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Detection of *Mycobacterium ulcerans* in *Mastomys natalensis* and Potential Transmission in *Buruli ulcer* Endemic Areas in Côte d'Ivoire

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Abstract

The endemicity of Buruli ulcer (BU), a non-tuberculous mycobacteria infection, has significantly increased in Côte d'Ivoire. The exact transmission mode remains unknown but DNA based evidence of *Mycobacterium ulcerans*, the causative agent, have implicated potential environmental reservoirs, similar to those suspected in the transmission to humans. The role of small mammals in transmission has recently received some research attention. Based on the hypothesis that the overlapping ecology of human and animal habitats would favour mycobacteria transmission, the study aimed to identify BU like infections in small mammals living in close proximity to humans, in endemic communities. One hundred and eleven animals were trapped within five communities in two major endemic areas, Daloa and Tabbo. Majority of trapped small mammals were mice, *Mastomys natalensis*, of which 8 animals had external lesions. PCR on organ and lesion samples identified, predominantly, mycobacterium spp, of which five mice were positive for IS2404. IS2404 sequencing confirmed infection in two mice as *M. ulcerans* strain Agy99. Our findings suggest that small terrestrial mammals could be susceptible to mycobacteria, particularly, *M. ulcerans*, within endemic communities in Côte d'Ivoire. It also consolidates earlier evidence asserting their role as potential environmental reservoirs of *M. ulcerans* in endemic communities. The potential public health threat from these small mammals warrants an "OneHealth" approach to investigating the ecology and transmission of *M. ulcerans* in endemic countries.

Keywords: Non-tuberculous mycobacteria; Small mammals; Transmission; Reservoirs; *Buruli ulcer*

Introduction

Mycobacterium ulcerans (MU) is the causative agent of *Buruli ulcer* (BU), a severe necrotizing skin infection, occurring in tropical and subtropical regions of the world. It is the third most common mycobacterial disease after leprosy and tuberculosis [1]. *M. ulcerans* is environmental like most non-tuberculous mycobacteria (NTM), which are ubiquitous opportunistic pathogens causing infections in both humans and animals [2]. Although transmission of BU is still unknown, a few hypotheses have been proposed [3,4]. BU is a serious public health problem in developing countries including Benin [1], Côte d'Ivoire [5,6] and Ghana [7,8] where several new cases have been reported in rural areas since 1980. Much of research efforts have focused on risk factors associated with environmental habitat of the pathogen. A case-control study in 3 BU endemic districts in Ghana associated the disease with water bodies [9]. Using variable number tandem repeat (VNTR) typing, a recent study in Ghana showed that BU patients could be infected from MU-contaminated water bodies [10]. Similarly, a study conducted in three BU endemic communities in Côte d'Ivoire associated water bodies used by rural populations to MU infection [11].

Ecological and molecular data, further, suggest contaminated water, soil, detritus and a variety of organisms including protozoans, fish, aquatic insects, mosquitoes, crustaceans, snails and wild amphibians as sources and/or reservoirs of *M. ulcerans* [12-20].

Mammals, especially rodents, living in close proximity to humans have been also implicated as potential reservoirs and/or hosts for mycobacteria, particularly *M. ulcerans* [21-23]. Indeed, the possible role of rodents in the ecology of *M. ulcerans* was also considered over 30 years ago in Uganda (East Africa), where researchers attempted but were unsuccessful to culture *M. ulcerans* from rodents collected in a BU endemic area [24]. Separately, studies in Australia observed skin ulcers on koalas, which were later isolated and confirmed as *M. ulcerans* and *M. scrofulaceum* [25,26]. Recent findings in the same country showed high concentrations of *M. ulcerans* DNA in possum faeces [23]. In West Africa, rodents and some insectivores like shrews may carry pathogenic mycobacteria [21] and this has been proven in experimental infections in animal models [27-30].

A study conducted in Benin detected different mycobacteria, but not *M. ulcerans*, in small mammals collected in BU endemic communities. The authors advocated for more research on small terrestrial mammals [22].

