15/34 (44%) isolates genotyped were identified to be similar using MIRU-VNTR and spoligotyping with a 0.84 HGDI.[17]

Discussion

A previous study of 64 *M. tuberculosis* isolates collected in 1991/1992 in French Polynesia found 38 different IS6110 restriction fragment length polymorphism patterns, with 83% of isolates grouped into 11 clusters.[10] These data suggested that, in addition to reactivation of latent tuberculosis, active TB circulation was still ongoing in French Polynesia. Twenty years later, this study indicates that the Euro–American lineage predominates in French Polynesia. We detected only one Beijing isolate in French Polynesia, whereas this lineage comprised 49% of isolates in Kiribati (Micronesia)[7] and 21.9% in Papua New Guinea (Melanesia).[8] The Beijing lineage is also largely represented in the Western Pacific (South East Asia), with 30% of the isolates in Cambodia[5] and 32.5% in Vietnam.[6] Nevertheless, the detection of one Beijing isolate in French Polynesia is in agreement with the global dissemination of the Beijing lineage of *M. tuberculosis*,[20] suggesting a need for increased awareness to limit its spread within the populations. One isolate was identified as belonging to the Manu lineage. The Manu lineage belongs to the Indo-Oceanic family and, along with the East African–Indian family, they are two ancestral families of the principal genetic group 1 characterized by an intact disseminated TbD1 locus.[21,22] In Egypt, the high proportion of this ancestral lineage led the authors to suggest that the Manu lineage could be the missing link between modern and ancestral lineages of *M. tuberculosis*.[23]

In the PICTs, molecular epidemiological data about *M. tuberculosis* are available for Kiribati (Micronesia subregion),[7] Papua New Guinea (Melanesia subregion),[8] and French Polynesia (Polynesia subregion, from this study). In

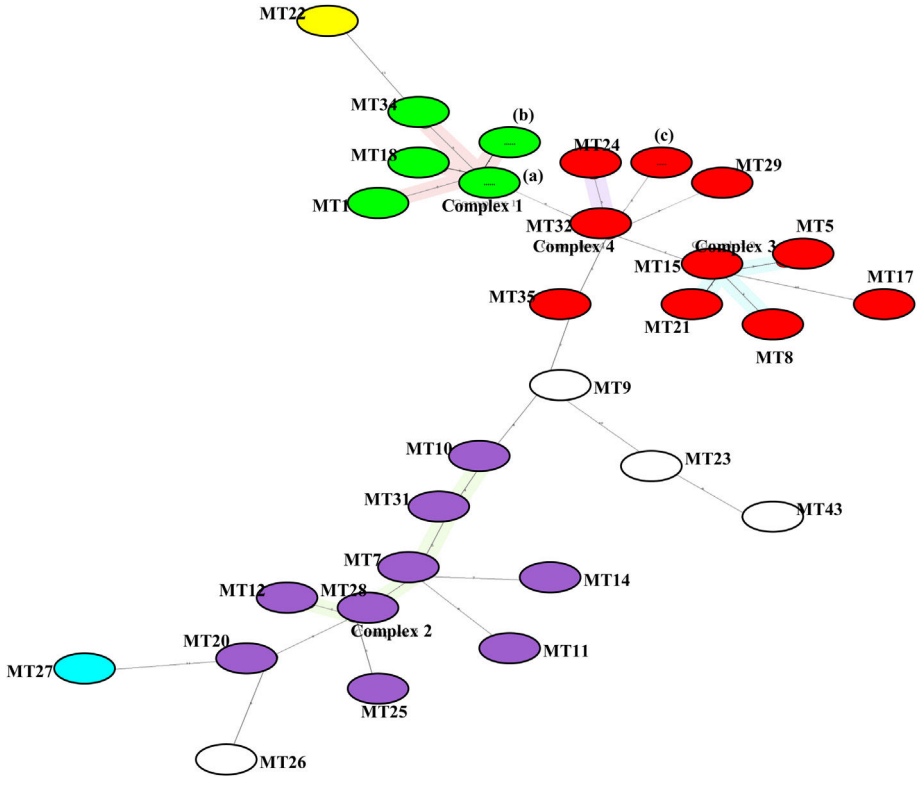

Figure 3. Minimum spanning tree calculated using MIRU-VNTRplus and a maximum locus difference within a clonal complex of five. Circles and color indicate, respectively, strain number and cluster, using the same color code as in Figures 1 and 2. The number between circles indicates distance between two strains. Circle (a) is constituted of three strains (MT2, MT16, and MT33); circles (b) and (c) contain, respectively, two strains: MT3, MT30 and MT4, MT19. MIRU = mycobacterial interspersed repetitive unit; VNTR = variable number of tandem repeat.

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these countries, the Euro—American lineage predominates with 49% of the isolates in Kiribati,[7] 76.9% in Papua New Guinea,[8] and 85.3% in French Polynesia. The Euro—American lineage also predominates in New Zealand (37.8% of the isolates). In New Zealand-born individuals, Maori and New Zealand—European share the same predominant lineages.[24]

In the PICTs, different percentages of the predominant lineage combined with the distribution of other lineages are in accordance with the high diversity among Pacific Islands in terms of population (Polynesian, Melanesian, Micronesia), country size, medical care capacities, socio-economical conditions, lack of global exchanges (French- and English-spoken countries, >1200 languages), and the large geographic area (22 Pacific Island Countries and Territories; approximately 25,000 islands and islets). The difference in the prevalence is principally recorded for the Beijing isolate, which represented 49% of the strains in Kiribati,[7] 2.9% in French Polynesia, and 21.9% in Papua New Guinea.[8]

Epidemiology is different in the Western Pacific (South East Asian) region in which the predominant lineage is the East African—Indian lineage with 59% of the strains in Cambodia[5] and 50.7% in Vietnam (East African—Indian—Vietnam genotype).[6] The East African—Indian lineage also predominates in Australia (28.5% of the isolates).[25]

Overall, these results suggest that two *M. tuberculosis* lineages predominate in the Pacific area, with the Euro—American lineage being the predominant lineage in the PICTs (Melanesia, Micronesia, and Polynesia subregions) and in New Zealand. The East African—Indian lineage is the predominant lineage in the Western Pacific and in Australia.

The low incidence of TB in French Polynesia, combined with the divergence in circulating strains, is consistent with epidemiological patterns encountered in developed countries. Modern and ancestral strains of *M. tuberculosis* are in circulation in French Polynesia. This study also allowed the detection of new spoligotypes that will be further characterized.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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CHAPITRE 3-AVANT-PROPOS

L'identification des espèces de mycobactéries était généralement effectuée par des tests biochimiques fastidieux et l'utilisation des sondes d'acides nucléiques a été ensuite proposée comme un moyen rapide (Shamputa, 2004). Le séquençage du gène de l'ARN ribosomique 16S (ARNr 16S) a émergé comme la meilleure méthode pour identifier rapidement les bactéries (Drancourt, 2000). Cependant cette méthode n'est pas suffisamment résolutive pour différencier les membres du complexe *Mycobacterium tuberculosis* en raison de la forte similarité de leurs séquences 16S rRNA (Warren, 2001). La récente augmentation du nombre de cas de tuberculose et l'émergence de souches multirésistantes ont démontré les faiblesses des techniques utilisées actuellement et souligné le besoin de méthodes plus rapides et plus précises de diagnostic de laboratoire (Organisation Mondiale de la Santé, 2014). Nous avons utilisé une technique protéomique rapide, la spectrométrie de masse couplant une source d'ionisation laser assistée par une matrice Matrix-Assisted Laser Desorption/Ionisation time-of-flight mass spectrometry (MALDI-TOF) (El Kéchine, 2011), pour l'identification d'une collection de 34 isolats cliniques de *M. tuberculosis* en Polynésie-Française.

Le résultat obtenu par profilage MALDI-TOF a permis d'identifier deux nouveaux isolats caractérisés ensuite par une mutation originale sur la séquence du gène 16S rRNA. Ainsi l'isolement et la caractérisation de nouveaux isolats jusqu'à alors non décrits dans cette partie du monde a été possible grâce à la combinaison de différentes méthodes.

Article 3

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Draft Genome Sequence of *Mycobacterium tuberculosis* Strain MT11, Which Represents a New Lineage

Djaltou Aboubaker Osman,[a,b] Michael Phelippeau,[a] Didier Musso,[c] Catherine Robert,[a] Caroline Michelle,[a] Olivier Croce,[a] Michel Drancourt[a]

Aix Marseille Université, URMITE, UMR CNRS 7278, IRD 198, INSERM 1095, Marseille, France[a]; Institut de Recherche Médicinale (IRM), Centre d'Études et de Recherche de Djibouti (CERD), Djibouti, Djibouti[b]; Institut Louis Malardé, Papeete, French Polynesia[c]

We sequenced the genome of *Mycobacterium tuberculosis* strain MT11, which exhibits a specific 16S rRNA gene mutation found in 6% of French Polynesian *M. tuberculosis* isolates. It comprises a 4,110,293-bp chromosome with 65.15% G+C content, and it encodes 3,949 proteins and contains 85 predicted RNA genes. The TbD1 region is absent in strain MT11 as in modern *M. tuberculosis* strains.

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Address correspondence to Michel Drancourt, michel.drancourt@univ-amu.fr.

