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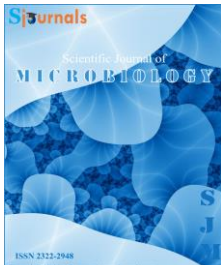

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Original article

Potentially pathogenic environmental strains of non-tuberculous mycobacteria from wastewater samples from the municipalities of Yopougon and Koumassi-Marcory in Abidjan

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ABSTRACT

Mycobacteria represent a large group of bacteria commonly found in the environment. They are involved in several infections ranging from lung infections to skin infections. In Côte d'Ivoire, very little information is available on these species apart from the best known, namely *M. ulcerans* and *M. tuberculosis*, responsible for Buruli ulcer and tuberculosis respectively. The cultivation of these species is a real challenge, especially in developing countries such as Côte d'Ivoire. However, there are reports in the literature of infections caused by these mycobacteria and few species have been described in cases of human or animal infections. Mycobacteriosis due to these mycobacteria is difficult to estimate because these diseases are not reportable illness. These pathologies are difficult to treat because of their resistance to most anti-tuberculosis antibiotics. The aim of our study was to identify the strains of potentially pathogenic non-ulcer and non-tuberculosis environmental mycobacteria circulating in the wastewater in the city of Abidjan. The strains isolated in this study were fast-growing mycobacteria and slow-growing mycobacteria. Thanks to the sequencing of the amplification product, 5 species of mycobacteria were identified, namely *mycolicibacterium*

fortuitum; *mycolicibacterium mageritense*; *mycolicibacterium europaeum*; *mycolicibacterium neworleansense* and *mycolicibacterium Brumae*. This study would be the first to identify these fast-growing and slow-growing species in Côte d'Ivoire.

1. Introduction

Non-tuberculous mycobacteria (NTM) or atypical mycobacteria are opportunistic pathogens whose reservoir is the environment (soil; water) (van Ingen et al., 2009). The term "atypical Mycobacteria" was applied in 1935 by Pinner to characterize Mycobacteria other than tuberculosis and leprosy bacilli that can cause pathologies in humans or animals (Pinner, 1935). Many other terms are also used: “Non-Tuberculosis Mycobacteria”, “Opportunistic Mycobacteria” and “Environmental Mycobacteria”. Recently, a new name for the genus *Mycobacterium* under the term *Mycolicibacterium* was created in 2018 following the revision of the genus *Mycobacterium* by Gupta et al. (2018). They are strict aerobes, non-spore-forming, immobile, non-pigmented. They are acid-alcohol-fast (AFB) on direct examination and naturally resistant to many antiseptics and certain antibiotics.

Depending on their growth period, a distinction is made between NTM that grow for more than 7 days, known as “slow-growing” (SGM) and NTM that grow in less than 7 days, known as “fast-growing” (RGM).

In Côte d'Ivoire, studies on environmental mycobacteria have focused more on slow-growing mycobacteria (*Mycobacterium ulcerans* and *Mycobacterium tuberculosis*), responsible for Buruli ulcer and tuberculosis respectively (Al Jarad et al., 1996; Aka et al., 2015). Thirty-nine percent (39%) of skin ulceration samples suspected of Buruli ulcer, analyzed by the National Reference Laboratory, the Pastor Institute of Côte d'Ivoire, had an etiology not identified by the techniques used. (Laurence et al., 2009; Aka et al., 2018). This suggests the probable involvement of other environmental mycobacteria in certain skin ulcerations in Côte d'Ivoire. Indeed, according to Ishii et al. (1998) and Gomez-Moyano et al. (2009), other mycobacteria are also responsible for skin infections. The treatment of infections depends significantly on the correct diagnosis of the condition and the crucial species identification at this level. Unfortunately, they are underestimated in tropical areas, because few laboratories are capable of culturing and identifying NTM. Identification in diagnostic laboratories is most often limited to common species (Varghese et al., 2017). The steady increase in mycobacterial species, the use of time-consuming techniques, and the lack of standardized identification methods make achieving this objective a demanding challenge. Due to the emergence or even the increase in cases, it is becoming important to know the non-ulcer and non-tuberculous mycobacteria involved in the pathology. Also, the main objective of this study was to identify strains of *non-ulcer* and *non-tuberculosis* environmental mycobacteria harboring pathogenic genes circulating in the city of Abidjan.

Thanks to the sequencing of a number of target genes, a more precise identification by species was possible, in particular the small subunit of the ribosomal gene (16S rRNA), which proves to be a robust, accurate and reproducible method that has been used for years (Oren and Trujillo, 2019).

