Internal Medicine: Open Access

Gregoire et al., Intern Med 2017, 7:3
DOI: 10.4172/2165-8048.1000241

Research Article Open Access

Molecular Typing using PCR-RFLP Reveals Diversity of Environmental Mycobacteria Agent of Buruli Ulcer in Ivory Coast, Cote d'Ivoire (West Africa)

Quinet Gregoire[1, 3], **Kakou Ngazoa E Solange**[1, 2*], **Aka Nguetta**[2], **Vakou Sabine**[1, 2], **Coulibaly Ngolo David1**[2], **Kouakou Helene1**[1, 2], **Sylla Aboubacar**[1], **Faye-Kette Hortense**[2], **Aoussi Serge**[1] **and Dosso Mireille**[1, 2]

[1]*Department of Technics and Technology, Platform of Molecular Biology, Pasteur Institute Abidjan, BP 490 Abidjan 01, Cote d'Ivoire*

[2]*Department of Bacteriology and Virology, Unit of Environmental Mycobacteria, Pasteur Institute Abidjan, BP 490 Abidjan 01, Côte d'Ivoire*

[3]*Department of Biochemical Engineering, National Institute of the Applied Sciences, Toulouse, France*

[*]**Corresponding author:** ES Kakou Ngazoa, Institut Pasteur Abidjan, BP 490 Abidjan, Cote d'Ivoire, Tel: 225 08240453; Email: ngazoa_solange@yahoo.fr

Received date: July 29, 2016; **Accepted date:** June 12, 2017; **Published date:** June 16, 2017

Abstract

Buruli ulcer is a neglected skin disease caused by Mycobacterium ulcerans (MU), and affects 1000 people each year worldwide and particularly in African countries. Eradication of Buruli ulcer is difficult because of the lack of early diagnostic in rural endemic regions, and the unknown of the disease in national health care system. In the rural wetlands and marshes in Central and West Africa, children are most affected. MU belongs to environmental Mycolactone Producing Mycobacteria (MPMs) and presents high genetic diversity of the circulating strains. Diagnoses were applied by culture and genome detection by Polymerase Chain Reaction (PCR). The PCR became a gold method to confirm clinical and environmental samples for MU. The MIRU-VNTR method and the Restriction Fragment Length Polymorphism (RFLP) combined with MIRU-VNTR markers were used to discriminate Mycobacteria from different sources. The aim of this study was to investigate the molecular diversity of different mycobacteria sources, from environment, culture strains, and clinical samples by using combined MIRU-VNTR-Typing and RFLP.

A total of 26 samples (water, sediment, mycobacteria strains, and swab) from endemic sites were first confirmed by IS2404 or Ziehl-Neelsen staining. The samples were analyzed by PCR typing for 4 specific markers (MIRU-1, VNTR6, VNTR19, ST-1) and the amplicons were digested with MspI restriction endonuclease and separated by 3% agarose gel electrophoresis for PCR-RFLP analysis.

Our results showed different amplification by VNTR-MIRU-typing. For environmental samples a low amplification was detected by 25% for PCR-MIRU-1. Culture strains and clinical samples had amplification rate of 35.7% and 62.5% respectively. ST-1 had the best amplified marker for culture strains by 71.4%, while clinical samples have good amplification rate by 62.5 % for all markers. PCR-RFLP-profiles of clinical samples were identical, while environmental and mycobacteria strains showed different PCR-RFLP-profiles.

We have developed a PCR-RFLP sensitive, easier and inexpensive approach to confirm genotyping of nontuberculosis mycobacteria in endemic countries for environmental screening. We suggest mutation in repeat loci of VNTR-MIRU sequence and the adaptation of mycobacteria from environment to human. This study confirms the circulation of several genotypes of mycobacteria in Ivory Coast.