Pulmonary tuberculosis remains one of most deadly infectious diseases (1). The responsible agent, *Mycobacterium tuberculosis*, is a genetically monomorphic species, owing to its low DNA diversity (2) and clonal evolution (3), but with a host adaptation characterized by specific clones in some geographic areas (4–6). Among 34 *M. tuberculosis* isolates obtained from French Polynesian patients (7), we recently observed two isolates, MT11 and MT14, the peptidic spectra of which were clustered by matrix-assisted laser desorption ionization–time of flight mass spectrometry. Of note, the 16S rRNA gene sequences of both isolates showed 99% sequence similarity to that of *M. tuberculosis* H37Rv (GenBank accession no. AL123456) and a unique mutation at position 1247. This mutation differed from all 16S rRNA genes previously reported in the other *M. tuberculosis* complex members. The partial *rpoB* gene sequences of MT11 and MT14 showed 100% similarity with that of *M. tuberculosis* H37Rv (GenBank accession no. AL123456) (8). Twenty-four mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) genotyping loci (9) and spoligotyping (10) identified the two isolates as belonging to the Haarlem lineage (O. D. Aboubaker, M. Phelippeau, M. Drancourt, and D. Musso, unpublished data). We thought that analyzing the whole-genome sequence of *M. tuberculosis* MT11 would help define the phylogenetic relationships within the *M. tuberculosis* complex and design tools for its advanced detection and identification.

Chromosomal DNA was isolated as previously described (11) and sequenced on the MiSeq Technology (Illumina, Inc., San Diego, CA, USA) through four runs using two mate-pair libraries with insert sizes of 10 and 3.3 kb in a 2 × 250-bp run for each barcoded library. The whole set of reads was trimmed using Trimmomatic (12) and assembled using the assembler software SPAdes (13, 14). Contigs were combined using SSPACE (15) and Opera (16), helped by GapFiller (17), and homemade tools in Python were used to refine the set.

The draft genome of *M. tuberculosis* MT11 consists of 16 contigs without gap containing 4,110,293 bp, which is the second smallest genome among *M. tuberculosis*, and a 65.15% G+C content. Noncoding genes and miscellaneous features were predicted using RNAmmer (18), Aragorn (19), Rfam (20), Pfam (21), and Infernal (22). Coding DNA sequences (CDSs) were predicted using Prodigal (23), and functional annotation was achieved using BLAST+ (24) and HMMER3 (25) against the UniProtKB database (26). The genome was shown to contain at least 85 predicted RNAs, including 3 rRNAs, 51 tRNAs, one transfer-messenger RNA (tmRNA), and 30 miscellaneous RNAs. A total of 3,949 genes were also identified, representing a coding capacity of 3,682,833 bp (89.6% coding density). Among these genes, 281 (7.12%) encode putative proteins, and 542 (13.72%) encode hypothetical proteins. Moreover, 2,776 genes matched a least one sequence in the Clusters of Orthologous Groups (COGs) database (27, 28) using BLASTp default parameters.

Nucleotide sequence accession numbers. The *M. tuberculosis* MT11 strain annotated genome sequence has been deposited at EMBL under the accession numbers CVMX01000001 to CVMX01000016.

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CHAPITRE 4-AVANT-PROPOS

L'analyse de la distribution de 20 régions variables des génomes de 100 souches appartenant au complexe *Mycobacterium tuberculosis*, isolées à partir de différents hôtes et différentes zones géographiques, a montré une délétion spécifique appelée TbD1 chez les souches des lignées Est-asiatique, y compris Beijing, Est-afro-indienne et Euro-américaine (Brosch, 2002). L'étude des souches circulant en Inde a suggéré une prédominance de génotypes «TbD1+» de *M. tuberculosis* dans le sous-continent indien, soutenant ainsi l'hypothèse que l'Inde est un ancien foyer endémique de la tuberculose (Guiterrez, 2006). Une autre étude menée en Egypte a montré une prédominance des souches de *M. tuberculosis* appartenant au génotype Manu «TbD1+». Les auteurs ont émis l'hypothèse que ce génotype pourrait être «un chaînon manquant» dans l'évolution des bacilles tuberculeux entre souches «TbD1+» et souches «TbD1-» (Helal, 2009). Le génotypage des isolats de la Polynésie Française nous a permis de décrire une souche «TbD1+» identique à celles circulant dans le continent sud-est asiatique. Nous avons décrit ainsi la circulation des lignées aussi bien «TbD1+» que «TbD1-» en Polynésie Française.

Article 4

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Draft Genome Sequence of *Mycobacterium tuberculosis* Strain MT43, a Representative of the Manu2 Genotype

Djaltou Aboubaker Osman,[a,b] Michael Phelippeau,[a] Didier Musso,[c] Catherine Robert,[a] Caroline Michelle,[a] Olivier Croce,[a] Michel Drancourt[a]

Aix Marseille Université, URMITE, UMR CNRS 7278, IRD 198, INSERM 1095, Marseille, France[a]; Institut de Recherche Médicinale (IRM), Centre d'Études et de Recherche de Djibouti (CERD), Djibouti, Djibouti[b]; Institut Louis Malardé, Papeete, French Polynesia[c]

We announce the draft genome sequence of *Mycobacterium tuberculosis* strain MT43, isolated from a pulmonary form of tuberculosis in French Polynesia. Analyzing its 4,145,007-bp, 65.17% G+C chromosome confirmed a fully antibiotic-susceptible Manu2 spoligotype.

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Address correspondence to Michel Drancourt, michel.drancourt@univ-amu.fr.

Mycobacterium tuberculosis strain MT43 was isolated from a female 56-year-old French Polynesian patient suffering from pulmonary and pleural tuberculosis (D. Aboubaker Osman, M. Phelippeau, M. Drancourt, and D. Musso, unpublished data). Spoligotyping indicated the spoligotype international type SIT1634 (Manu2), which is one "ancestral" lineage of *M. tuberculosis* comprising only two isolates from the United States, one from Indonesia, and one from Taiwan in the SITVIT database. Recently, at least nine isolates belonging to SIT1634 were described in China, and this spoligotype has been reported to be associated with isoniazid susceptibility testing discrepancies (1). Therefore, we thought that analyzing the whole-genome sequence of *M. tuberculosis* MT43 could help to determine the phylogenetic relationships within the *M. tuberculosis* complex and assess for genetic determinants of antibiotic resistance.

Genomic DNA extracted from *M. tuberculosis* MT43 grown in MGIT Middlebrook liquid culture (Becton Dickinson, Le Pont-de-Claix, France) at 37°C by the cetyl-trimethylammoniumbromide method (2) was sequenced on the Illumina MiSeq platform throughout three runs using 5-kb mate-pair libraries in a 2 × 250-bp run for each barcoded library. The whole set of reads was trimmed using Trimmomatic (3) and assembled with the assembler software Spades (4, 5). Contigs were combined together by SSPACE (6) and Opera (7), with help from GapFiller (8), and refined with homemade tools in Python. Finally, the draft genome of *M. tuberculosis* MT43 strain consists of 15 contigs without gap for a total of 4,145,007 bp and a 65.17% G+C content. Noncoding genes and miscellaneous features were predicted using RNAmmer (9), ARAGORN (10), Rfam (11), Pfam (12), and Infernal (13). Coding DNA sequences (CDSs) were predicted using Prodigal (14), and functional annotation was achieved using BLAST+ (15) and HMMER3 (16) against the UniProtKB database (17).

The genome was shown to encode at least 76 predicted RNAs, including 3 rRNAs, 45 tRNAs, 1 tmRNA, and 27 miscellaneous RNAs. A total of 2,786 genes spanning over 3,722,052 bp were also identified, representing a coding percentage of 89.8%. Among these genes, 274 (6.88%) were assigned as putative proteins and 560 (14.05%) were assigned as hypothetical proteins. Moreover, 2,786 genes matched at least one sequence in the Clusters of Orthologous Groups (COGs) database (18, 19) with BLASTp default parameters. Genomic analysis confirmed a Manu2 spoligotype. To identify known drug-resistance markers, we used the online TB profiler database (http://tbdr.lshtm.ac.uk); no SNPs conferring resistance were found, confirming the *in vitro* data that *M. tuberculosis* strain MT43 is fully susceptible to antibiotics.

Nucleotide sequence accession numbers. The genome sequence of *M. tuberculosis* strain MT43 has been deposited with its annotations at EMBL under the accession numbers CVMY01000001 to CVMY01000015.

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CHAPITRE 5-AVANT-PROPOS

Les mycobactéries non-tuberculeuses sont des organismes environnementaux, trouvées dans les sources d'eau et le sol et responsables d'infections opportunistes. Ces mycobactéries sont responsables de plus en plus de pathologies infectieuses, dont les infections pulmonaires qui sont souvent les plus décrites (Johnson, 2014). Ainsi, comme peu de données étaient disponibles en Polynésie Française sur ces mycobactéries dont l'impact sur la santé publique est non négligeable, nous avons analysé 87 isolats de mycobactéries non-tuberculeuses. La spectrométrie de masse couplant une source d'ionisation laser assistée par une matrice Matrix-Assisted Laser Desorption/Ionisation time-of-flight mass spectrometry (MALDI-TOF) (El Kéchine, 2011) combinée aux amplifications géniques des séquences 16S rRNA, *rpob* et *hsp65*, nous a permis de caractériser 42/87 des isolats comme appartenant au complexe *Mycobacterium fortuitum*, 28/87 dans le complexe *Mycobacterium abscessus*, 8/87 dans le complexe *Mycobacterium mucogenicum* et 5/87 dans le complexe *Mycobacterium avium*. Trois isolats ont été identifiés comme *Mycobacterium acapulcensis, Mycobacterium phocaicum* et *Mycobacterium cosmeticum.*

Un dernier isolat non caractérisé par aucune de trois méthodes est représentatif d'une nouvelle espèce que nous avons nommée *Mycobacterium massilipolynesiensis*.