2. Materials and methods

2.1. Study sites and scope

The study was carried out in Abidjan (Côte d'Ivoire) precisely in the municipalities of Yopougon, Koumassi and Marcory. These sites are considered non-endemic areas for Buruli ulcer according to the national Buruli ulcer control program in Côte d'Ivoire. The biological and microbiological analyzes in this study were carried out at the Pastor Institute of Côte d'Ivoire.

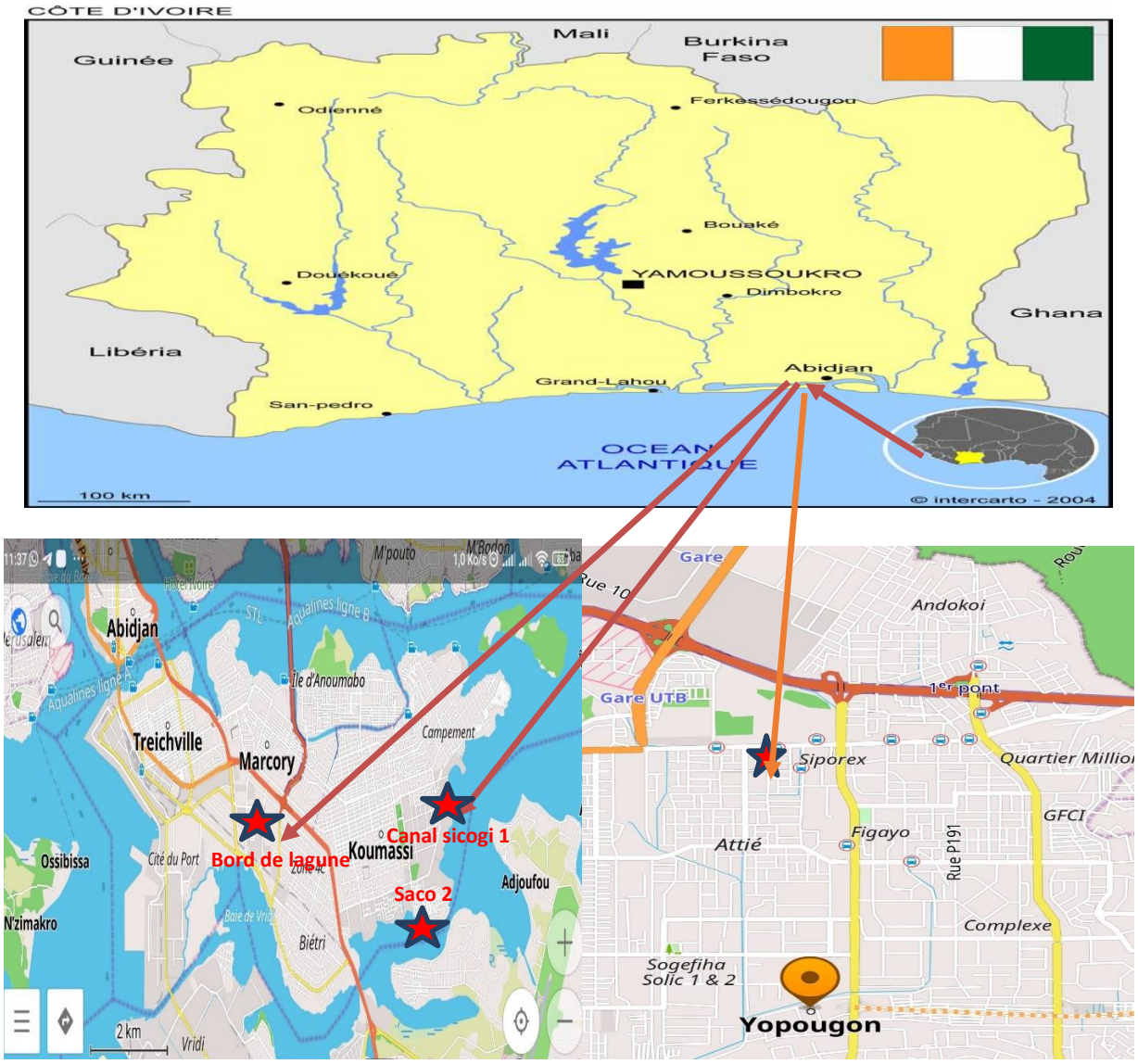

Fig. 1. Study site (Vakou et al., 2022).

