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Phenotypic Profile of Isolated Strains of Environmental Mycobacteria in the Buruli Ulcer Endemic Zones in Cote d`Ivoire (2015)

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Abstract: Non-tuberculous mycobacteria (NTM) are microorganisms of the genus Mycobacterium. They are widely present in the environment (soil, water, sediment, aquatic plants ....). They are responsible for many infections as reported by several authors. The purpose of this study is to isolate and identify mycobacteria in the water and sediments found in hyperendemic areas and hypoendemic of Buruli ulcer. A total of 473 samples were obtained. As follows, 251 samples from water and 222 from sediment distributed according to sampling sites. The sample decontamination was performed with Cetylpiridium Chloride (CPC), followed by neutralization with phosphate buffer. A total of 8 species (12.3%) were identified in our study. 50.77% of species identified were found in hyper endemic zones against, 49.23% of species at the hypo-endemic zones. *Species like M. peregrinum, like M. smegmatis, like M. peregrinum, M. immunogenicum, M. chelonae, M. mucogenicum, M. abscessus, M. sp*. were isolated in this study. The species *M, peregrinum* (13.84%) was the most common in all sites, except in the sites of Bodo and Bouaké. This study reveals the presence of fast growing mycobacteria such as *M. peregrinum* in water and in sediment in Côte d`Ivoire, which represents the potential risk of contamination in humans especially in people who are in permanent contact with water.

Keywords: Mycobacterium, Buruli Ulcer, Non-tuberculous Mycobacteria, Culture

1. Introduction

Mycobacteria are found everywhere in the environment like: soil, water, aerosols, plants, aquatic animals ... They cause lung, skin or lymph infections [1-5]. They are not among serious pathogens, therefore no serious attention are being paid to by the scientific community, who rather committed more attention to tuberculosis epidemic. These are bacteria that appear red on blue background according to *Ziehl-Neelsen:* they are called Acid- Alcohol Resistant Bacillus (BAAR) [6]. This is an extremely polymorphic bacterial genus, they include fast-growing species (less than 7 days), and slow growing species (7-60 days) and non-cultivable species outside animals, *Mycobacterium leprae*, leprosy agent [7]. Mycobacteria are grouped into two major groups namely *tuberculosis complex* and *nontuberculous mycobacteria* (NTM) also called environmental mycobacteria (mycobacteria of leprosy and atypical

mycobacteria) [8-10]. The common laboratory protocols that are used to isolate mycobacteria in clinical samples are becoming less suitable to environmental mycobacteria [11]. Culturing, however, remains the most effective way to describe species, understand their physiology and to test their sensitivity to antibiotics [12, 13]. There are currently more than hundreds of atypical mycobacteria species [14]. The most common are *M. avium, M. intracellulare, M. kansasii, M. xenopi, and M. abscessu*s [15, 16]. The distribution of commonly isolated species is in constant change in most countries where studies have been carried out and new species emerged [17]. They would be part of non-pigmented genes atypical mycobacteria group such as *M. abscessus, M chelonae, M fortuitum, M. smegmatis* [18]. The Buruli ulcer (BU), caused by *Mycobacterium ulcerans*, is a real public health problem in Ivory Coast, almost all areas are affected. The mode of transmission and environmental sources are unknown. To be able to understand the level of prevalence of mycobacterial infections, it is very important to identifier the species involved in *ulcerations*. The main objective of this study is to isolate and identify the *mycobacteria* in water and in sediments in Buruli Ulcers hyper endemic and hypo endemic zones in Ivory Coast.

2. Materials and Methods

2.1. Sites and Study Environment

The study was conducted in sites considered to be BU hyper endemic areas (Adiopodoumé, Tiassalé (Sokrogbo and Bodo), Adzopé) and hypo-endemic areas (Agboville, Bouaké (Loka) Aghien) according to the National Programme on fight against BU in Côte d`Ivoire (Fig 1). The Bacteriological part of this study was carried out at the Pasteur Institute of Côte d`Ivoire.