Keywords: Genotyping; PCR-RFLP; Mycobacterium ulcerans; Buruli ulcer; Cote d'Ivoire

Introduction:

Environmental mycobacteria are responsible for many infections in humans. One of them, Mycobacterium ulcerans (MU), is the agent of a skin disease called Buruli ulcer (BU). BU infection is the third most important mycobacteria infection worldwide after Leprosis and Tuberculosis [1]. BU occurs in rural regions in West and Central Africa, Latin America, Australia and Asia [2, 3]. Since 2009, over 1000 cases of BU have been reported in Côte d'Ivoire each year [4]. Several studies have demonstrated the evidence of environmental transmission of MU from environmental samples [5-6]. MU genomes have been detected in endemic sites in aquatic environment, like rivers, wetlands and stagnant water spots [7-14] and in small mammals [15, 16]. MU is a slow growing mycobaterium and the first positive results appears between 6 to 12 weeks, after several culture transition in various media [17]. For a faster diagnosis of MU, molecular detection is gold standard [18-20]. The virulence gene of MU is coded for Mylolactone, a toxin responsible for the destruction of skin tissues [21]. Further genetic analyses have confirmed high genomic diversity of MU and other MPMs, by using Variable Number Tandem Repeat (VNTR) and Mycobacterial Interspersed Repetitive Units (MIRU) [22-25] has demonstrated the high specificity and sensitivity of several VNTR-MIRU markers in clinical and environmental matrices.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis is a widely applied method to detect gene mutations, which allows distinguishing mutant-type and wild-type sequences via destructing or generating enzyme restriction sites through PCR and subsequent electrophoresis separation of differential fragments [26]. Compared to other methods, PCR-RFLP

offers a simple operation, higher sensitivity and reproducibility, and no complex equipment requirements [27].

The objective of this study is to elucidate the genomic diversity of environmental Mycobacteria in several matrices, by using combined PCR-RFLP-method applied on four MIRU-VNTR markers.

Ethics Statement:

This work was carried out with the approval of the National Buruli Ulcer Control Program in Ivory Coast (PNLUB) and the CNR Buruli at Pasteur Institute no further permissions were required for the environmental sampling and clinical samples.

Materials and Methods

Sampling

Clinical strains from suspected patients in different endemic sites (Daloa, Divo, Kong, Toumodi, Zouehele, Abidjan) were fresh collected from the National Buruli Control program (PNLUB) and transferred to the National Reference Center (CNR Buruli) in Pasteur Institute between September and November 2015. Two swabs were collected for each patient from ulcers. 500 mL environmental samples (water and sediment) were collected in 6 endemic villages in two points for each site between March and October 2015. The sites were located as follows: Adiopodoume 1 (latitude 5.339201; longitude -4.124042), Adiopodoume 2 (latitude 5.339079; longitude -4.12426), Adzope (latitude 6.107145; longitude -3.855350), Aghien (latitude 5.3887570; longitude -3.9954967), Bodo (latitude 5.916666; longitude -4.683332), Tiassale (latitude5. 904262; longitude -4.826142) (Table 1).

No	Source	Strains ID	Geographic origin	Date collection
1	Environmental	Adios1	Adiopodoume 1	Sep-15
2	Environmental	Adios1	Adiopodoume 1	Sep-15
3	Environmental	Adios2	Adiopodoume 2	Sep-15
4	Environmental	Adios2	Adiopodoume 2	Sep-15
5	culture	Tias1	Tiassale	Apr-15
6	culture	Ad2	Adiopodoume 2	Apr-15
7	culture	Ad3	Adzope	May-15
8	culture	Ag4	Agboville	Jun-15
9	culture	Adz5	Adzope	Jul-15
10	culture	Adz6	Adzope	Aug-15
11	culture	Adz7	Adzope	Jun-14
12	culture	Agh8	Aghien	Jun-15
13	culture	Agh9	Aghien	Mar-15
14	culture	B10	Bodo	Jun-15
15	culture	E1-N	Adiopodoume 1	Oct-15
16	culture	E1-L	Adiopodoume 1	Oct-15
17	culture	E1-G	Adiopodoume 2	Oct-15
18	culture	S2-L	Adiopodoume 2	Oct-15
19	clinical	181UB15	Daloa	Sep-15
20	clinical	226UB15	Divo	Oct-15
21	clinical	235UB15	Abidjan	Oct-15
22	clinical	236UB15	Tiassale	Oct-15
23	clinical	249UB15	Zohouele	Oct-15
24	clinical	277UB15	Kong	Oct-15
25	clinical	287UB15	Daloa	Oct-15
26	clinical	297UB15	Toumodi	Oct-15

Table 1: Samples used in this study.