The reservoirs and source of transmission to humans remains unclear and whether small mammals have a role in transmission is an important question, which has received little attention. To address this question, surveys of small mammals are urgently required in BU endemic areas. The cases of natural infections reported in wild and domesticated animals (koalas, possums and an alpaca) in Australia,

suggest that BU may cause lesions clinically identical to those observed in humans. Both humans and animals are probably infected through contact with environmental sources of *M. ulcerans* [31]. To extend findings from studies in Ghana [10], suggesting that transmission of MU depends on the overlap between human and environmental habitat of the pathogen, we aimed to identify BU like infections in small mammals which lived in close proximity to human environments, including homes and water bodies used by the communities in endemic areas. We present data, both clinical and molecular evidence, suggesting that small mammals may be susceptible to *M. ulcerans* infections and may be acting as reservoirs.

Materials and Methods

Study communities and animal collection sites

Study sites were selected based on data provided by the National Program for BU control (PNLUB) and BU endemicity. They were located in two major BU endemic areas in Côte d'Ivoire with characteristic aquatic ecosystems (Figure 1). The first area, Daloa (Haut-Sassandra District), is located in the western centre, 406 km from Abidjan, the economic capital. This area is covered largely with forest and has the Sassandra River tributaries, which are used for agriculture irrigation and fishing activities. Gorodi and Zaïbo were the two selected rural communities. The second area, Taabo (Tiassalé Department), is located in the south eastern, 160 km from Abidjan. This area has three communities, Sokrogbo, Léléblé and Ahondo, with reported high BU incidences. Also, peculiar to this second area were the different environmental modifications, attached to Bandama River, including the hydroelectric dam construction, displaced populations with attached socio-economic activities.