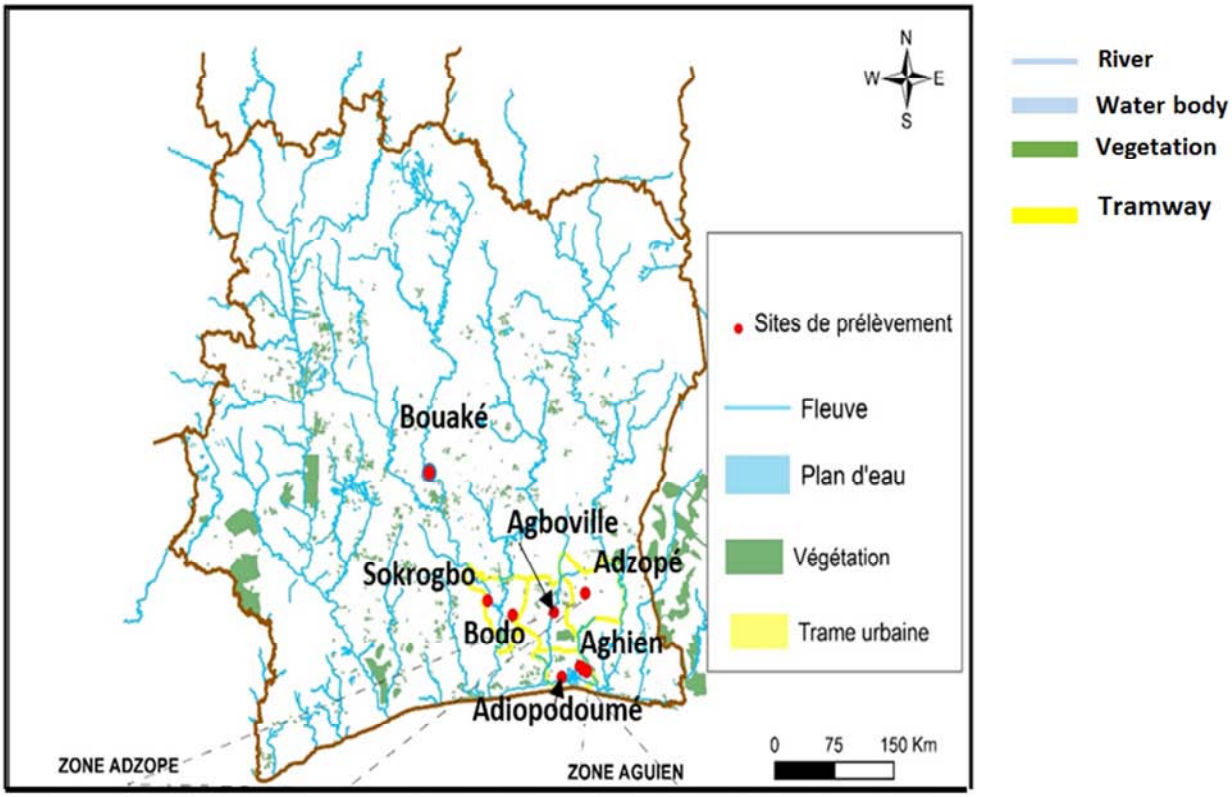

Figure 1. *Sampling sites.*

2.2. Biological Material

The biological material consisted of samples from water and sediment obtained from different sites.

3. Methodology

3.1. Sample

A monthly sample was taken from June 2014 to June 2015. A total of 22 sites were selected, as follows 11 Aghien Lagoon, 3 swamps in Adzopé. 2 sites each were selected in Sokrogbo, Bodo and Adiopodoumé respectively. 1 site was selected at the entrance of Agboville town and on swamp in Loka at Bouaké.

The sediment samples were collected using dump Eckmann at big water points and a sampler at the banks [19]. A 5 liter capacity bucket spout allowed to draw water at the bank of water points and a hydrological bottle 1.5L capacity for drawing water far away from the banks. The samples were then kept refrigerated at 4°C during transportation, protected from light and taken to the laboratory within 24 hours of sampling.

3.2. Culturing

3.2.1. Sediments

In the laboratory, 500g of each sediment sample were collected. In a Falcon tube of 50 mL capacity, 10g of sediment were mixed with 40 mL of sterile distilled water using the method described by [20] slightly modified. After mixture of the sediment and distilled sterile water, the supernatant was recovered in a new Falcon tube. Decontamination of supernatant recovered was carried out with the chloride Cetylpiridium (CPC) [21] followed by neutralization with phosphate buffer.

3.2.2. Waters

100 mL of water drawn were centrifuged at 13,000 rpm for 20 min. Decontamination of the pellet was carried out with the chloride Cetylpiridium (CPC) **[**21], followed by neutralization with phosphate buffer.

3.2.3. Seeding

The different culture medium: Loweinstein Jensen (LJ), Mac Conkey without crystal violet, ordinary agar and Middlebrook 7H10 were used for seeding. Samples in LJ and Middlebrook 7H10 agar were seeded in duplicate. 1 lot of each of them was packed in aluminum foil for the photo induction test.