Article 5

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Revised version

Epidemiology of nontuberculous mycobacteria in French Polynesia

Running title: Nontuberculous mycobacteria, French Polynesia

Michael Phelippeau[1], Aboubaker Osman Djaltou[1], Didier Musso[2] and Michel Drancourt[1] #

1. Aix-Marseille Université, URMITE, UMR CNRS 7278, IRD 198, Inserm 1095, Marseille, France.
2. Pôle de recherche et de veille sur les maladies infectieuses émergentes, Institut Louis Malardé, Tahiti, Polynésie Française

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Nontuberculous mycobacteria, MALDI-TOF-MS, *rpoB,* French Polynesia

Corresponding author : Michel Drancourt

Unité de Recherche sur les Maladies Infectieuses et Tropicales Émergentes,

Faculté de médecine, 27 Boulevard Jean Moulin, 13385 Marseille cedex 05, France

tel : +33 491 385 517, Fax : +33 491 387 772

michel.drancourt@univ-amu.fr

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ABSTRACT

As few data are available in the Pacific countries and territories of the Oceania region regarding nontuberculous mycobacteria, we retrospectively identified 87 such isolates from French Polynesia from 2008 to 2013 by hybridization using DNA strip, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) and partial *rpoB* gene sequencing. Partial *rpoB* gene sequencing classified 42/87 (48.3%) isolates inthe *Mycobacterium fortuitum* complex, 28 (32.2%) in the *Mycobacterium abscessus* complex, 8 (9.2%) in the *Mycobacterium mucogenicum* complex and 5 (5.7%) in the *Mycobacterium avium* complex. Two isolates were identified as *Mycobacterium acapulcensis* and *Mycobacterium* cosmeticum by partial 16S rRNA gene sequencing. One isolate, unidentified by MALDI-TOF-MS and yielding less than 92% and 96% sequence similarity with *rpoB* and *hsp65* reference sequences respectively, was regarded as a potentially new species. Three patients exhibiting ≥ two *Mycobacterium porcinum* isolates and one patient with emphysema and a lung abscess exhibiting two *Mycobacterium senegalense* isolates, fulfilled the American Thoracic Society microbiological criteria for nontuberculosis mycobacterial lung infection. Remote geographic areas such as French Polynesia are potential sources for the discovery of new mycobacteria species.

Revised version

INTRODUCTION

Nontuberculous mycobacteria (NTM) are emerging as opportunistic pathogens worldwide [1]. They comprise several species mainly grouped into the *Mycobacterium avium* [2], *Mycobacterium abscessus* [3] and *Mycobacterium fortuitum* [4] complexes. Hundreds of reports from the five continents address both environmental and clinical NTM. However, NTM continue to be overlooked in some remote areas [5], obscuring the overall understanding of these organisms as well compromising the diagnosis and medical care of exposed patients.

French Polynesia (FP) is a remote French territory located in the South Pacific. It is divided in five archipelagoes, which consists of 118 islands; of them 67 are inhabited (274,000 inhabitants). It is one of the 22 Pacific Island Countries and Territories of the Oceania region. Very little is known about the epidemiology of NTM is this wide region because, due to the lack of laboratory facilities. In these countries, mycobacteria infections diagnosis relies mostly only on acid-fast bacilli analysis of the samples and culture is not routinely performed.

Apart from one case of *M. abscessus* infection [6], this is the first study on NTM in French Polynesia as data previously reported from this country have been limited to the *Mycobacterium tuberculosis* complex [7-11] and leprosy [12].

We herein report the result of retrospective identification of 87 NTM isolates in FP over a 5-year period.

MATERIALS AND METHODS

Patients and ethical statements

The study was conducted between 2008 and 2013 and all the patients were living in FP. This study was approved by the French Polynesia Ethics Committee under reference N°61/CEPF.

Clinical isolates

Clinical samples collected from patients from four archipelagoes (Society Islands, Tuamotu,

Gambier and Marquesas) of FP suspected of pulmonary or urinary tuberculosis were sent to the Institut Louis Malardé, a French Polynesian health and research institute in charge of infectious diseases control and surveillance which receive samples from the whole of French Polynesia. We received no samples from the Austral archipelago inhabited by less than 1.5% of the French Polynesia population. All specimens were received with a confidential medical questionnaire form. Diagnosis criteria for NTM lung disease were those of the American Thoracic Society (ATS)/Infectious Diseases Society of America (IDSA) recommendations [13]: symptomatic patient, radiographic abnormalities, more than 2 positive sputum samples,

The study was conducted on all NTM strains isolated in the Institut Louis Malardé, strains isolated in the other French Polynesian laboratories were not included in this study.

In FP, microscopic examination of the samples was performed by Ziehl-Neelsen staining, and specimens were inoculated onto Löwenstein-Jensen medium (bioMérieux, La Balme-les-Grottes, France) at 37°C in a 5% CO_2 atmosphere. Positive acid-fast bacilli colonies were hybridized using *Mycobacterium tuberculosis* complex probes (AccuProbe *M. tuberculosis* complex, bioMérieux). Colonies which did not hybridize (considered as nontuberculous mycobacteria) were then tentatively identified by a second test (GenoType® *Mycobacterium* CM/AS, HainLifescience, Nehren, Germany). A 87-isolate collection of NTM was then sent to the Mycobacterium Reference Laboratory, at the Institut Hospitalier Universitaire Méditerranée Infection in Marseille, France for molecular and matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) identification. All specimens were subjected to DNA extraction and subcultured on Middlebrook 7H10 agar incubated at 37°C in a 5% CO_2 atmosphere. MALDI-TOF-MS identification was performed on sub-cultured colonies with the Bruker protocol (BrukerDaltonics, Bremen, Germany), as previously described [14, 15]. An isolate was considered to have been identified at the species level when log scores reached ≥1.6 in comparison with the Mycobacteria Bruker Library v2.0 (BrukerDaltonics). All DNA extracts were further identified by partial *rpoB* gene sequencing as

previously described [16], and in the presence of negative controls. Partial *rpoB* gene sequences were compared to those available in the GenBank library (August, 2014) using Blast software (NCBI). An isolate exhibiting > 98.3% partial *rpo*B sequence similarity with that of the same species (type strain) listed in the ten first Blast results was considered as having been identified at the species level [16]. Unidentified isolates were further characterized by PCR-amplification and sequencing the complete 16S rRNA and partial *hsp65* genes sequences as previously described [17]. Sequences were compared with homologous sequences in GenBank using Blast software. Clustal W for multiple alignment and MEGA 5 (Kimura two-parameter protocol) were used to construct phylogenetic trees.

RESULTS

Clinical samples

Eighty-three patients living in four FP archipelagos yielded 87 clinical NTM isolates over a five-year period spanning from October 2008 to May 2013 (Figure 1). These isolates were isolated from sputum specimens (n=64), bronchoalveolar lavages (n=20), gastric aspirates (n=2) and urine (n=1). During the same period, 42 *M. tuberculosis* complex isolates were isolated in the same laboratory [11], the percentage of NTM isolates was 67.4%.

NTM identification

Partial *rpoB* gene sequencing identified 83/87 (95.4%) NTM isolates: 42 (48.3%) in the *M. fortuitum* complex, 28 (32.2%) in the *M. abscessus* complex, eight (9.2%) in the *M. mucogenicum* complex, five (5.7%) in the *M. avium* complex (Table 1), four miscellaneous isolates were identified by 16S rRNA gene sequencing. MALDI-TOF-MS identified correctly 34/58 (58.6) of the sub-cultured isolates. GenoType® *Mycobacterium* CM/AS probes identified correctly 31/87 (35.6%) isolates,

M. fortuitum complex isolates

According to *rpoB* partial gene sequencing the 42 *M. fortuitum* complex isolates comprised of 16

Mycobacterium porcinum, 12 *Mycobacterium senegalense,* 10 *M. fortuitum* subsp. f*ortuitum,* three *M. houstonense* and one *M. mageritense* isolates. GenoType® *Mycobacterium* CM/AS probes identified correctly only 7 *M. fortuitum* among these 42 isolates, all belonged to the *M. fortuitum* subsp. f*ortuitum*. Hybridization erroneously identified 5/12 *M. senegalense*, 2/3 *Mycobacterium houstonense* and 4/16 *M. porcinum* as *M. fortuitum* and one *M. porcinum* as *M. mucogenicum* and failed to identify the other isolates. Twelve *M. senegalense rpoB* partial sequences shared 100% sequence similarity with that of *Mycobacterium conceptionense,* resulting in ambiguous identification. Among sub-cultured isolates, MALDI-TOF-MS identified 9/11 *M. senegalense* isolates, 10/10 *M. fortuitum* isolates, 2/15 *M. porcinum* isolates but none of the three *M. houstonense* and the *M. mageritense* isolates. The three *M. houstonense* isolates were erroneously identified as *M. fortuitum* subps. a*cetamidolyticum* by MALDI-TOF-MS but these species are very closely related and there was only one spectrum of*M. houstonense* in the Bruker Mycobacteria Library v2.0. Surprisingly, only two *M. porcinum* isolates were identified with a ≥1.6 log score. Accordingly, only two *M. porcinum* spectra are available in this commercial MALDI-TOF-MS spectra database.