DNA Extraction from environmental and clinical samples

The Mobio Powersoil Kit (MOBIO, USA) was used with minor modifications to extract DNA from water and sediment samples. Briefly, 400 µL of samples were added to 60 µL of lysis C1 solution, following by horizontally shaking with the Mobio Vortex and then centrifuged for 30 seconds, 10000 rpm at room temperature. After several incubation by washing and centrifugation, DNA was resuspended in 100 µL of elution buffer. DNA extraction from clinical samples were performed by alcaline heat shock following by ethanol precipitation [28]. The samples were suspended in 2 ml sterile water and centrifuged for 10 min, 15000 rpm at 4°C. 200 µL of pellet were performed in DNA extraction. Each sample was performed twice in DNA extraction. All DNA templates were eluted with 50 µL DNA/Rnase fee H2O and stored at −20°C until use. Negative and positive extraction samples were applied to verify the presence of inhibitors in the samples.

Culture of Mycobacteria from environmental samples

Cultures were performed at 22°C on 3 Lowenstein-Jensen (LJ) solid media: a simple LJ medium, a LJ medium supplemented with NaCl and LJ supplemented with Glycerol. All media were inoculated with 150 µL of inactivated samples. The samples were placed in the dark and in the light for 21 days. Each sample was performed twice in dark and in light incubation. Positive cultures were analyzes using Ziehl-Neelsen (ZN) staining test with microscopy for acid fast bacilli specific for mycobacteria [17].

MIRU-VNTR typing

5 µL of extracted DNA were added to 45 µL PCR-mix containing µM each specific primer for MIRU-1, ST-1, VNTR6, VNTR19, 0.2 mM dNTPs (Sigma, USA), 1X Flexi Taq-polymerase buffer, 1.5 mM MgCl2 and 1Unit Go-Flexi Taq-Polymerase (Promega, Germany). The PCR reaction was described in previous studies [11, 28]. PCR were tested by the 1/10 dilution of DNA, to test the presence of PCR inhibitors in the samples. Negative control (sterile DNase/RNase-free water) and positive control (181UB15 genomic DNA) were included in all PCR runs for the test quality and the detection of decontamination. The PCR was running in a thermocycler (GenAmp 9700, Applied Biosystems, USA).

PCR-RFLP for MIRU-VNTR

The analysis of MIRU-VNTR sequences of 4 markers was performed in the NCBI bank data, and analyzed with Geneious and RestrictionMapper 3.0 software (Geneious 8.1.7; http://www.restrictionmapper.org/). Restriction maps performed with various restriction enzymes were compared to find the best fitting enzyme in order to have the clearest specific profile after digestion. MspI was found to make better DNA profiles. The RFLP was applied with MspI amplified DNA from PCR reactions of MIRU-1, VNTR6, VNTR19 and ST-1.

The amplified DNAs from PCR were purified by using Qiaquick kit (Qiagen, Germany) with manufactor's instructions. 5 µL purified PCR product sequence were added to 15 µL restriction digestion-Mix containing 1X enzyme buffer, 10µg/mL BSA and 0.1U/µL MspI (Promega, Germany). The reaction was incubated for 24 hours at 37°C.

Electrophoresis of DNA amplified products

5µL PCR-product were analyzed by electrophoresis in 1.5% Agarose gel, and migrated at 100 Volts. The size was estimated by comparison with a 100 bp ladder (Promega, Germany). For RFLP-product, 20 µL of digested with MspI PCR-product were analyzed by electrophoresis in 3% Agarose gel at 85 Volts.

Results

Culture of mycobacteria

From 24 samples in culture, 62.5% (15/24) were positive and confirm by ZN staining specific for mycobacteria (Table 2). Water samples have the most positive cultures. All positive cultures were observed after 6 days, rapid growing mycobacteria, no slow-growing culture were positive from environmental samples. All positive mycobacteria were from endemic sites.

Genotyping by MIRU-VNTR

ST-1 was the most amplified locus by 57.4% (15/26) of the samples, while 42.3% (11/26), 30.8% (8/26) and 23.1% (6/26) for MIRU-1, VNTR6, and VNTR9 respectively (Table 2).