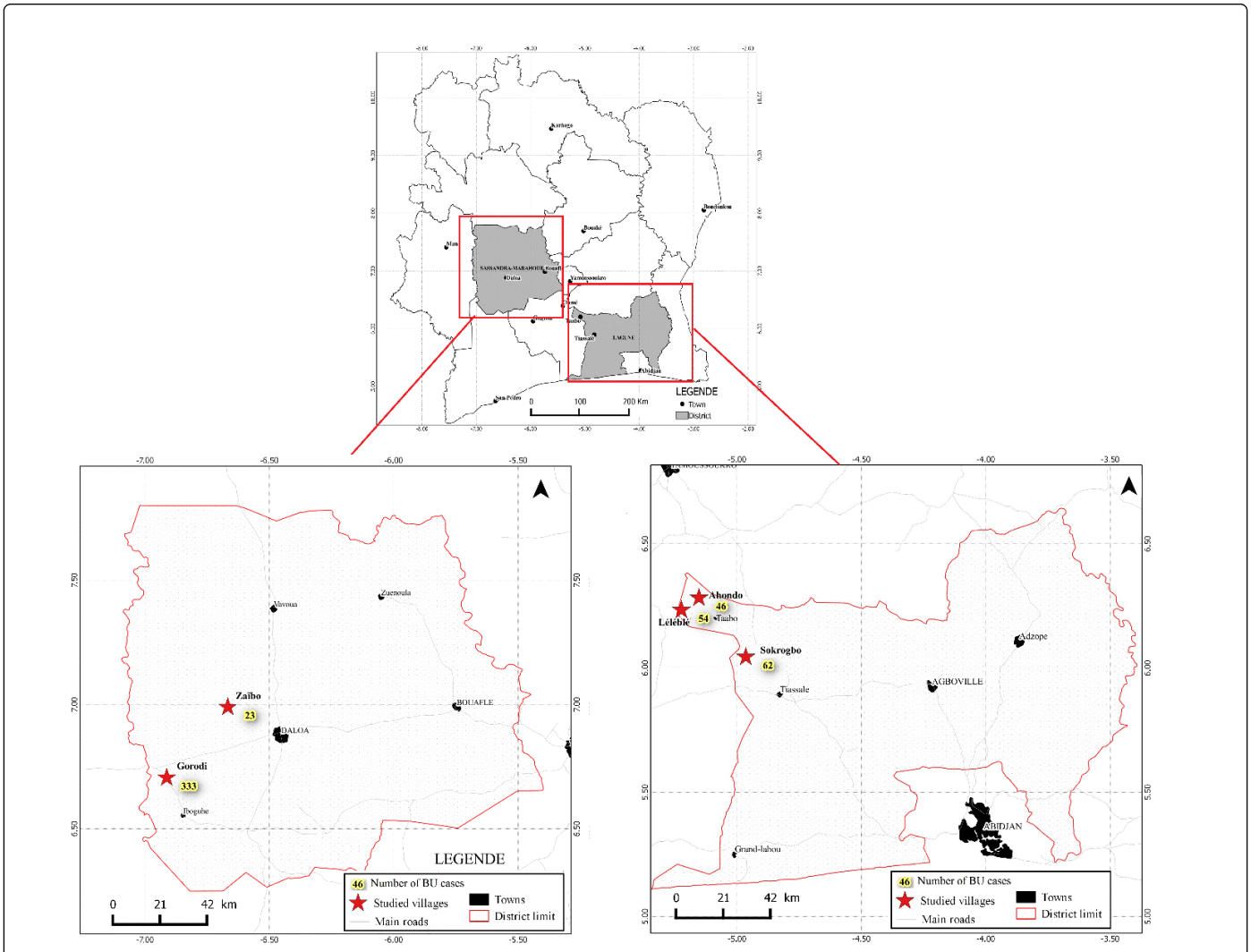

Figure 1: Location of the sampling sites with BU incidence for last 4 years. Daloa and Taabo areas are located in the western centre and south eastern, respectively, in Côte d'Ivoire. Studied sites selected had a considerable number of BU cases registered from health centers within the communities; Zaïbo, Gorodi (lower left) and Léléblé, Sokrogbo, Ahondo (lower right).

Social and demographic characteristics of the population as well as contact rates of the community members with various animal types and aquatic environments were estimated by the use of structured questionnaires.

Questionnaire administration

Five hundred questionnaires were administered in the study communities. The questionnaires inquired mainly the demographic characteristics of the community inhabitants, main occupations of households, common diseases, knowledge of BU, contact with animals (types, raised and captured), and the prevention and treatment of *Buruli ulcer*. Communities were stratified into subsets based on population and reported cases of BU. Questionnaires were mainly administered to a member of the household in the French language. Local languages were used only to verify unclear responses using the help of a trained local community aid.

Small mammals trapping, identification and specimen preservation

Small mammals were investigated simultaneously in two different habitat types at each site (water bodies usually used by the communities and within the villages, precisely in houses). Animal trapping was done in the communities following standard live-trapping techniques [32] and protocol described by Narh et al. [10]. Examination for suspected lesion and organ harvesting were performed as described elsewhere [10,22]. For each animal, sample specimen were collected and labelled individually.

DNA extraction, gel-based PCR and sequencing analysis

Prior to laboratory analysis animal samples were processed in a Biosafety hood (CYTAIR Equipements Scientifiques & Industriels S.A.) following the protocols described elsewhere [10,22]. Total genomic DNA was extracted from lesion and organ specimen of the eight small rodents following the protocol described by Williamson et al. [19]. DNA extracts were screened for *Mycobacterium sp.* by PCR, which included negative and positive controls. All PCR reactions were performed in a A206 gradient thermal cycler (LonGene®). Samples were initially screened for bacteria species using the 16S primer as described by Narh et al. Then, 16S positive samples were tested for the presence of bacteria which harbour the IS2404 insertion sequence using IS2404-PCR. Subsequently, positive samples from the latter were tested for the presence of pMUM001 using ER-PCR [10]. Sequencing of 16S and IS2404 PCR products was performed using the forward primers as described elsewhere [10]. Sanger sequencing was performed by Macrogen Inc (Netherlands).

Data analysis

Data was recorded and stored in Microsoft Excel. The mean trapping success in all selected sites was calculated and compared. Sequences were aligned using NCBI BLAST [33].

Results

Human activities related to animal

Of the 500 questionnaires, 288 (57.6%) were administered to males and 212 (42.4%) to females. Ages of respondents were ranged from 18 to 98 years old. The proportions of respondents involved in animal related-economic activities are shown in Figure 2. A higher proportion of respondents hunted for animals in the wild compared to those having domestic animals. Mostly animals hunted were rodents (85% for grass-cutters and 72% for rats).