The biological material consisted of wastewater samples from the municipalities mentioned above.

2.2. Sampling method

The water samples were collected from the surface using the technique described in (Britton and Gresson, 1987). The samples taken were packaged in glass bottles of one-liter capacity and kept in a cooler containing ice packs in order to maintain them at a temperature of approximately + 4°C until arrival at the laboratory, protected from light within 24 hours of collection (ISO 5667-15, 2009).

In the laboratory, these collected samples were treated according to the method described by Stinear et al. (2004) and Kankya et al. (2011). Decontamination of water samples was performed with cetylpiridium chloride (CPC), followed by neutralization with phosphate buffer. The different culture media: Loweinstein Jensen (LJ) and Middlebrook 7H10 agar were used for inoculation. The samples were inoculated in duplicate into the different LJ and Middlebrook, 7H10 media. The incubations were carried out at 23 and 37°C. in the ovens. Daily observations were made until colonies were obtained. An optical microscope (Zeiss®) was used for the observation of Bacilli resistant to acid alcohol after Ziehl-Neelsen staining (Barksdale and Kim, 1977). The classification according to the growth period of the species was made according to the method described by Runyon et al. (1959). The biochemical identification of mycobacteria was carried out according to the method described by Metchock

(1995). Briefly, for the biochemical identification, the colonies obtained after culture were observed under the microscope. The acid-alcohol-fast Bacilli were cultured in the presence and in the absence of light on LJ medium for the photo-induction test. Microscopic techniques will consist of Ziehl-Neelsen and Gram stains. The phenotypic and biochemical characters taken into account in the culture will be those described by (Runyon et al., 1959; Euzeby, 2010).

2.3. Molecular identification of environmental Mycobacteria

2.3.1. Extraction of DNA from isolated strains

DNA extraction was performed with the DNeasy Blood and tissue kit (QUIAGEN) following the manufacturer's procedures. Sterile distilled water served as a negative control.

2.3.2. Conventional PCR

The IS6110 gene, present in the 16S RNA of the majority of Mycobacteria, was searched for by conventional PCR using MycgenF/Mycgen R primer pairs (Wilton and Cousins, 1992), a mastermix (5X FirepolMasterMix RTL from Solisbiodyne). The mastermix contains the enzyme, the DNTPs, the magnesium and the colored buffer. DNA extracted from wastewater samples was then added. Amplification was performed in a Veriti® thermal cycler from AppliedBiosystem®. The size of the amplicons sought was 1030 base pairs.

2.3.3. Sequencing of the amplification product of the isolated strains

The amplification products of a size corresponding to that of IS6110 were sequenced by the CRCHU sequencing and genotyping platform of Quebec-CHUL. Forward and reverse primers of all genes are used for sequencing. The sequences are aligned using a local sequence alignment program (BLAST, NCBI).

3. Results and discussion

A total of 45 water samples were collected, including 15 water samples taken from 3 sites. After decontamination and culture, the isolated strains were fast-growing colonies appearing on Lowenstein-Jensen medium at 7 days (at 37°C and 32°C) and slow-growing (2 to 3 weeks of incubation at 37°C). These strains were then subcultured on Middlebrook 7H10 agar; the colonies had a smooth, unpigmented appearance. They were acid-alcohol-fast under the microscope after Ziehl Neelsen staining. The amplification of the IS6110 sequence of the isolated strains made it possible to confirm the belonging of the strains to the genus *mycobacterium* (fig 2).

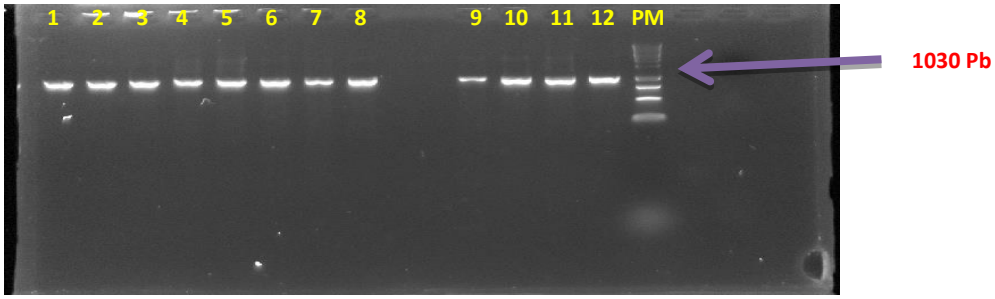

Fig. 2. Electrophoresis in 1% agarose gel containing 0.5 µg/ml of BET of 16S RNA amplification products from the DNA of isolated bacterial strains. PM lane: Smart Ladder molecular weight marker 1 Kb; lanes 1 to 12: DNA of strains obtained after culture.