Variables			PCR Analysis (bp size)			
Samples ID	Source	Geographical origin	MIRU-1	ST-1	VNTR 6	VNTR 19
Adios1	sediment	Adiopodoume 1	-	-	-	-
Adiow1	water	Adiopodoume 1	-	-	-	-
Adios2	sediment	Adiopodoume 2	-	-	-	-
Adiow2a	water	Adiopodoume 2	530	-	-	-
Total 1 (%)			1/4(25%)	0/4(0%)	0/4(0%)	0/4(0%)
Tias1	water	Tiassale	-	-	-	-
Ad2b	water	Adiopodoume 2	-	420	-	-
Ad3	water	Adzope 3	380	420	-	-
Ag4	water	Agboville	-	-	-	-
Adz5	water	Adzope 3	-	420	-	-
Adz6c	water	Adzope 3	-	420	-	350
Adz7	water	Adzope	-	-	-	-
Agh8	water	Aghien	-	420	-	-
Agh9d	water	Aghien	-	-	500	-
B10	water	Bodo	-	420	500	-
E1-N	water	Adiopodoume 1	380	420	-	-
E1-L1e	water	Adiopodoume 1	380	420	-	-
E1-G2e	sediment	Adiopodoume 1	530/380	420	-	-
S2-L	sediment	Adiopodoume 2	530	420	-	-
Total 2 (%)			5/14 (35.7%)	10/14 (71.4%)	2/14 (14.3%)	1/14 (7.1%)
181UB15	swab	Daloa	530	420	500	350

226UB15	swab	Divo	-	-	500	350
235UB15	swab	Abidjan	-	420	500	-
236UB15f	swab	Tiassale	530	420	500	350
249UB15	swab	Zohouele	-	-	-	-
277UB15	swab	Kong	530	420	500	350
287UB15	swab	Daloa	530	420	500	350
297UB15	swab	Toumodi	480/380	-	-	-
Total 3 (%)			5/8 (62.5%)	5/8 (62.5%)	6/8 (75%)	5/8 (62.5%)
Global (%)			11/26 (42.3%)	15/26 (57.7%)	8/26 (30.8%)	6/26 (23.1%)

Table 2: MIRU-VNTR typing of Mycobacteria from different matrices.

Adiow2a, Ad2b, Adz6c, Agh9d, E1-L1e, E1-G2e, 236UB15f were selected for RFLP-analysis.

By clinical samples from BU patients all genotyping markers were amplified MIRU-1, VNTR19, ST-1, by 62.5% (5/8), and by 75% (6/8) for VNTR6 (Table 2). MIRU-1 was detected in clinical samples with 3 profiles corresponding to one copy (380 bp), 3 copies (486 bp) and 5 copies (530 bp).

By environmental samples, only MIRU-1 was detected in water samples. In positive cultures of mycobacteria, ST-1 was most amplified by 71.4% (10/14) and by 35.7 % (5/14), 14.3 % (2/14), 7.1% (1/14), for MIRU-1, VNTR6 and VNTR19 respectively (Table 2).

RFLP-Analyzing

From all including samples, only positive PCR-typing was analyzed in RFLP-Analyzing. We compared the digestion profile of the 500 bp or 380 bp of MIRU-1 from 3 matrices.

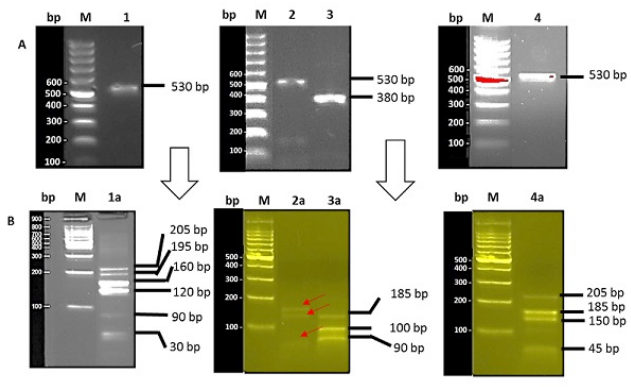

Figure 1: PCR-RFLP analysis with MIRU-1 for clinical, culture and environmental samples. M: 100 bp DNA ladder. (A) PCR-product of MIRU-1; 1: clinical sample (236UB15), 2-3: cultures samples (E1-G, E1-L); 4: environmental sample (Adiow2); (B) Digested with endonuclease MspI; 1a: clinical sample (236UB15); 2a-3a: culture samples (E1-G, E1-L); 4a: environmental sample (Adiow2).

RFLP from MIRU-1 by clinical samples showed an identical profile with DNA bands of 205, 195, 160, 120, 90 and 30 bp (Figure 1). For environmental samples and culture 3 major bands by 185, 100, 50 bp or 205,185, 150, 45 bp were observed respectively (Figure 1). RFLP from ST-1 by clinical sample (236UB15), had an identical profile with DNA fragments 190, 90, 60, and 30 bp (Figure 2). For culture strain (Ad2), the RFLP showed 2 corresponding bands of 190 and 90 bp. No environmental sample has been analyzed in RFLP-method for ST-1.

