Am. J. Trop. Med. Hyg., 101(3), 2019, pp. 491–493
doi:10.4269/ajtmh.19-0137

Disseminated *Mycobacterium ulcerans* Infection in Wild Grasscutters (*Thryonomys swinderianus*), Côte d'Ivoire

Nassim Hammoudi,[1,2] Agui Sylvestre Dizoe,[3,4] Sofiane Regoui,[1,2] Bernard Davoust,[1,2] Michel Drancourt,[1,2] and Amar Bouam[1,2]*

[1]*IHU Méditerranée Infection, Marseille, France;* [2]*Aix-Marseille-Univ., Institut de Recherche pour le Développement, Microbes, Evolution, Phylogénie et Infection, Institut Hospitalo-Universitaire Méditerranée Infection, Marseille, France;* [3]*Kongouanou Care Center, Yamoussoukro, Côte d'Ivoire;* [4]*National Buruli Ulcer Control Plan, Abidjan, Côte d'Ivoire*

Abstract. Buruli ulcer is an infectious disease provoking chronic, disabling skin ulcers in mammals and humans. Buruli ulcer is caused by *Mycobacterium ulcerans*, an environmental mycobacterium synthesizing a toxin called mycolactone responsible for the pathogenicity. The reservoirs and the modes of transmission of *M. ulcerans* remain elusive, limiting the prophylaxis capabilities in rural areas in endemic countries. In Australia, several studies have demonstrated the probable role of possums as reservoirs. In Côte d'Ivoire, some studies have speculated on the potential role of grasscutters in the transmission cycle of *M. ulcerans*. In this study, we detected *M. ulcerans*–specific sequences in rectal contents and spleens collected in wild grasscutters hunted in Buruli ulcer–endemic area in Côte d'Ivoire, but not in farmed negative control animals and in domesticated animals, namely, pigs, goats, cattle, and dogs, living in close contact with the local population. Some grasscutters exhibited the same sequence pattern in the feces and spleen. These observations confirm the asymptomatic gut carriage of *M. ulcerans* in this mammal species. Moreover, these observations suggest the dissemination of *M. ulcerans* from the gut to the spleen in grasscutters. These observations suggest that, in some mammals, *M. ulcerans* is not only an inoculated pathogen but also a translocating invasive pathogen.

INTRODUCTION

Mycobacterium ulcerans is responsible for a neglected devastating tropical infection named Buruli ulcer.[1] This WHO-notifiable infection has been reported by 34 different countries in the tropical belt, mainly located in West Africa where Côte d'Ivoire notifies the largest number of cases, along with southeast coastal China, Japan, Australia, and the Atlantic coast of South America.[2] In humans, this noncontagious infection slowly evolves from an edematous lesion of the skin at the putative portal of entry of the pathogen toward extensive cutaneous and subcutaneous ulcers and fibrosis, which is responsible for deformities.[3] Buruli ulcer most likely results from direct contact of unprotected skin with an environment contaminated with *M. ulcerans* after insect and ectoparasite bite, or by direct contact with animal excrements.[4,5]

Indeed, *M. ulcerans* has been detected and reported in water filtrates, biofilms, soil, detritus, aquatic plants, aquatic bugs, mosquitoes, wild amphibians, fishes, and small mammals, such as possums and grasscutters.[2] More convincingly, *M. ulcerans* has been isolated from moss, aquatic Hemiptera insects, possums, and *Thryonomys swinderianus* (grasscutters).[6–8] Accordingly, skin ulcers have been diagnosed in nonhuman mammals, including goats, dogs, and possums, in Buruli ulcer–endemic areas.[9–11] *Mycobacterium ulcerans* has also been firmly confirmed by *M. ulcerans* DNA detection and isolation from two koalas in Raymond Island, Australia, and from 20/27 reported possum cases between 1998 and 2011 in Buruli ulcer–endemic areas of Victoria.[9,10,12] These observations comforted the hypothesis that *M. ulcerans* is an environmental organism and that its transmission is not species specific.

One step forward, it has been proposed that domestic animals in close contact with populations in endemic areas could act as amplifiers of the pathogen, thereby intervening in the dissemination of the pathogen.[13] In Australia, it has been observed that possums excreted *M. ulcerans* DNA in feces and presented clinical symptoms of Buruli ulcer indistinguishable from those reported in patients.[12] Likewise in Côte d'Ivoire, *M. ulcerans* DNA has been repeatedly detected in several animal samples[2] and *M. ulcerans* has been cultured once from *T. swinderianus* feces.[7] Therefore, in this West-African country, *T. swinderianus* and *Mastomys natalensis* are suspected to be part of the epidemiological chain of *M. ulcerans*.[14,15]

Here, we observed that *M. ulcerans* DNA could be detected in the rectal contents and spleens of wild *T. swinderianus*, suggesting that *M. ulcerans* is disseminating in this mammal species.

MATERIALS AND METHODS

Sample collection. Wild *T. swinderianus* (grasscutters) have been captured by hunters in the Yamoussoukro area, Côte d'Ivoire, as part of their routine hunting activities as grasscutters are sold as bushmeat and damage growing rice cultures. This wild animal species is huntable because its International Union for Conservation of Nature conservation status is a "least concern."[16] Hunted grasscutters were eviscerated for domestic usage (bushmeat), and the intestine and spleen of each animal were separately put in sterile bags. The rectum was sectioned using a sterile scalpel to sample the content. A small piece of spleen was sampled using sterile scalpels. The samples were placed separately in sterile tubes containing a homemade sterile Trans *Mycobacterium ulcerans* (MU) transport medium.[7] Feces from domesticated animals, namely, pigs, goats, cattle, and dogs, living in the same environment as the local population was collected separately in sterile tubes. Tubes were preserved at +4°C until used for molecular analyses.