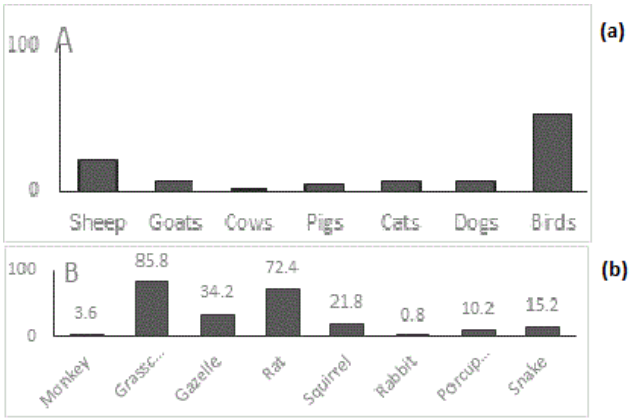

Figure 2: Percentage of respondents involved in animal related economic activities. (a) Animals reared in households. (b) Animals hunted for by respondents.

Trapping effort and traps success

After performing animal capture during two nights, a total of 111 animals were caught representing 11.46% of capture success (Table 1).

Community	Trapping effort			Trapping success	Performance (%)
	Night 1	Night 2	Total		
Zaïbo	100	99	199	11	5.53
Gorodi	100	90	190	34	17.89
Léléblé	100	90	190	31	16.32
Sokrogbo	100	100	200	14	7
Ahondo	95	95	190	21	11.05
Total	495	474	969	111	11.46

Table 1: Relative density of small mammals captured in BU endemic areas in Côte d'Ivoire.

Small rodent community analysis

The diversity of small mammals caught in the 5 communities is summarised below (Table 2). Different species of small rodents were identified among those trapped including *Mastomys natalensis* (74.78%), *Crocidura sp.* (11.71%), *Rattus sp.* (9.01%), *Mus musculoides* (1.80%), *Praomys rostratus* (1.80%) and *Iophuromys sikapusi* (0.90%). The majority of animals trapped were *Mastomys natalensis*.

Distribution and data of animal presenting external lesions within communities

The proportion of small rodents observed with external lesions were 7.2% (N=111) (Table 3). Animals presented lesions were identified as *Mastomys natalensis* (Table 4) and lesions were observed on the tail, hind foot, foot, abdomen, lateral side and bottom. Identical numbers of animals observed with lesions were found for both female and male rodents. At least, one small rodent with lesion was present in each study community and all were of adult age. They were trapped within the community (homes).

Species	Number	Percentage (%)
Mastomys natalensis	83	74.78
Crocidura olivieri	9	8.11
Crocidura poensis	4	3.60
Rattus rattus	9	8.11
Rattus norvegicus	1	0.90
Mus musculoides	2	1.80
Praomys rostratus	2	1.80
Lophuromys sikapusi	1	0.90
Total	111	100

Table 2: Diversity of capture within small rodents community.

Community	No external lesions	External lesions
Zaïbo	10	1
Gorodi	32	2
Léléblé	30	1
Sokrogbo	11	3
Ahondo	20	1
Total	103	8

Table 3: Distribution of small rodents with external lesions per community.

Area	Community	Trap Location	Animal Code	Species	Sex/Age	Lesion location	Sample specimen
Daloa	Zaïbo (Niessoko)	House	ZN1	*Mastomys natalensis*	Female Adult	Not specified	Lesion biopsy
							Lesion swab
	Gorodi (Ponagbeu)	House	GP3a	*Mastomys natalensis*	Female Adult	Tail	Lesion biopsy
		House	GP6	*Mastomys natalensis*	Female Adult	Hind foot	Lesion biopsy
							Lesion swab
Taabo	Léléblé (Djandjikro)	House	LDj3a	*Mastomys natalensis*	Male Adult	Foot	Lesion biopsy
							Lesion swab
	Sokrogbo (Troko)	House	ST1	*Mastomys natalensis*	Male Adult	Abdomen	Lesion biopsy
	Sokrogbo (Kpala)	House	SK3a	*Mastomys natalensis*	Male Adult	Lateral side	Lesion swab
	Sokrogbo (Kpala)	House	SK3b	*Mastomys natalensis*	Female Adult	Foot (swelling)	Fine needle aspiration
	Ahondo (Awlobo 1)	House	AA1-2a	*Mastomys natalensis*	Male Adult	Bottom	Lesion swab

Table 4: Data of small rodents with external lesions.