Sequence analysis of the amplified 16S rRNA confirmed the presence of fast-growing and slow-growing mycobacteria, including *mycolicibacterium fortuitum; mycolicibacterium mageritense; mycolicibacterium europaeum; mycolicibacterium neworleansense and mycolicibacterium Brumae.* These different species isolated are distributed according to the different sampling site in Table 1.

Mycolicibacterium fortuitum was the most isolated strain in the wastewater of Koumassi municipal followed by *mycolicibacterium mageritense* and *mycolicibacterium europaeum* while in the Yopougon municipal, only one species was identified, *mycolicibacterium Brumae*. *mycolicibacterium fortuitum; mycolicibacterium mageritense* were also isolated from the edge of the lagoon in Marcory.

Table 1
Genetic profile of Mycobacteria species isolated according to site after 16S RNA sequencing.

Provenance	ARN 16S	Scientific name	Query cover	E. Value	Percent identITY	Accession
Lagoon edge, (marcory)	POS	*Mycolicibacterium fortuitum subsp. fortuitum DSM 46621 = ATCC 6841*	95%	0.0	98.62%	NR_042912.1
Lagoon edge, (marcory)	POS	*Mycolicibacterium mageritense*	93%	0.0	98.94%	NR_115232.1
Saco II (Koumassi)	POS	*Mycobacterium europaeum*	94%	0.0	98.94%	NR_125568.1
Canal Sicogi I (Koumassi)	POS	*Mycolicibacterium fortuitum subsp. fortuitum DSM 46621 = ATCC 6841*	94%	0.0	96.92%	NR_042912.1
Saco II (Koumassi)	POS	*Mycolicibacterium mageritense*	96%	0.0	97.12%	NR_115232.1
Canal Sicogi (Koumassi) I	POS	*Mycolicibacterium fortuitum subsp. fortuitum DSM 46621 = ATCC 6841*	94%	0.0	99.05%	NR_042912.1
Canal Sicogi I (Koumassi)	POS	*Mycolicibacterium mageritense*	95%	0.0	98.82%	NR_115232.1
Canal Sicogi I (Koumassi)	POS	*Mycolicibacterium neworleansense*	93%	0.0	97.75%	NR_115113.1
Canal Sicogi I (Koumassi)	POS	*Mycolicibacterium fortuitum subsp. fortuitum DSM 46621 = ATCC 6841*	95%	0.0	98.31%	NR_042912.1
Siporex (Yopougon)	POS	*Mycolicibacterium brumae*	95%	0.0	99.26%	NR_025233.1

Nontuberculous mycobacteria (NTM) are of great importance due to their pathogenesis in the occurrence of severe ulcerations in animals and humans (Fyfe et al., 2010). Nontuberculous mycobacteria (NTM) include over 200 species and subspecies. In Asia, 31% of infectious diseases associated with NTM are caused by rapidly growing mycobacteria (RGM) (Shinnick, 1994; Hoefsloot, 2013) and generally require long-term treatment with polyvalent antibiotic regimens. They are often refractory to treatment and have a high probability of relapse (Park, 2015). Therefore, a better understanding of their epidemiology and ecology is important for controlling the spread of the diseases that these mycobacteria could cause, in particular ulcerations similar to those caused by *M. ulcerans* in the case of Buruli ulcer (BU). Note that Buruli ulcer is a neglected and endemic disease in more than 30 countries. The disease can be treated but leaves irreversible sequelae in patients. With approximately 1,500 cases per year, Côte d'Ivoire is the most affected country in West Africa according to the WHO (WHO, 2019). This study was undertaken to identify species of Mycobacteria other than *ulcerans* in wastewater in the city of Abidjan, at the following sites; Marcory, Koumassi and Yopougon. This study was conducted to prevent potential risks related to the presence of potentially pathogenic species of environmental mycobacteria other than *M. ulcerans* and also, to support the hypothesis that "wastewater could be a potential source of contamination to the population vulnerable to mycobacteriosis". The wastewater used in this study is one of the most polluted water in Abidjan with an average daily production of more than 7000 kg of BOD5 (Soro et al., 2010). These conditions could generate many health problems whose consequences on families are measured at different levels: proliferation of waterborne diseases, high infant mortality, economic difficulties.