M. abscessus complex isolates

According to *rpoB* partial gene sequencing, the 28 *M. abscessus* complex isolates comprised 26 (92.8%) *M. abscessus* subp. a*bscessus*, one (3.6%) *Mycobacterium massiliense* and one (3.6%) *M. bolletti* isolates. GenoType® *Mycobacterium* CM/AS probes correctly identified 19/26 (73.1%) isolates, all belonged to the *M. abscessus* subp. *abscessus* species; it erroneously identified one *Mycobacterium massiliense* isolate as *M. abscessus* and failed to identify the *M. bolletti* isolate. Among sub-cultured isolates, MALDI-TOF-MS erroneously identified the *M. massiliense* isolate as *M. abscessus* subp. *abscessus.* Using the standard 3,000-15,000 m/z range [14,15], *M. abscessus* subsp. *abscessus* isolates were identified with a 1.754 +/- 0.11 log-score analyzing the standard 3,000-15,000 m/z range spectrum; significantly lower than the 1.888 +/- 0.12 log-score obtained

using a 2,000-5,000 m/z range (P=0.004; Student's T-test). Fourteen *M. abscessus* subsp. *abscessus* isolates were collected from March to May 2013 from 14 unrelated patients who had undergone bronchoscopy in the same center for lung diseases. No further epidemiological investigation was conducted.

M. mucogenicum complex isolates

According to *rpoB* partial gene sequencing, the eight *M. mucogenicum* complex isolates comprised seven (87.5%) *M. mucogenicum* and one (12.5%) *Mycobacterium phocaicum* isolates. Noteworthy, the 98.72% *rpo*B sequence similarity value observed for isolates in this *M. mucogenicum* complex was significantly lower than the 99.71% value for isolates identified in the other NTM complexes (P=0.01, Student's T-test). GenoType® *Mycobacterium* CM/AS probes identified only three *M. mucogenicum* isolates and erroneously identified one *M. phocaicum* isolate as *M. mucogenicum*. The only-sub-cultured isolate was not identified by MALDI-TOF-MS.

M. avium complex isolates

According to *rpoB* partial gene sequencing, the five *M. avium* complex isolates comprised three (60%) *Mycobacterium chimaera* one (20%) *Mycobacterium avium* and one (20%) *Mycobacterium intracellulare* isolates. Two *Mycobacterium chimaera* isolates were erroneously identified as *Mycobacterium intracellulare* by the GenoType® *Mycobacterium* CM/AS probes (Table 1). MALDI-TOF-MS failed to identify all sub-cultured isolates.

Miscellaneous NTM isolates

Four isolates, MT25, MT26, MT50 and MT80, were not identified at species level using *rpoB* gene sequencing and MALDI-TOF-MS (Table 2). Isolate MT25 was finally identified as *Mycobacterium cosmeticum*, exhibiting 93.52% *rpoB* sequence similarity with that of *Mycobacterium farcinogenes*

NCTC 10955^T (GenBank:AY262742), 100% 16S rRNA gene sequence similarity and 99.55% *hsp*65 sequence similarity with that of *M. cosmeticum* LTA-388^T (GenBank:NR025787 and AY449730, respectively). The urine isolate MT50 was finally identified as *M. phocaicum*, exhibiting 98.24% *rpoB* sequence similarity, 99.56% 16S rRNA gene sequence similarity and 99.09% *hsp65* gene sequence similarity with that of *M. phocaicum* CIP 108542^T (GenBank:AY859693, AY859682 and AY859676, respectively). Isolate MT80 was finally identified as *Mycobacterium acapulcensis*, exhibiting 100% 16S rRNA gene sequencing similarity with that of *M. acapulcensis* ATCC14473^T (GenBank:AF480575) but 98.05% *rpo*B sequence similarity and 99.29% *hsp*65 sequence similarity with that of *Mycobacterium flavescens* CIP 104533^T (GenBank:AY859698 and AF547831, respectively). At the time of the study, no *M. acapulcensis rpoB* and *hsp65* sequences were available in the GenBank database. Isolate MT26 remained unidentified, exhibiting 91.47% *rpoB* gene sequence similarity with that of *Mycobacterium rhodesiae* CIP 106806^T (GenBank: EU109302), 98.93% 16S rRNA gene sequence similarity with that of *Mycobacterium smegmatis* ATCC 19420^T (GenBank: NR115233) and 95.59% *hsp65* gene sequence similarity with that of *M. conceptionense* CIP 108544^T (GenBank:AY859678). Isolate MT26, exhibiting yellow-colored colonies, could not be identified as being phylogenetically related to *M. flavescens* and *M. rhodesiae* (Figure 2). MT26 was, therefore, regarded as being representative of a potential new species.

Phylogenetic analyses confirmed the identification of isolates MT25, MT50 and MT 80 as *M. cosmeticum*, *M. phocaicum* and *M. acapulcensis* respectively (Figure 2).

Clinical relevance of NTM in French Polynesia

Mean age and sex ratio for patient with NTM isolates are reported in Table 3. Based on ATS)/IDSA recommendations [13], 4/83 (4.8%) patients were regarded as presenting a NTM respiratory tract infection (microbiological and clinical criteria) and 78/83 (94%) patients were regarded as suffering from colonization of the respiratory tract. One female patient yielded a unique urinary isolate and could not be classified as having infection or colonization because there are no

agreed upon criteria for NTM urinary tract disease. *M. porcinum* isolates were isolated throughout three archipelagos of FP from the sputum samples of patients aged from 31 to 74. From three patients aged around 40 (two women and a man living in two different islands), *M. porcinum* was isolated in at least two consecutive sputum specimens and these patients fulfilled ATS/IDSA criteria for *M. porcinum* respiratory infection. From a fourth 74-year-old patient suffering for emphysema and chronic obstructive lung insufficiency, *M. senegalense* was isolated from two separated sputum specimens, while a computed tomography (CT) scan identified a necrotic abscess in the lower lobe of the right lung. This patient also fulfilled the ATS/IDSA criteria for *M. senegalense* respiratory infection. Clinical status improved, while the two-month CT monitoring scan showed that the abscess had regressed, after the patient had been treated with amoxicillin-clavulanate plus levofloxacin for three weeks.

DISCUSSION

In the case of a NTM infection, a precise NTM identification is useful to guide the therapeutic options [13]. Here, the CM/AS hybridization probes identified correctly only 31/87 (35.6%) NTM isolates at the species level. In particular, this assay lacked sensitivity and specificity in the *M. fortuitum* complex with only 7/42 (16.7%) strains correctly identified when compared with partial *rpoB* gene sequencing. Erroneous NTM identification using commercial hybridization tests have been previously reported, especially for less frequently encountered species [18]. Further, MALDI-TOF-MS identified only 34/58 (58.6%) of NTM isolates studied. This is due in part to an incomplete database which comprises only 2.4 +/- 2.6 spectra per species. Nevertheless, there was no statistical correlation between the identification rate and the number of spectra per species in the Bruker Mycobacteria Library (P=0.36; Pearson's correlation). Interestingly, *M. abscessus* complex isolates exhibited a higher log-score analyzing the spectrum through a 2,000-5,000 m/z range rather than the standard 3,000-15,000 m/z range [14,15]. This observation had been made previously in a study

identifying three sub-species included in the *M. abscessus* complex, *M. abscessus*, *M. bolletii* and *M. massiliense* [19]. The most accurate identification tool was partial *rpoB* gene sequencing that firmly identified 83/87 (95.4%) NTM isolates at the species level. However, we observed that this molecular tool failed to identify isolates in the *M. fortuitum* group. Indeed, we observed that *M. senegalense* CIP 104941^T and *M. conceptionense* CIP 108544^T [4], which were used as references, unexpectedly shared 100% *rpoB* sequence homology, resulting in ambiguous identification of 12 isolates. The current confusion in *rpo*B-based delineation of both taxa is responsible for difficulties in the *rpo*B-based identification we observed. Resolving this issue was obviously not in the spectrum of the present study but warrants further analyses including complete genome sequencing of reference type strains. Altogether, these data suggest using MALDI-TOF-MS and an expanded database as a first line identification tool and partial *rpo*B sequencing as the second line tool for isolates non-identified by the MALDI-TOF-MS. We would not recommend the use of CM/AS in French Polynesia. Additional sequencing and whole genome sequencing could be used to resolve unusual isolates which could be representative of new species.

The prevalence of NTM (67.4%) among identified mycobacteria is high in our study but the prevalence of NTM infections in Oceania is unknown. In Asia, similar prevalence has been reported in Taiwan in 2007 (45.8%) [20], in a region in which the prevalence of NTM infections is increasing [21].

In this study, the majority of isolates belonged to the *M. fortuitum* complex. Indeed, *M. porcinum* has mainly been reported in water supplies in tropical or subtropical areas in the USA [22, 23], Iran [24], Brazil [25] and Mexico [26]. We report *M. porcinum* isolates identified in three patients with *M. porcinum* lung infection according to the ATS criteria [13], which is consistent with other cases reported previously, particularly in sub-tropical areas such as Texas and Florida, where clonal dissemination was observed, particularly through water systems [22,27]. Here, the source of *M. porcinum* isolates was not traced in the three archipelagos of FP because NTM identification was

performed retrospectively.