Detection of *Mycobacteria*/*M. ulcerans* in animals presenting external lesions

PCR analysis of 16S rRNA and IS2404 markers for lesion samples detected bacteria DNA in 5 mice (Figure 3).

The amplification of enoyl reductase (ER) marker from lesion did not show any positivity for the protocol we used. Besides that, 16S rRNA positivity was observed for some organs harvested from the animals with lesions (Table 5).

Animal Code	Lesion site	IS2404 lesion positivity	ER lesion positivity	16S rRNA lesion and organ positivity	Organism (% Sequence identity)
GP3a	Tail	LB	none	LB, SP, KI, LI, LU, STO, AS, SI, CS, HT	LB, *M. ulcerans* Agy99 (98%)
GP6	Hind foot	LB, LS	none	LB, LS, SP, KI, LI, LU, STO, AS, HT	LB, *Corynebacterium kutscheri* strain MYL-3 (86%) LS, *Arthrobacter spp.* (75%)
ST1	Abdomen	LB	none	LB, SP, KI, LI, LU, STO, AS, CS, HT	LB, *Corynebacterium kutscheri* strain MYL-3 (99%)
SK3b	Foot	Pus FNA	none	FNA, SP, KI, LI, LU, AS, HT	FNA, *M. ulcerans* Agy99 (94%)
AA1-2a	Bottom	LS	none	LS, SP, KI, LI, STO, AS, CS	LS, *Corynebacterium kutscheri* strain MYL-3 (98%)
SP: Spleen, KI: Kidney, LI: Liver, LU: Lung, STO: Stomach, AS: Anal Swab, SI: Small Intestine, CS: Caecum, HT: Heart, LB: Lesion Biopsy, LS: Lesion Swab, FNA: Fine Needle Aspiration.					

Table 5: Data of small rodents with external lesions tested positive for bacteria/NTM.

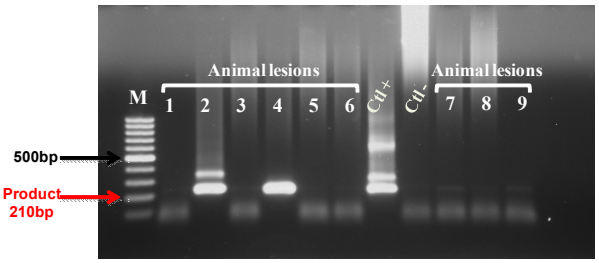

Figure 3: Identification of NTM/*M. ulcerans* from animals with suspected lesions DNA after IS2404 PCR. M: 100bp DNA ladder; Ctl^+: positive control (MU positive culture); Ctl^-: negative control (water); lanes 1-6, 7-9: animal lesions specimens tested.

Sequence analysis of the amplified 16S rRNA and IS2404 from the lesion samples confirmed bacteria as shown previously. *Corynebacterium kutscheri* strain MYL-3 was detected in 3 lesions on hind foot, abdomen and bottom sites respectively for animals with GP6, ST1 and AA1-2a test codes. *Arthrobacter spp.* was also identified in a lesion infected by *Corynebacterium kutscheri* strain MYL-3 (GP6 animal test code); the sequence matching with *Arthrobacter spp.* S/4 partial 16S rRNA gene, suggesting co-infection. Furthermore, 2 lesions sampled on the tail (GP3a) and foot (SK3b) (Figure 4) were confirmed as *M. ulcerans*. The IS2404 sequence from these two animal lesions collected from Gorodi (Daloa) and Sokrogbo (Taabo) showed 98% and 94% sequence identity to *M. ulcerans* Agy99 (Accession N° CP000325.1, GenBank).

Discussion

Our data presents *M. ulcerans* infection in the African small rodent *Mastomys natalensis*. These rodents were trapped from Gorodi (Daloa) and Sokrogbo (Taabo), two BU endemic communities in the western centre and south-eastern of Côte d'Ivoire. The majority of animal trapped within the study sites were *Mastomys natalensis*. This species is widespread and common in Sub-Saharan Africa. It may be a natural carrier of non-human pathogens due to its semi-commensal habit and specific factors as its high propagation rate [34].