The strains isolated in this study are fast-growing mycobacteria and slow-growing mycobacteria. They are all acid-alcohol resistant and the majority showed no pigmentation except *m. brumae*, which is a non-photochromogenic mycobacterium, with rough, pale yellow colonies. Amplification of the 16S rRNA gene, common to all mycobacteria, makes it possible to distinguish between members of the mycobacterium tuberculosis complex and opportunistic pathogenic mycobacteria, thanks to minor variations of this gene identified by the following primers: Mycgen-F and Mycgen-R. these primers generate a band of 1030 bp common to all mycobacteria. According to the results of this study, all the isolated strains show their belonging to the genus *mycobacterium*. The sequencing of the amplification product has allowed 5 species of mycobacteria to be identified, namely *mycolicibacterium fortuitum*; *mycolicibacterium mageritense; mycolicibacterium europaeum;*

mycolicibacterium neworleansense and *mycolicibacterium Brumae.* This study would be the first to identify these fast-growing and slow-growing species in Côte d'Ivoire. The mycobacterial 16S rRNA (16S rRNA) gene (encoding 16S DNA) is of great evolutionary importance and has been widely used to trace phylogenies, differentiate NTM isolates (Portaels et al., 1996; Clarridge, 2004; Janda et al., 2007) and resolve ambiguities in bacterial nomenclature (Janda et al., 2007).

According to literature, these species found are responsible for several infections ranging from pulmonary infections to skin infections (Tortolis et al., 2011; Pourahmad et al., 2012; Kothavade et al., 2013; Phelippeau et al., 2015). Lung infections from *M. fortuitum* are rare, but *Mycobacterium fortuitum* can cause local skin disease, osteomyelitis (bone inflammation), joint infections, and eye infections after trauma. *Mycobacterium fortuitum* has a worldwide distribution and can be found in natural and treated water, sewage and dirty water.

M. mageritense, found in the Marcory and Koumassi municipal, is generally isolated from water and soil (Wallace et al., 2002; Gira et al., 2004). A few cases of skin infection caused by *Mycolicibacterium mageritense* have been reported to date. (*M. mageritense*) is a rare fast-growing mycobacterium related to *Mycobacterium fortuitum*. It is of low virulence among RGM and was initially discovered from human sputum in 1997 (Domenech, 1997) and from human samples collected from surgical wounds, blood, sinuses, and joint fluid (Wallace et al., 2002). *Mycolicibacterium neworleansense*, found in Koumassi, is a fast-growing nontuberculous species belonging to the *Mycobacterium fortuitum* complex (Asmar et al., 2015). These organisms can cause respiratory infections, a range of soft tissue and skeletal infections, bacteraemia and disseminated diseases (Schinsky et al., 2004). *Mycolicibacterium Brumae* is a fast-growing, non-photochromogenic microorganism found in Yopougon-Siporex wastewater. It was isolated for the first time from water samples, soil, and human sputum from Barcelona, Spain (Luquin et al., 1993). Apart from a 2004 report of catheter-related blood infection, no other infections with this organism have been reported (Luquin et al., 1993).

The identification of mycobacteria is very important for the management of patients. This identification is usually achieved through phenotypic tests based on a panel of biochemical tests, pigmentation and growth characteristics (Katoch et al., 2007). Today, with the emergence of immunodeficiency diseases resulting from HIV, cancers, and the uncontrolled use of immunosuppressive drugs, opportunistic microorganisms such as nontuberculous mycobacteria (NTM) are becoming common pathogens in patients (Hartsman et al., 2006; Azadi et al., 2016). Skin infections are usually caused by *M. abscessus*, *M. chelonae*, *M. fortuitum* and *M. kansasii* (Gazeroglu et al., 2004). To this end, a case of co-infection between SARS-COV and *Mycobacterium abscessus* was noted in Florida (Rodriguez et al., 2021).

4. Conclusion

In the current context of the Coronavirus (Covid 19) pandemic known throughout the world, these species capable of also causing pulmonary infections must be known to all in order to better guide the diagnosis of pulmonary infections in Côte d'Ivoire. It is important to have an open mind in order to assess other differential factors such as superimposed infections, especially in the immunocompromised population, as appropriate antimicrobial treatment may alter the results. The isolation of potentially pathogenic Mycobacteria therefore makes it possible not to rule out the hypothesis of a biological risk to the population in mycobacterial infections. It would therefore be wise to continue the study of the distribution of these species in Côte d'Ivoire and to detect the various sources of direct contamination to the vulnerable population and indirect by the drinking water distribution system.

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