3.2.4. Incubation

Incubations were made at 23°C and 37°C in ovens on special racks, tilted. The tubes were closed until after evaporation of the liquid, the medium have to be dry but not dried out so that mycobacteria can grow. Agar should be incubated at least 8 weeks and up to 12 weeks [22]. **A** daily observation was made until colonies were formed.

3.3. Identification and Classification

The presence of mycobacteria was confirmed by *Ziehl Neelsen* coloration. An optical microscope (*Zeiss*®) was used for the observation of Acid- Alcohol Resistant Bacillus after Ziehl-Neelsen coloration [23]. Classification of species was made according to the method described by Runyon and collaborators [24]. According to the classification of Runyon, the following characteristics were tested: the incubation temperature, the growth time (- 8 days for rapidly growing mycobacteria, + 8 days for slow growing mycobacteria) colonies morphology, observed under the microscope, either eugonic colonies (rough, progressive development in colonies of up to 1 cm in diameter) type "R" or "S" or dysgonic colonies (smooth, always remaining small colonies of around 1 millimeter (mm) in diameter, they are sometimes embedded in agar) type "R" or "S" of photochromogens (colonies whose pigmentation occurs as a result of exposure to light) of scotochromogens (colonies whose pigmentation occurs in the dark), the achromogens (non-pigmented colonies), also according to the pigmentation, chamois beige, yellow, orange, pink or red or not [7].

4. Results

A total of 473 samples were obtained distributed as follows, 251 from water and 222 from sediment collected from several sites (Table 1).

Table 1. *Number of samples per sampling site.*

SAMPLES COLLECTION SITE								
	hyper endemic site				**hypo endemic site**			
	Adzopé	**Adiopodoumé**	**Tiassalé**		**Agboville**	**Aghien**	**Bouake**	**TOTAL**
			Sokrogbo	**Bodo**				
Eaux	45	18	18	6	15	143	6	251
Sédiments	16	18	18	6	15	143	6	222
TOTAL	61	36	36	12	30	286	12	473

All decontaminated samples were cultured and observed on a daily basis up to colonies development. After pigmentation and microscopic observation of colonies, 65 were BAAR positive (13.74%), 408 were BAAR negative (86.25%). Of the 65 identified as BAAR positive, 34 were from hyper endemic sites (52.3%) and 31 from hypo-endemic sites (47.69%). According to colonies appearance, 8 colonies were Rough (R) (12.3%), they are from Aghien and Adzopé sites and 57 colonies were smooth (S) (87, 69%). None of the colonies have both rough and smooth appearance. Based on pigmentation of colonies to light and dark, no strain was scotochromogenic, 4 were photochromogenic, they are from Adzopé sites, Bodo, Agboville and Bouake (loka) and 61 were non Chromogenic. According to the growth of colonies in the presence of the substrate used, the addition of NaCl to LJ medium was equally favorable to the growth of mycobacteria likewise LJ without NaCl, 76.92% of strains were Tween 80 negative.

Based on the characteristics of culture, morphological appearance and biochemical, isolated strains were all rapidly growing mycobacteria. No slow growing mycobacteria have been isolated. A total of 8 species (12.3%) were identified in our study. About 50.77% of identified isolated strains were from the hyper endemic sites as against, 49.23% of species from the hypo-endemic sites. Species like *M. peregrinum species, like M. smegmatis, like M. peregrinum, M. immunogenicum, M. chelonae, M. mucogenicum, M. abscessus, M. sp*. were isolated in this study. The specie *M. peregrinum* (13.84%) was the most common in all sites, except in the sites of Bodo and Bouaké.

In the hyper endemic sites sediments were the most

contaminated by mycobacteria with 60% against 43.3% in water samples. In hypo-endemic areas, water sample was the most contaminated with a rate of 53.57% against 43.24% in sediments sample (Table 2).

Table 2. *Number of isolates per site according to the type of sample.*

	Number of isolated strains per sample							
	Sites hyper endémiques				**Site hypo endémiques**			**TOTAL**
	Adzopé	**Adiopodoumé**	**Tiassalé N(%)**		**Aghien**	**Agboville**	**Bouaké**	
	N(%)	**N(%)**	**Sokrogbo**	**Bodo**	**N(%)**	**N(%)**	**N(%)**	
Eaux	5 (16, 66)	3 (10)	3(10)	2 (6,66)	12 (40)	3 (10)	0	28 (43, 07)
Sédiments	7 (20)	4 (11, 42)	6 (17,14)	4 (11,42)	11 (31, 4)	4 (11, 42)	1	37 (59, 92)
TOTAL	12 (18, 46)	7 (10, 76)	9(13,84)	6(9,23)	23(35, 38)	7 (10, 7)	0	65

The identified species in our study were classed according to the type of sample. From the hyper endemic zone Adzopé site has the most contaminated sample with a rate of 66.6% while from hypo-endemic zone Agboville site has the most contaminated sample.