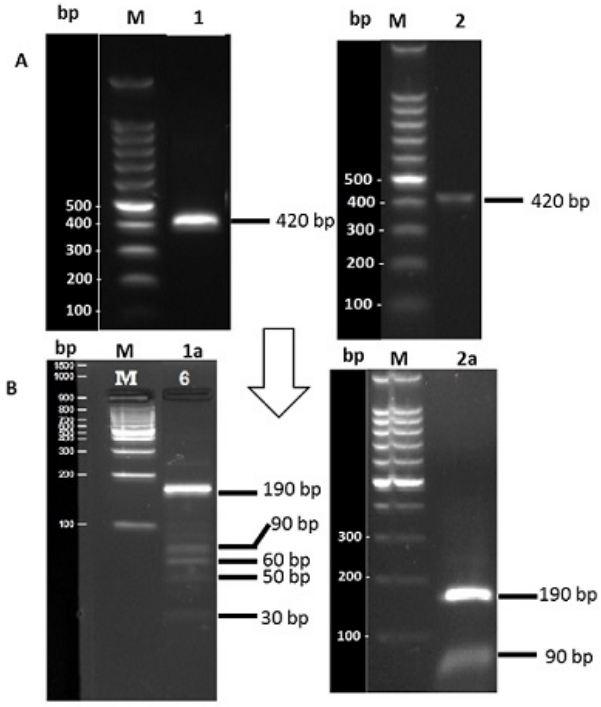

Figure 2: PCR-RFLP analysis with ST-1 for clinical and culture samples. M: 100 bp DNA ladder. (A) PCR-ST-1 product; 1: clinical sample (236UB15); 2: culture sample (Ad2); (B) Digested with endonuclease MspI; 1a: clinical sample (236UB15); 2a: culture sample (Ad2).

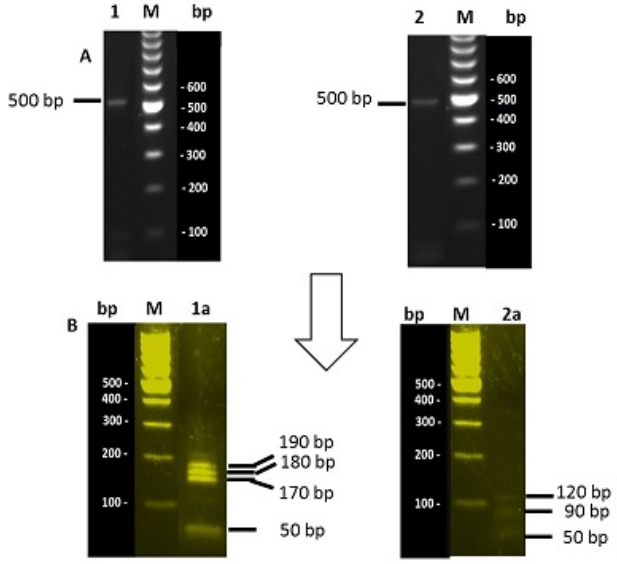

Figure 3: PCR-RFLP-analysis with VNTR6 for clinical and culture samples. M: 100 bp DNA ladder. (A) PCR-VNTR6 product; 1: clinical sample (236UB15); 2: culture sample (Agh9); (B) Digested with endonuclease MspI; 1a: clinical sample (236UB15); 2a: culture sample (Agh9).

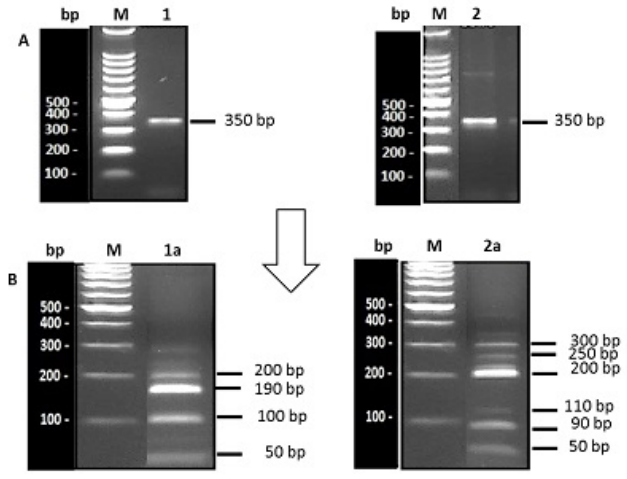

Figure 4: PCR-RFLP analysis with VNTR19 for clinical and culture samples. M: 100 bp DNA ladder. (A) PCR-VNTR19-product; 1: clinical sample (236UB15); 2: culture sample (Adz6); (B) Digested with endonuclease MspI; 1a: clinical sample (236UB15); 2a: culture sample (Adz6).