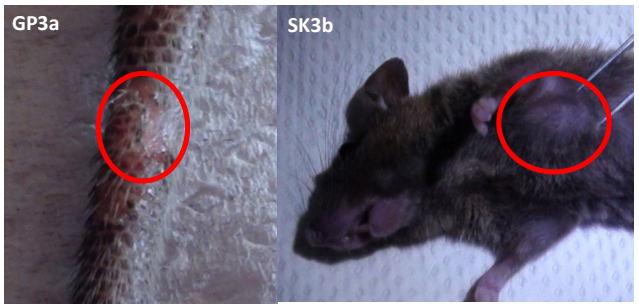

Figure 4: Suspected lesions observed with small rodents trapped from houses. Left: lesion on tail; right: swelling on foot.

After 16S rRNA and IS2404 PCR positivity, IS2404 sequencing showed 98% and 94% identity to *M. ulcerans* strain Agy99 for two lesions specimens on tail and foot. Besides that, 16S rRNA sequencing of suspected lesions confirmed presence of other bacterial strains like Corynebacterium kutscheri and Arthrobacter spp. The detection of bacterial infections in small mammals in this study, particularly mycobacterial infections, reflects previous findings [21,22,35]. Indeed, studies conducted in Benin in order to identify reservoirs for *M. ulcerans* in terrestrial mammals have shown the presence of other mycobacteria in African rodents and insectivores [21] and more particularly different mycobacteria were detected in small mammals within endemic areas for BU [22]. In this setting, although earlier studies conducted in a BU endemic village (Ananekrom) in the Ghana Ashanti Region failed to detect *M. ulcerans* in the organs or faeces of rodents and shrews, the hypothesis that these small terrestrial mammals may be part of the reservoir of *M. ulcerans* was not rejected [35].

As shown previously, small mammals in BU endemic areas could also present external lesions similar to those observed in humans. This suggests common sources of infection, the environment [10]. Additionally, experimental trials with grasscutters also revealed that

this small rodents developed lesions characteristic of BU when infected with *M. ulcerans* [27]. In Australia, other terrestrial mammals were implicated in BU transmission; *M. ulcerans* DNA was detected in the faeces of possum leaving in BU endemic sites [23]. These emphasize the major role of small mammals in the ecology and transmission of *M. ulcerans*.

Durnez et al. [21] did not observed *M. ulcerans* DNA in the organs of *Rattus rattus* trapped in Benin. An Australian study suggested that the rodents could be infected with *M. ulcerans* from direct environment shared with other animal species susceptible to *M. ulcerans*, a similar source of *M. ulcerans* could be absent in Benin [21]. In the same way, Fyfe et al proposed that infected mammals may ingest *M. ulcerans* from the environment or get infected by an insect vector [23].

Thus, transmission to humans may occur via these vectors or from direct contact with contaminated environment. These findings strengthen the hypotheses that both humans and animals could probably be infected through contact with environmental sources of *M. ulcerans* [31]. Thereby, regarding the possible role of small mammals in the dissemination of *M. ulcerans*, the mode of contamination from environment to humans could be linked to overlapping and sharing of ecological habitats. Several field observations during the study and data gotten from questionnaire, suggest that inhabitants' lifestyle, activities and socio cultural behaviours may be risk factors for MU infection; then the detection of *M. ulcerans* in these rodents may enhance these risk factors. One more point may be to determine if as in a disease reservoir, the pathogen could be permanently maintained and if infection could be transmitted from them to target population (other vector, human) [36]. Regarding the few number of animals presented BU-like lesions observed, trapping effort should be increase within our studies communities. The harvested organs collected from animal which did not present lesions should also be processed for mycobacterial screening as proceed for the ones with lesions. Investigations for potential environmental reservoirs of *M. ulcerans* within small mammals should also be done in other endemic communities in Côte d'Ivoire.

Conclusion

In light of our findings, the role of small terrestrial mammals as potential environmental reservoirs for *M. ulcerans* in sub-Saharan Africa, particularly in West African communities, should not be neglected. The model of 'One Health' concept which has been used in this study highlights the importance to consider an approach taking into account human, animal and environment they share, to understand manage and prevent disease transmission. As suggested by previous studies, the precise role of small mammals particularly rodents in BU transmission has to be clarified in order to develop efficient strategies for BU outbreaks, control and prevention.

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