As for the *M. abscessus* complex, it comprised 14 *M. abscessus* isolates from respiratory tract specimens in 2013, in addition to 12 *M. abscessus* strains isolated over the three preceding years. In fact, these 14 *M. abscessus* strains were isolated from 14 patients who all underwent bronchoscopy in the same medical center, where an additional isolate was made from a sink in the center. The investigation concluded that the pseudo-epidemic was due to contamination of a bronchoscope by tap water which had been used to clean it. Similarly, *M. abscessus*, an intra-amoeba mycobacterium [28], has been found in the drinking water supply in tropical areas of the Northern Australia [29].

We observed one *M. senegalense* lung abscess case, according to the ATS/ISDA criteria for NTM lung infection [13]. *M. senegalense*, the agent of bovine farcy [30], is rarely involved in human soft tissue infections [31] and has possibly been implicated in pulmonary infections in Texas [32]. *M. senegalense* is usually susceptible to ciprofloxacin [32, 33] and the patient reported here was cured by a three-week regimen including a fluoroquinolone antibiotic. This is the second case which reports the successful treatment of a *M. senegalense* human infection using a fluoroquinolone antibiotic [33].

We report the first description of *M. phocaicum,* isolated from the urine of a 39-year-old woman presenting with polyuria in 2009, which was not resolved after consecutive antibiotic therapies. Because this species has usually been isolated from sputum [34], it is an original clinical presentation.

Finally, *M. acapulcensis* was isolated on one occasion in sputum collected from a patient living in a central atoll in the Tuamotu archipelago. This very rarely described species followed the seminal description of strains isolated from the sputum of patients with history of pulmonary tuberculosis [35]. The *M. acapulcensis* ATCC 14473^{T} 16S rRNA gene sequence (GenBank:AF480575) was deposited in 2002 but no other gene sequences were available at the time of this study. This work highlights the ability of *M. acapulcensis* to be isolated particularly from respiratory tract specimens from people living in the South Pacific area.

263 **CONCLUSIONS**

264 Over a five-year period, 87 NTM strains isolated from clinical specimens of patients living in
265 FP were identified at the species level. With the exception of one case report of *M. abscessus*
266 pneumonia [6], this is the first report of NTM in FP. Previous reports dealt with *M. tuberculosis* [7-
267 11]. This study led to discover that *M. fortuitum* complex was the most prevalent NTM in this part of
268 the world. This observation warrants further environmental and clinical investigations to precise the
269 sources of contamination and the clinical relevance of these NTM. Furthermore, the present study led
270 to the discovery of a potential new NTM species which remains to be formally described on the basis
271 of its whole genome sequence. Microbiological studies conducted in remote areas may lead to the
272 discovery of new taxa in bacteriology, and new clinical entities due to known taxa.
273

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Revised version

388 **Acknowledgments**

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390 et Emergentes (URMITE), Marseille, France.

Revised version

Figure legends:

Figure 1. Geographical distribution of the 87 nontuberculous mycobacterial isolates made in French Polynesia within the 2008–2013 period.

Legend: MABSC, *Mycobacterium abscessus* complex; FORT, *Mycobacterium fortuitum* complex; MAC, *Mycobacterium avium* complex; MUCO, *Mycobacterium mucogenicum* complex; and OTHER, other nontuberculous mycobacteria.

Figure 2. Phylogenetic tree based of the four *rpoB* gene sequences of non identified nontuberculous mycobacteria collected in French Polynesia from 2008 to 2013, constructed using the neighbor-joining method (bootstrapped 1000 times) and Kimura's two parameters distance correction model. Bootstraps values above 90% are given at nodes. *Mycobacterium bovis* strain BCG Pasteur was used as an outgroup. The scale bar represents 2% difference in nucleotide sequences. GenBank accession numbers are given into parenthesis.

Revised version

404 **Table 1.** Partial *rpoB* gene sequencing identification of 83 NTM isolates in French Polynesia.

Complex	*rpoB* identifications (number of isolates)	Correct CM/AS identifications	*rpoB*-amplicon length	Reference type strain	% Similarity
M. fortuitum	*M. fortuitum* complex (42)	7			
	M. porcinum (16)	0	723	*M. porcinum* CIP 105392^T	99.98
	M. senegalense (12)	0	721	*M. senegalense* ATCC 35796^T	99.53
	M. fortuitum (10)	7	726	*M. fortuitum* subsp. *fortuitum* ATCC 6841^T	99.71
	M. houstonense (3)	0	744	*M. houstonense* ATCC 49403^T	99.82
	M. mageritense (1)	0	704	*M. mageritense* ATCC 700351^T	99.15
M. abscessus	*M. abscessus* complex (28)	19			
	M. abscessus (26)	19	730	*M. abscessus* subp. *abscessus* CIP 104536^T	100
	M. bolletii (1)	0	734	*M. bolletii* CIP 108541^T	99.72
	M. massiliense (1)	0	732	*M. massiliense* CCUG 48898^T	99.73
M. mucogenicum	*M. mucogenicum* complex (8)	3			
	M. mucogenicum (7)	3	722	*M. mucogenicum* ATCC 49650^T (n=6) and 49651^T (n=1)	98.83
	M. phocaicum (1)	0	695	*M. phocaicum* CIP 108542^T	98.6
M. avium	*M. avium* complex (5)	2			
	M. chimaera (3)	0	728	*M. chimaera* CIP 107892^T	100
	M. avium (1)	1	725	*M. avium* subp. *avium* ATCC 25291^T	99.72
	M. intracellulare (1)	1	739	*M. intracellulare* ATCC 13950^T	99.32

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Revised version

407 **Table 2.** MALDI-TOF mass spectrometry identification results (analysis comprised comparison to the Bruker Mycobacteria Library

408 v2.0 through the 2,000—5,000 m/z and 3,000—15,000 m/z ranges) into the 62 isolates group that subcultured.

Complex	Species	mean log scores through the 3,000 -15,000 m/z range (correct MALDI-TOF identifications / number of MALDI-TOF profiles)	mean log scores through the 2,000 - 5,000 m/z range (correct MALDI-TOF identifications / number of MALDI-TOF profiles	Log scores' comparison into correctly identified, *P*= (Student's T-test)
M. fortuitum	*M. senegalense*	1.756 (9/11)	1.486 (1/11)	0.002
	M. fortuitum	1.919 (10/10)	1.69 (7/10)	0.3
	M. houstonense	1.653 (0/3)	1.645 (0/3)	ND[a]
	M. porcinum	1.472 (2/15)	1.536 (1/15)	0.6
	M. mageritense	1.311(0/1)	1.486 (0/1)	ND
M. abscessus	*M. abscessus*	1.754 (13/13)	1.888 (13/13)	0.004
	M. bolletii	ND	ND	ND
	M. massiliense	1.672 (0/1)	1.857 (0/1)	ND
M. mucogenicum	*M. mucogenicum*	1.287 (0/1)	1.423 (0/1)	ND
	M. phocaicum	ND	ND	ND
M. avium	*M. avium*	ND	ND	ND
	M. chimaera	1.327 (0/3)	1.331 (0/3)	ND
	M. intracellulare	1.164 (0/1)	1.248 (0/1)	ND
Other	M25 (*M. cosmeticum*)	1.237 (0/1)	1.458 (0/1)	ND
	M26 ('*M. massilipolynesisensis*')	No comparative profile	No comparative profile	ND
	M80 (*M. acapulcensis*)	No comparative profile	No comparative profile	ND

[a] Not determined

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412 **Table 3.** Mean age and sex ratio for patients with NTM isolates, French Polynesia.
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Complex	Mean age	Sex ratio male/female
M. fortuitum	48.12 +/- 17.7	22/20
M. abscessus	49.46 +/- 11.73	13/15
M. mucogenicum	59.5 +/- 10	1/7
M. avium	57.8 +/- 9.04	3/2

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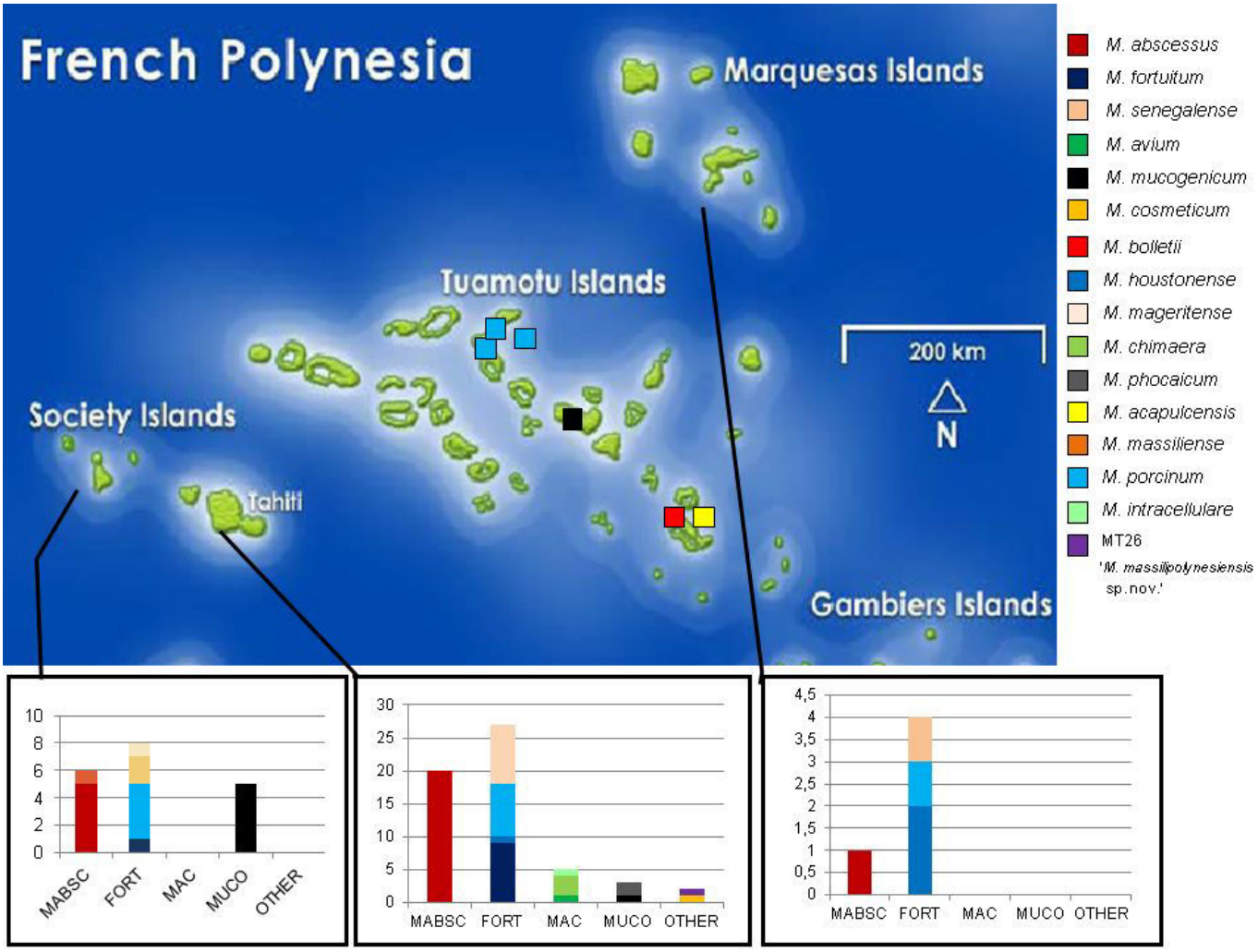