RFLP for VNTR6 showed an identical profile for clinical samples with different DNA bands with 190, 180 and 170 bp (Figure 3). For culture sample (Agh9), 3 DNA bands with 190, 90 and 50 bp were detected (Figure 3). No environmental sample was analyzed in RFLP-method for VNTR6. The RFLP-VNTR19 detected 4 DNA bands (200, 190, 150, 50 bp) for clinical sample (236UB15), while in culture sample (Adz6), 5 bands were detected with different approximated size of 300, 250, 200, 110, 90, and 50 bp (Figure 4). No environmental sample was analyzed in RFLP-method for VNTR19.

Discussion

M. ulcerans, the agent of BU is endemic in Ivory Coast, and its epidemiology unknown. The evidence of environmental transmission of MU and MPMs from molecular studies were essential to discriminating clinical and environmental or their interaction. In this study, we have demonstrated that specific markers from mycobacteria were not detected in different samples in the environment and in clinical samples. The high amplification rate by PCR was detected for ST-1 by 57% for all samples. Our results were similar with the findings of Kakou et al. [28] for the polymorphism of MU for clinical samples in Ivory Coast. The authors have found the circulation of 2 clones in the same endemic sites and the high amplification rate of ST-1 by 85%. Previous studies have detected different genotypes and profiles of MU in West Africa [13]. In this study, the using of endonuclease enzyme MspI to confirm the DNA profiles of amplified PCR-VNTR has demonstrated that clinical and environmental samples have difference in genotyping's markers.

We have detect only rapidly growing mycobacteria, in contrast of findings of Aboagye et al. [29], the have detected 60% of slow growing mycobacteria in Ghana. This could be explain by the use of another decontamination method in this study and the difficulties of the growth mycobacteria from environmental samples [29]. In Ivory Coast, molecular tests have confirm the BU incidence in the country [19] and reveals the diversity of circulating strains in humans in endemic sites, but the goal is to elucidate the correlation between all nontuberculosis mycobacteria in the environment involved in the incidence of BU in the country.

Recently, Ablordey et al. [30] have demonstrated using whole genome sequencing that two exotic genotypes were found in an endemic site in Ghana with highly restricted intra-strain genetic variation. Our finding shown the profiles difference between genotyping markers. MIRU-VNTR are variable repeat sequences with some new deletion or insertion in the repeat loci, we suggest the evidence of mutation in the MIRU-VNTR loci from the environment in human. This can been explain the difference in RFLP-method from clinical, culture or environmental matrices. VNTR-MIRU could be an adaptation genotyping marker of mycobacteria during infection from environment to human. The using of RFLP-method is consistent to detect low rate of gene mutations in cancer cells [31].

The high specificity and sensitivity of genotyping markers for MU and MPMs cannot be the evidence for detection in all matrices and in particular in endemic sites of MPMs. This study confirmed those VNTR profile variability between MU clinical strains in one hand, but also between environmental mycobacteria strains. The use of RFLP with MspI to confirm each amplified marker, gives better resolution of MIRU-VNTR-typing method. The RFLP-profiles confirm the difference in MIRU-VNTR markers. Chemlial et al. [32] has demonstrated the high diversity of MU strains by using RFLP with IS2404. IS2404 is located both in chromosome and in plasmid in MU and was abundant copies in mycobacteria. Our study is only focused on chromosome genome for all genotyping markers to elucidate the polymorphism of mycobacteria.

Conclusion

Buruli ulcer is endemic in Ivory Coast, and the need to control and understand the transmission from the environment to human is very important. We used molecular tools to elucidate the diversity of circulating strains in different matrices. Our results confirm the existence of polyclonal infection of BU in the high endemic country and the spread of several mycobacteria strains in the environment. We have developed first a PCR-RFLP sensitive, easier and inexpensive approach to confirm genotyping of nontuberculosis mycobacteria in endemic countries for environmental screening. In perspective, we will explore the whole genome sequencing for circulating strains in Ivory Coast.