5. Discussion

In this study, 473 samples were analyzed. The most contaminated sample by the presence of mycobacteria with 60% was sediments against 43.3% for water samples in hyper endemic areas. In hypo-endemic areas, water sample was the most contaminated with a rate of 53.57% against 43.24% in sediment. However other studies, such as Mohammad *et al*. found the rate of isolated mycobacteria in water to be lower than in soils [25]. This could be explained by the presence of parameters favorable to the growth of mycobacteria in the sediment [26]. The environmental mycobacteria prevalence rate was 13.74%. This rate was relatively lower than the 15.5% observed by Kankya *et al*. [20] in Uganda and 18.2% reported par Mohammad *et al*. after treatment with 3% SDS combined with 1% NaOH [25]. This result could be explained due to the utilization of different decontaminating compounds having variables effects on the growth of mycobacteria [27]. The most suitable method used in this study was the 0.1% CPC added to 0,067 M phosphate buffer pH 6.8. Indeed, there are several decontamination methods available [28]. The treatment with Cetylpyridium chloride (CPC) detergent stimulates the growth of many NTM with low contamination by interfering bacteria [21]. The nontoxic nature of the compound and its bactericidal properties makes it an exceptionally versatile and valuable disinfectant. However, the efficacy of decontaminants depends on their concentration and contact time with sample [29]. These treatments can convert viable living cells into a state of viable bacteria but non-culturable [27]. The concentration and decontamination stages often result to loss of culturable mycobacteria, and the culture medium to be used are still relatively selective because they can't reveal some non-culturable organisms [30]. Thus Pickup *et al*. noted that several species of NTM in the river Tall (GB) could not be isolated by culture [31]. Parashar *et al.* also observed variation in the effectiveness of decontamination methods based on the origin of the samples [32]. Indeed it is known that mycobacterial species do not have the same resistance to different decontamination procedures [32]. All 65 isolated strains were Bacillus acid-alcohol resistant (BAAR). However these BAAR are also found in other *genus*, not well described *Dietzia, Gordona and Tsukamurella* [7] [33, 34]. These procedures therefore represent the first step in the identification of mycobacteria, however less specific concerning the Mycobacterium genus [35]. Identifying of species therefore requires additional biochemical tests on growth [7] in the presence of certain compounds, and resistance to some chemicals.

Concerning the colonies observed, 87.69% of the strains were Smooth (S), against 12.30% R (rough). They were mostly dysgonic colonies and not pigmented. According to the Runyons classification, all isolated mycobacteria belong to the group of rapidly growing mycobacteria (group IV). Several studies have also revealed the presence of rapidly growing mycobacteria in soil [11]. The following species have been identified in our study *M. peregrinum, M. chelonae, M. abscessus, M. mucogenicum, M. immunogenum, like M. smegmatis, like M. peregrinum and M. sp*. In our study a prevalence rate of 50.77% was observed, in the BU hyper endemic areas against 49.23% in the BU hypo endemic areas. *M. peregrinum* specie (13.84%) was more frequent in all the sites, except in Bodo and Bouaké. Tsukamura *et al.* found that *M. fortuitum* specie was the most common in soil samples [36]. The epidemiology of NTM would be different from one country to another and from one continent to the other. For example *M. xenopi* appeared to be the second mycobacterium isolated in Europe and the first in some areas, like in the southeast of Britain, while the same specie represent less than 0.01% of isolated MNT in the US [1, 17, 37].

All these species identified in Côte d`Ivoire causes skin infection in some countries according to report, *M. peregrinum*, *M. chelonae*, are responsable for skin infections, *M. abscessus* are responsable for skin and soft tissue infection with formation of abscess or skin nodules, often appearing after trauma or surgical operation done with infected material [1, 3, 38, 39] and various other infections caused by *M. lentiflavum and*, *M. intracellulare* in Zambia [40]. There could be a relationship between the species present in the environment and the high endemic ulcerations in Côte d`Ivoire. This study revealed the presence of rapidly growing mycobacteria such as *M. peregrinum* in water and sediment.

6. Conclusion

This study describes the potential risk of contamination in

humans especially those who are constantly in contact with the environment. The risk of transmission of cutaneous mycobacteriosis would be equally present in hypo-endemic area as well as in hyper endemic areas like Williamson *et al.* reported in the case of Buruli Ulcer [41]. Further studies should be carried out in other to explain the conditions for spreading of these species in the environment.

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