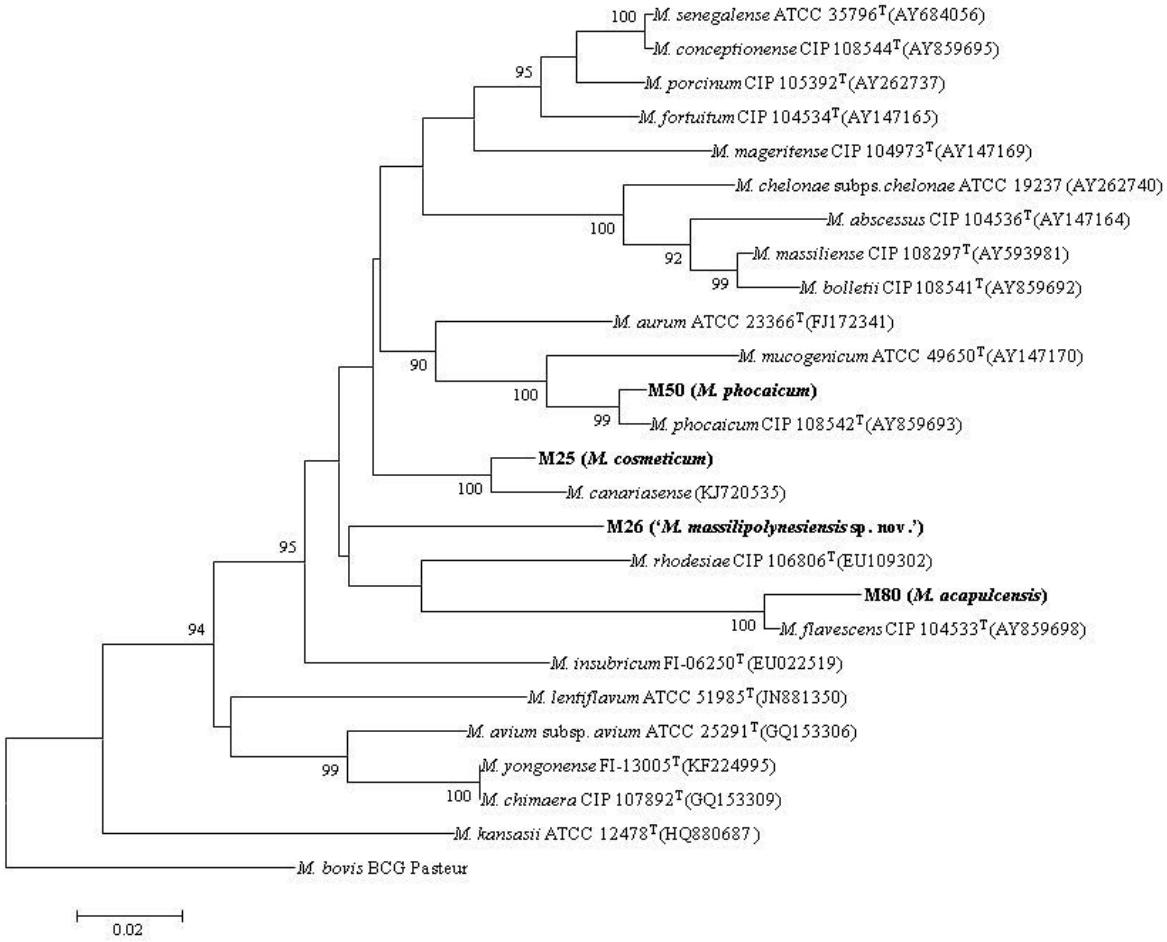

CHAPITRE 6-AVANT-PROPOS

La spectrométrie de masse couplant une source d'ionisation laser assistée par une matrice Matrix-Assisted Laser Desorption/Ionisation time-of-flight mass spectrometry (MALDI-TOF) (El Kéchine, 2011) combinée aux amplifications géniques des séquences 16S rRNA, *rpob* et *hsp65* n'a pas permis de caractériser un isolat M26 dans la collection de 87 isolats réalisée en Polynésie Française. En effet, M26 présente 91.47% de pourcentage de similarité dans sa séquence *rpoB* avec *Mycobacterium rhodesiae* CIP106806^{T} (GenBank: EU109302) et 95.59% de pourcentage de similarité dans sa séquence *hsp65* avec *Mycobacterium conceptionense* CIP108544^{T} (GenBank: AY859678) Nous avons nommé ce dernier *Mycobacterium massilipolynesiensis* représentatif d'une nouvelle espèce.

Article 6

Manuscrit en préparation

Mycobacterium massilipolynesiensis sp.nov., a rapidly-growing mycobacteria in French Polynesia

Michael Phelippeau, Shady Asmar, Olivier Croce, Catherine Robert,

Aboubaker Osman Djaltou, Didier Musso and Michel Drancourt

CHAPITRE 7-AVANT-PROPOS

Les bacilles tuberculeux humains lisses, dont "*M. canettii*" sont régulièrement isolés chez les patients suspects de tuberculose et exposés à la République de Djibouti et les pays limitrophes formant la «Corne de l'Afrique» (van Soolingen, 1997, Koeck, 2011). Ces mycobactéries se caractérisent par une croissance rapide et une production des colonies lisses, brillantes (van Soolingen, 1997). Le temps de génération de So93, la première souche bien caractérisée est de 17 heures ce qui est significativement plus court (P <0,0001) que le temps de génération de *M. tuberculosis stricto sensu* (24 heures) et de *Mycobacterium bovis* (20 heures) (van Soolingen, 1997). Cet aspect a été confirmé pour cinq souches représentatives de ce groupe par des tests de croissance en milieux liquides et en milieux solides (Supply, 2013). L'absence de transmission interhumaine suggère un réservoir dans l'environnement mais qui est inconnu pour le moment (Koeck 2011, Blouin, 2014). Les données cliniques obtenues dans notre revue ainsi que quelques données expérimentales non-publiées suggèrent l'eau potable et de la nourriture contaminés comme des sources potentielles d'infection (Aboubaker Osman et al., 2015 soumis pour publication).

Cette hypothèse soulève la question de savoir si ces bacilles pourraient être inactivés par la chaleur qui est le procédé d'inactivation des agents pathogènes dans les produits d'origine alimentaire et de prévenir ainsi les infections (Silva, 2014; Pflug, 2000). Il n'existe pas des données disponibles concernant cette question. Nous avons donc étudié la tolérance thermique de trois souches de '*M.canettii*'. Nous avons pour cela mis en place un protocole pour mesurer la viabilité de ces mycobactéries soumis à une gamme de températures et différents temps d'exposition.

Chapitre 7

Manuscrit en préparation

Dry heat inactivation of '*Mycobacterium canettii*'

Djaltou ABOUBAKER OSMAN[1, 2], Eric GARNOTEL[3], Michel DRANCOURT[1],

[1] Aix Marseille Université, URMITE, UMR CNRS 7278, IRD 198, INSERM 1095. Faculté

Médecine, Marseille 13005, France

[2]Institut de Recherche Médicinale (IRM), Centre d'études et de Recherche de Djibouti (CERD), Djibouti

[3]Hopital d'Instruction des Armées LAVERAN, 34 Boulevard Laveran 13384 MARSEILLE

Les bacilles tuberculeux lisses, y compris *"Mycobacterium canettii"* sont régulièrement isolés chez les patients suspects de tuberculose et exposés à la République de Djibouti et les pays voisins dans la Corne de l'Afrique. (van Soolingen, 1997, Koeck, 2011). Les bacilles tuberculeux lisses se caractérisent par une croissance rapide et des colonies lisses (van Soolingen, 1997). Le temps de génération de la souche So93 *"M. canettii"*, le premier isolat bien caractérisé, est de 17 heures, ce qui est nettement plus court que le temps de génération de *Mycobacterium tuberculosis* et *Mycobacterium bovis* (van Soolingen, 1997). Cet aspect a été confirmé pour cinq souches représentatives de bacilles tuberculeux lisses par des essais de croissance à la fois dans les milieux liquides et les milieux solides (Supply, 2013). De telles caractéristiques sont rares au sein du complexe M. *tuberculosis* (CMT). A ce jour aucune transmission interhumaine n'a été observée pour ces souches lisses, un réservoir inconnu dans l'environnement a été suggéré (Koeck 2011, Blouin, 2014). Les données cliniques obtenues avec les quelques données expérimentales suggèrent l'eau potable et la nourriture contaminées comme des sources potentielles (Aboubaker Osman 2015 Soumis).