Acknowledgments

We thank Rousseau Djaouka for critical review of the analysis methods and Miriam Eddyani for the providing of positive strains. Financial funding of RIIP (Institute Pasteur Network) for Gregoire Quinet.

Author Contributions

Conceived and designed the experiments: KNS, DM. Performed the experiments: GQ, VS, SA, HK, KS. Analyzed the data: GQ, KNS, VS. Contributed reagents/materials/analysis tools: AK, AS, CDD. Wrote the paper: KNS, GQ.

References

1. Walsh DS, Portaels F, Meyers WM (2010) Recent advances in leprosy and Buruli ulcer (Mycobacterium ulcerans infection). Curr Opin Infect Dis 23: 445-455.
2. WHO (2010). Number of new cases of Buruli ulcer reported, 2010.
3. Buruli ulcer: progress report, 2004-2008 (2008). Wkly Epidemiol Rec 83:145-156.
4. WHO Global health observatory data. Available: http://www.who.int/gho/neglected_diseases/buruli_ulcer/en/. Accessed 26 June 2015
5. Portaels F, Meyers WM, Ablordey A, Castro AG, Chemlal K, et al. (2008). First cultivation and characterization of Mycobacterium ulcerans from the environment. PLoS Negl Trop Dis. 2: e178.
6. Merritt RW, Walker ED, Small PLC, Wallace JR, Johnson PDR, et al. (2010) Ecology and transmission of Buruli ulcer disease: a systematic review. PLOS Negl Trop Dis. 4: e911.
7. Marsollier L, Robert R, Aubry J, Saint Andre JP, Kouakou H, et al. (2002). Aquatic insects as a vector for Mycobacterium ulcerans. Appl Environ Microbiol 68: 4623-4628.
8. Williamson HR, Benbow ME, Nguyen KD, Beachboard DC, Kimbirauskas RK, et al. (2008) Distribution of Mycobacterium ulcerans in Buruli Ulcer endemic and non-endemic aquatic sites in Ghana. PLOS Negl Trop Dis 2: e205.
9. Doannio JM, Konan KL, Dosso FN, Kone AB, Konan YL, et al. (2011). Micronecta sp (Corixidae) and Diplonychus sp (Belostomatidae), two aquatic Hemiptera hosts and/or potential vectors of Mycobacterium ulcerans (pathogenic agent of Buruli ulcer) in Cote d'Ivoire. Med Trop 71: 53-57.
10. Marion E, Deshayes C, Chauty A, Cassisa V, Tchibozo S, et al. (2011) Detection of Mycobacterium ulcerans DNA in water bugs collected outside the aquatic environment in Benin. Med Trop 71: 169-172.
11. Williamson HR, Benbow ME, Campell LP, Johnson CR, Sopoh G, et al. (2012) Detection of Mycobacterium ulcerans in the environment predicts prevalence of Buruli ulcer in Benin. PLOS Negl Trop Dis 6: e1506.
12. Konan KL, Doannio JM, Coulibaly NG, Ekaza E, Marion E, et al. (2015) Detection of the IS2404 insertion sequence and ketoreductase produced by Mycobacterium ulcerans in the aquatic Heteroptera in the health districts of Dabou and Tiassalé in Côte d'Ivoire. Med Sante Trop 25: 44-51.
13. Narh CA, Mosi L, Quaye C, Dassi C, Konan DO, et al. (2015). Source tracking Mycobacterium ulcerans infections in the Ashanti region, Ghana. PLOS Negl Trop Dis 9: e0003437.
14. Tian RBD, Niamke S, Tissot-Dupont H, Drancourt M, (2016) Detection of Mycobacterium ulcerans DNA in the Environment, Ivory Coast PLOS ONE 11: e0151567.
15. Fyfe JAM, Lavender CJ, Handasyde KA, Legione AR, O'Brien CR, et al. (2010). A major role for mammals in the ecology of Mycobacterium ulcerans. PLOS Negl Trop Dis 4: e791.
16. Dassi C, Mosi L, Akpatou B, Narh CA, Quaye C, et al. (2015) Detection of Mycobacterium ulcerans in Mastomys natalensis and Potential Transmission in Buruli ulcer Endemic Areas in Côte d'Ivoire Mycobact Dis 5: 3.