Cette hypothèse soulève la question de savoir si les souches lisses sont inactivées par la chaleur qui est le procédé utilisé habituellement pour l'inactivation des agents pathogènes dans les aliments et de prévenir les maladies d'origine alimentaire (Silva, 2014; Pflug, 2000). Il n'existe pas de données concernant cette question. Nous avons donc étudié la tolérance thermique des souches lisses et nous avons mis en place un protocole pour mesurer la viabilité de ces mycobactéries soumis à une gamme de températures et de temps d'exposition. Nous avons utilisé pour ce faire, la souche de référence *"M. canettii"* CIPT140010059, la souche de référence *M. tuberculosis* H37Rv et deux isolats cliniques de bacille tuberculeux lisses dans cette étude. Un séquençage partiel des gènes *gyrB* et *ropB* comme décrit précédemment (ADEKAMBI, 2005, Goh, 2006) a permis de confirmer l'identification des isolats. Les mycobactéries ont été mises en culture dans un bouillon Middlebrook 7H9 (Becton Dickinson, Le Pont-de-Claix, France) complémenté avec l'acide oléique-albumine-dextrose-catalase (OADC) (Becton Dickinson) pendant deux semaines pour *M. tuberculosis* H37Rv et six jours pour les souches avec une incubation à 37°C sous atmosphère à 5% de CO_2.

Les mycobactéries ont été calibrées à une concentration finale de 10^5 mycobactéries/mL dans du tampon phosphate salin (PBS) stérile avec le turbidimètre Biolog (Biolog, Hayward CA, USA) à 590 nm. Pour éviter les agrégats de colonies, les bactéries ont été dispersées sept fois avec une aiguille de calibre 26 stérile fixée à une seringue de 1 mL (Becton Dickinson). Un volume de 0,1 mL de cette suspension mycobactérienne a été mélangée avec 0,9 mL de bouillon Middlebrook 7H9 enrichi avec de l'OADC à température ambiante. Chaque microtube a été placé dans un système de bloc chauffant (Grant Instruments, Cambridge, Ltd, Angleterre) dans une plage de températures de 25°C à 75°C avec une incrémentation de 10 degrés. Quatre durées d'exposition (15, 30, 60 et 90 min) ont été testées pour chaque température, en duplicate. A la fin du temps d'exposition à la chaleur, 0,1 mL de la suspension chauffée a été immédiatement ensemencé sur agar Middlebrook 7H10 (Becton Dickinson) supplémenté en OADC et le reste a été conservé à 4°C. La présence de mycobactéries viables a été déterminée par comptage des colonies de mycobactéries formées sur gélose Middlebrook 7H10 après deux dilutions en série et incubation pendant six semaines à 37°C sous une atmosphère à 5%de CO_2.

Les mycobactéries ont été confirmées par la coloration de Ziehl-Neelsen et la spectrométrie de masse masse couplant une source d'ionisation laser assistée par une matrice (MALDI-TOF) (El Kéchine, 2011).

Pour étudier l'inactivation thermique des mycobactéries dans les aliments, nous avons utilisé des cylindres d'agar (Bellara, 1999) et le lait en suivant les modèles publiés (Alpas, 2000). Les cylindres de gélose ont été préparés et inoculés à l'aide d'une seringue 50 mL (Becton Dickinson). Un volume de 150 mL d'agar 7H10 (Becton Dickinson) à 50°C a été inoculé avec 1,5 mL d'une suspension de mycobactérie à 10^4/mL et 50 mL de ce mélange ont été rapidement versés dans la seringue. Les expériences d'inactivation thermique ont été effectuées avec les cylindres de gélose préparées comme ci-dessus. Les seringues ont été chauffées à 15, 30, 60 et 90 min d'exposition en les immergeant dans un bain d'eau circulant (Memmert GmbH, Schwabach, Allemagne) fixé à 70°C. La seringue a été maintenue à la température ambiante pour permettre à la gélose de se solidifier afin d'être découpée en portion de diamètre uniforme. Les tranches ainsi ont été incubées à 37°C sous une atmosphère à 5% de CO_2 pendant six semaines. Toutes les expériences d'inactivation thermique ont été dupliquées.

Un microscope à fluorescence MZ-FLIII (Leica, Nanterre, France) équipé d'un filtre Green Fluorescent Protein (GFP) et une caméra numérique ICA (Leica) a été utilisé pour détecter les colonies de mycobactéries autofluorescentes (Ghodhbane, 2014; Patino, 2008). Un volume de 0,9 mL d'un lait pasteurisé a été inoculé avec 0,1 mL d'une suspension mycobactériene à 10^4/mL et chauffé dans un bain sec (Grant Instruments) à 70°C pendant 15, 30, 60 et 90 min d'exposition. Un volume de 0,1 mL a été ensemencé immédiatement sur de la gélose Middlebrook 7H10 (Becton Dickinson) supplémenté avec de l'OADC et le reste a été conservé à 4°C à la fin de chaque temps de traitement thermique. Les colonies viables ont été comptées comme ci-dessus.

Toutes les expériences ont été répétées deux fois. Les données ont été analysées avec le test ANOVA en utilisant le logiciel en ligne Biosta TGV (UPMC, Paris, France) pour déterminer s'il y avait des différences significatives ($P < 0,05$) dans les valeurs moyennes des unités formant colonies (UFC).

Le nombre de bactéries viables de souches lisses testées indique que ces bactéries sont thermiquement tolérantes entre 25°C et 45°C après 90 minutes d'exposition mais inactivées pour des températures ≥ 55°C et en temps d'exposition ≥ 15 minutes.

Ces résultats préliminaires indiquent que les souches lisses du complexe *M. tuberculosis* peuvent survivre jusqu'à 45°C ce qui est compatible avec un réservoir environnemental dans certaines régions de la Corne de l'Afrique, compte tenu des températures observées dans ces régions du monde où la température peut excéder 45°C. Par ailleurs, ces données préliminaires indiquent que les aliments cuits ne constituent très probablement pas une source de contamination pour les hommes.

Mots clés**: "*Mycobacterium canettii*", bacilles tuberculeux lisses, tolérance thermique, aliments, réservoir environnemental.**

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CONCLUSIONS ET PERSPECTIVES

Dans notre travail nous avons décrit les mycobactéries du complexe *Mycobacterium tuberculosis* et les mycobactéries non-tuberculeuses qui circulent en Polynésie Française. L'utilisation de différentes techniques nous a permis d'identifier des souches qui n'étaient pas décrites jusqu'à aujourd'hui. Cependant nous n'avons pas pu identifier de souche lisse du complexe *Mycobacterium tuberculosis*. Les souches lisses du bacille tuberculeux humain constituent une singularité au sein du complexe *M. tuberculosis.* D'un point de vue épidémiologique, les cas de tuberculose dus à cette mycobactérie sont marginaux. Sa restriction géographique à la Corne de l'Afrique vient renforcer cet aspect. Cependant, les trois souches séminales ont été isolées en dehors de cette région. Comment expliquer cette répartition géographique? L'absence de description par G. Canetti des premières souches a laissé place à de nombreuses hypothèses. En effet, les enquêtes auprès de la population Djiboutienne n'est pas assez exhaustive pour tenter d'expliquer pourquoi les expatriés sont les plus touchés par cette forme de la tuberculose. A Djibouti, les patients sont pris en charge par deux structures dont les moyens de prise en charge ne sont pas comparables.

L'Hôpital Médico-chirurgical (HMC) Bouffard français assure la santé des forces Françaises à Djibouti ainsi que leurs familles, celle des forces armées Djiboutiennes et une frange de la population dont les moyens permettent d'être pris en charge; tandis que l'Hôpital Dr Chakib (anciennement Paul Faure) prend en charge tous les patients indistinctement. Dans ce dernier, le diagnostic de la tuberculose repose essentiellement sur les signes cliniques et l'examen microscopique du crachat après coloration de Ziehl-Nielseen. Or l'aspect des bacilles des souches lisses après une coloration au Ziehl-Nielseen est différent de celui de *M. tuberculosis stricto sensu* qui présente lui un aspect cordé. Ceci constitue un premier biais dans l'échantillonnage. Même les renforcements des capacités de l'Hôpital Dr Chakib avec l'installation d'un laboratoire P3 et la dotation en équipements *ad hoc* ne permettent pas d'effectuer la culture des mycobactéries en routine pour tous les patients. Alors que pour le HMC Bouffard la situation est tout autre. Ainsi la difficulté de prendre en charge tous les patients est un biais dans l'échantillonnage qui pourrait être à l'origine d'une sous-estimation des cas dans la population Djiboutienne. De plus, la tuberculose étant une maladie non immunisante, les patients Djiboutiens qui sont le plus en contact avec ce pathogène devraient développer la maladie.