17. Portaels F, De Muynck A, Sylla MP (1988) Selective isolation of mycobacteria from soil: a statistical analysis approach. J Gen Microbiol 134: 849-855.
18. Fyfe JAM, Lavender CJ, Johnson PDR, Globan M, Sievers A, et al. (2007) Development and application of two multiplex real-time PCR assays for the detection of Mycobacterium ulcerans in clinical and environmental samples. Appl Environ Microbiol 73: 4733-4740.
19. Ngazoa-Kakou ES, Ekaza E, Aka N, Coulibaly-N'Golo D, Coulibaly B, et al. (2011) Evaluation of real-time PCR for Mycobacterium ulcerans in endemic region in Côte d'Ivoire. Af J Microbiol Res 5: 2211-2216.
20. Narh CA, Mosi L, Quaye C, Tay SCK, Bonfoh B, et al. (2015) Genotyping Tools for Mycobacterium ulcerans-Drawbacks and Future Prospects. Mycobact Dis 4: 149.
21. Stinear TP, Mve-Obiang A, Small PL, Frigui W, Pryor MJ et al. (2004) Giant plasmid-encoded polyketide synthases produce the macrolide toxin of Mycobacterium ulcerans. Proc Natl Acad Sci 101: 1345-1349.
22. Ablordey A, Swings J, Hubans C, Chemlal K, Locht C, et al. (2005). Multilocus Variable-Number Tandem Repeat Typing of Mycobacterium ulcerans. Journ Clin Microbiol 43: 1546-1551.
23. Stragier P, Ablordey A, Meyers WM, Portaels F, (2005). Genotyping Mycobacterium ulcerans and Mycobacterium marinum by Using Mycobacterial Interspersed Repetitive Units. Journ Bacteriol 1639-1647.
24. Hilty M, Yeboah-Manu D, Boakye D, Mensah-Quainoo E, Rondini S, et al. (2006) Genetic diversity in Mycobacterium ulcerans isolates from ghana revealed by a newly identified locus containing a variable number of tandem repeats. Journ Bacteriol 1462-1465.
25. Lavender CJ, Stinear T, Johnson PDR, Azuolas J, Benbow ME, et al. (2008) Evualation of VNTR typing for the identification of Mycobacterium ulcerans inenvironmental samples from Victoria, Australia. FEMS Microbiol Lett 287: 250-255.
26. Loda M (1994) Polymerase chain reaction-based method for the detection of mutations in oncogenes and tumor suppressor genes. Hum Pathol. 25: 564-571.
27. Ota M, Fukushima H, Kulski JK, Inoko H, (2007). Single nucleotide polymorphism detection by polymerase chain reaction-restriction fragment length polymorphism. Nat Protoc. 2: 2857-2864.
28. Kakou ES, Coulibaly ND, Aka N, Vakou S, Aoussi S, Dosso M, et al. (2015) Clonality of Mycobacterium ulcerans by Using VNTR-MIRU Typing in Ivory Coast (Côte d'Ivoire), West Africa. International Journal of Tropical Disease & Health 7: 163-171.
29. Ablordey AS, Vandelannoote K, Frimpong IA, Evans K, Eddyani M, et al. (2015) Whole Genome Comparisons Suggest Random Distribution of Mycobacterium ulcerans Genotypes in a Buruli Ulcer Endemic Region of Ghana. PLOS Neglected Tropical Diseases 9: e0003798.
30. Aboagye SY, Danso E, Ampah KA, Nakobu, Asare P, et al. (2016) Isolation of Nontuberculous Mycobacteria from the Environment of Ghanian Communities Where Buruli Ulcer Is Endemic. Appl Environ Microbiol 14: 4320-4329.

31. Li WM, Hu TT, Zhou LL, Feng YM, Wang YY, et al. (2006). Highly sensitive detection of the PIK3CAH1047R mutation in colorectal cancer using a novel PCR-RFLP method. BMC Cancer 16:454.

32. Chemlal K, Huys G, Fonteyne PA, Vincent V, Lopez AG, et al. (2001). Evaluation of PCR-Restriction Profile Analysis and IS2404 Restriction Fragment Length Polymorphism and Amplified Fragment Length Polymorphism Finger printing for Identification and Typing of Mycobacterium ulcerans and M. marinum. Microbiol 39: 3272–3278.