Il est important de mettre en place une enquête épidémiologique auprès de la population Djiboutienne, même si les conditions de réalisation ne sont pas toutes réunies (absence de système informatisé,…) pour comprendre au mieux cette maladie.

Dans ce cadre, l'utilisation des écouvillons pour prélever les selles pour une recherche de mycobactéries pourrait constituer un moyen simple qui doit être évalué (Bonnave, 2003; El Kéchine, 2009).

Les souches lisses sont également exceptionnelles de part leur aspect génotypique. Contrairement à *M. tuberculosis,* les souches lisses sont caractérisées par une diversité génotypique. Ceci les place comme des 'outgroup' au sein du CMT. Leur possible réservoir environnemental est appuyé par l'étude de la structure de leurs génomes. En effet, les souches sont décrites comme des mycobactéries échangeant du matériel génétique avec d'autres pathogènes inconnus. Une forte présomption de ce réservoir est l'eau. Ainsi, une piste de recherche est l'échantillonnage de l'eau à Djibouti pour détecter par des techniques moléculaires la présence de ces pathogènes. Nous avons dans ce but et grâce à la disponibilité des génomes, mis au point une PCR quantitative. Si la présence est détectée, l'étape suivante sera d'isoler les souches lisses notamment par des co-cultures amibiennes.

Il serait en parallèle intéressant d'étudier la flore présente dans l'eau pour expliquer la présence des fragments exogènes chez les souches lisses. En plus de l'eau, nous avons également avancé au cours de notre travail que les aliments pourraient également constituer une source de contamination. A l'exception des produits de la pêche, la majorité de l'alimentation est importée à Djibouti notamment en provenance de l'Ethiopie pour les fruits, les légumes et le bétail. En se basant sur les enquêtes, des aliments peuvent être ciblés pour une recherche des souches lisses. Au regard des données obtenues, un premier travail crucial est la collecte des données cliniques exhaustives à Djibouti. Sur cette base, des sites et de population pour l'échantillonnage peuvent être ciblés. Ainsi, il sera possible d'appliquer les méthodes développées pour la caractérisation des souches circulant en Polynésie Française, aux souches de Djibouti. Ce travail pourrait faire l'objet d'un programme de recherche dont la trame est proposée en annexe.

Dans ce travail nous nous sommes également intéressés aux mycobactéries non tuberculeuses (MNT) qui ne sont pas des pathogènes obligatoires de l'homme. Considérées comme pathogènes opportunistes, la source d'infection est environnementale.

Au total, 87 MNT ont été isolées à partir d'échantillons cliniques de patients vivant en Polynésie Française sur une période de cinq ans et identifiées au niveau de l'espèce. Cette étude a permis de découvrir que le complexe *M. fortuitum* est le plus répandu dans cette partie du monde et que 93% (81/87) des isolats sont des mycobactéries à croissance rapide. Ces résultats doivent être complétés par des études supplémentaires pour confirmer qu'ils sont exhaustifs de l'épidémiologie des MNT en Polynésie Française et écarter tout autre biais (technique, échantillonnage…). D'autre part, il serait intéressant de voir si des facteurs climatiques pourraient favoriser les MNT à croissance rapide. Il ressort également de notre étude que l'eau est la source principale de contamination. La présente étude a également conduit à la découverte d'une nouvelle espèce de MNT qui reste à être décrite sur la base de la séquence du génome entier. La disponibilité de nouveaux génomes et le développement des outils de biologie moléculaire permettent de mieux détecter les nouveaux microorganismes et une meilleure prise en charge du patient. En effet, le traitement des MNT est long et nécessite l'usage combiné de plusieurs antibiotiques. L'étude des génomes est cruciale afin de comprendre la pathogénicité de ces microorganismes.

En conclusion, cette thèse a contribué à améliorer les connaissances sur l'épidémiologie des mycobactéries circulant en Polynésie Française et impliquées dans des maladies chez l'homme avec la description d'un nouveau clone de *Mycobacterium tuberculosis* et une nouvelle espèce de mycobactérie non tuberculeuse.

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ANNEXES

PROGRAMME DE RECHERCHE (draft)

Partie I: Compréhension et diagnostic de l'infection

Le diagnostic de la tuberculose à Djibouti repose essentiellement sur des signes cliniques (altération de l'état général, signes cliniques respiratoires et extra-respiratoires). Les examens complémentaires sont la radiologie et l'examen du crachat. Cependant, du fait d'un contexte épidémiologique particulier[(1)], le diagnostic bactériologique de la tuberculose a été amélioré via le programme national. Ainsi la mise en culture (automate BACTEC 960 et milieu solide Löwenstein-Jensen), les méthodes d'amplification génique (GenoType MTBDRplus, Xpert MTB/RIF), le test antigéniques (TB*Ag* MPT64) et les tests de sensibilité aux antituberculeux de première ligne sont venus renforcer la capacité du laboratoire. Mais ces examens ne sont pas entrepris pour chaque patient tuberculeux. Pour comprendre au mieux cette infection, il est important de mener des enquêtes épidémiologiques auprès des patients Djiboutiens et des expatriés (voir formulaire en annexe).

Il serait nécessaire de cibler la population touchée par les adénopathies tuberculeuses et procéder à des examens bactériologiques des ponctions.

[1]Selon les chiffres du Programme national djiboutien de lutte contre la tuberculose (PNLT), l'incidence de la pandémie, toutes formes confondues, est estimée à Djibouti à 620 cas pour 100.000 habitants par an, avec un taux de mortalité estimé à 71 pour 100. 000 habitants.

Partie II: Recherche de '*Mycobacterium canettii'* dans les selles

Chez l'enfant, la tuberculose est difficile à confirmer. Car souvent l'atteinte est extrapulmonaire et le prélèvement de matériel pour un examen bactériologique est difficile ou impossible à obtenir. Pour les enfants atteints de formes pulmonaires, ces derniers ne produisent pas d'expectorations. Un protocole standardisé pour la détection des membres du complexe *Mycobacterium tuberculosis* dans les selles a été mis en place par El Kéchine et al 2009. Les données indiquent l'utilité de selles comme un autre spécimen pour le diagnostic de la tuberculose pulmonaire. Dans cette partie, un large échantillonnage des selles des patients sera effectué pour la recherche de "*Mycobacterium canettii"* dans les selles.

Partie III: Sources alimentaires

Les données cliniques suggèrent une voie d'entrée digestive du pathogène. Dans cette perspective, Il semble que les aliments et les eaux de consommation humaines infectés pourraient constituer une source d'exposition au pathogène chez les humains. L'échantillonnage des aliments et d'eau pour la recherche de "*Mycobacterium canettii*" fait l'objet de cette troisième partie.

Partie IV: Echantillons environnementaux

Koeck et al. ont montré que les caractéristiques cliniques de l'infection par les bacilles tuberculeux lisses suggèrent l'existence d'un réservoir environnemental (Koeck et al. 2000). D'autres travaux au sein de l'URMITE ont montré que les mycobactéries du complexe *Mycobacterium tuberculosis* sont des organismes résistant aux amibes (Felix Mba Medie, et al 2011). Ghodbane, et al 2014 ont quant à eux montré la survie à long terme de ces dernières dans le sol. Les amibes peuvent ainsi représenter un réservoir pour les mycobactéries. Daffé et al., 1987; Lemassu et al., 1992 ont étudié la composition lipidique de la paroi cellulaire des quatre souches lisses du bacille tuberculeux connues à cette époque (CIPT 1400100-59,60, -61 et -62) et ont caractérisé des lipides qui étaient proches de certains présents dans *Mycobacterium bovis* et *Mycobacteriun kansasii*, mais pas dans *Mycobacterium tuberculosis*. Et un nouveau scénario évolutif est proposé pour l'émergence de *Mycobacterium tuberculosis* impliquant la progression du pathogène d'un organisme de l'environnement, tel que *Mycobacterium kansasii*, par le biais des bacilles tuberculeux lisses (Wang, 2014; Wang, 2015; Minnikin, 2015).

Dans cette partie, il s'agit de collecter des échantillons d'eau et de sols pour isoler *Mycobacterium canettii* et notamment par des cocultures d'amibes.

Informations patient:

Lieu et date de naissance :

Profession (Occupée dans les 2 années passées) :

Nationalité :

Femme ❒ Homme ❒

Clinique

Symptômes

Formes cliniques

Pulmonaire : Cavitaire ❒ ….. Miliaire ❒

Extra-pulmonaire : Adénopathie ❒ Effusion pleurale ❒ Neurologique ❒
Viscérale ❒

Traitement :

Durée :

Complété ❒ Perdu-de-vue ❒ Décédé ❒

Echantillon

Expectoration ❒ ……Sang ❒ ……. Urines ❒

Culture BK : Identification BK :

Alimentation

Type d'eau consommée :

Précisez si différentes eaux consommées :

Consommation de produits frais : Oui ❒ Non ❒

Si oui le(s)quel(s) :

Consommations des produits de la mer : Oui ❒ Non ❒

Si oui le(s)quel(s) :

Cuisson : Oui ❒ Non ❒

Contact avec les animaux

Animal domestique : oui ❒ non ❒

Lequel :

Profession obligeant d'être en contact d'animaux : oui ❒ non ❒

Le(s)quel(s) :

Déplacement au cours de 5 dernières années

Lieu(x) : Durée du séjour :

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