DNA extraction protocols. Total DNA was extracted from rectal contents of 51 wild *T. swinderianus* (grasscutters) and from fecal samples collected in 23 *T. swinderianus* grasscutters from livestock farming using a stool extraction kit according to the manufacturer's instructions (QIAmp®, DNA Stool; Qiagen, Stochach, Germany). Total DNA was extracted

* Address correspondence to Amar Bouam, MEPHI, IHU Méditerranée Infection, Marseille, France. E-mail: amarbouam@yahoo.fr

from 51 *T. swinderianus* grasscutter spleen specimens and from fecal samples of 45 domesticated animals (Table 1). After manual lysis in a Trans MU medium using sterile disposable pestles and vigorous vortexing, 200 µL of the mixture was added to 200 µL of lysis buffer G2 and 20 µL of proteinase K (Qiagen, GmbH, Germany) and incubated at 56°C for 3 hours. A small amount of glass powder was added to the mixture and put through three runs of FastPrep (6.5 m/second) followed by centrifugation for 1 minute at 13,000 × *g*. Two hundred microliters of the supernatant was transferred into a new tube and total DNA extraction was performed on the EZ1 machine (Qiagen, Hilden, Germany). Extracted DNA was stored at –20°C until use.

***Mycobacterium ulcerans* DNA detection.** Total DNA extracted from the 51 rectal content and 51 spleens from wild *T. swinderianus* (grasscutters), 23 fecal samples of domesticated *T. swinderianus* (grasscutters), and 45 fecal samples of different animals were subjected to real-time polymerase chain reaction (PCR) assays to detect IS2404 and IS2606 insertion sequences and the ketoreductase-B domain of *M. ulcerans* plasmid (KR-B) as previously described.[17] Five microliters of RNase-DNase–free water (Invitrogen, Villebon sur Yvette, France) was incorporated instead of extracted DNA in the reaction mixture as negative controls. Also, total DNA extracted from a 6-week old *M. ulcerans* strain CU001 culture was used as a positive control to generate a calibration curve. Any sample was considered to be PCR positive for *M. ulcerans* when the negative control was undetectable and at least one of the two insertion sequences IS2404 and IS2606 and the KR-B gene were detected with a Ct ≤ 33 cycles.

RESULTS

Positive controls used in our real-time PCR assays were positive for all targeted sequences, namely, IS2404, IS2606, and KR-B, and negative controls introduced into each real-time PCR batch remained negative. PCR assays on 51 rectal specimens collected from wild grasscutters hunted by the local population revealed 10 samples (19.6%) positive for *M. ulcerans* DNA, including two samples (20%) positive for all three systems IS2404, IS2606, and KR-B sequences; six samples (60%) positive for IS2404 and KR-B; and two samples (20%) positive for IS2606 and KR-B. Twenty-one samples (40.3%) were positive only for one sequence (IS2404, IS2606, and KR-B, 35.3%, 17.6%, and 70.6%, respectively). Two samples (3.8%) were positive for IS2404and IS2606. All the feces samples collected from 23 *T. swinderianus* (grasscutters) from livestock farming and feces samples from 45 other animals living in close contact with local populations remained negative for *M. ulcerans* DNA (Tab. 02).

As for spleen specimens, 6/51 (11.7%) of those collected in wild *T. swinderianus* (grasscutters) yielded a positive amplification for all three systems tested, 38/51 (74.5%) were PCR positive for both (IS2404 and KR-B), and no spleen specimen was PCR positive for (IS2606 and KR-B). Furthermore, 6/51 (11.7%) spleen specimens were positive for both (IS2404 and IS2606) but not for KR-B gene and one spleen specimen was positive for IS2404 only (1.9%). Thus, according to our criteria, 44/51 (86.2%) spleen specimens were positive for *M. ulcerans* DNA. Moreover, four animals exhibited the same sequence pattern (IS2404 and KR-B) in feces samples and spleen samples (Supplemental Table 1).

TABLE 1

Results from PCR tests to detect *Mycobacterium ulcerans* in the rectal contents and feces of wild and domesticated animals from Yamoussoukro area, Côte d'Ivoire

Animals	Number	Ketoreductase-B	IS2606	IS2404
Wild grasscutters	51	20	7	10
Farmed grasscutters	23	0	0	0
Goat	18	0	0	0
Pork	16	0	0	0
Sheep	2	0	0	0
Bovine	3	0	0	0
Hens	1	0	0	0
Total	114	20	7	10

DISCUSSION

Data here reported indicate that in a large proportion of wild *T. swinderianus* (grasscutters) captured in the Yamoussoukro area, Côte d'Ivoire, *M. ulcerans* DNA was detected in the rectal content of these animals, as documented in this report by the detection of three DNA sequences in which quantitative co-detection is routinely used as biomarkers for the specific detection of this opportunistic pathogen.[17] Our report, supported by the negativity of the negative controls and the reproducibility of the results, agrees with previous reports by our team and others that *M. ulcerans* DNA could be detected in *T. swinderianus* (grasscutters) feces.[5,7,14] In one instance, we were able to culture one colony of *M. ulcerans* after using an appropriate protocol for decontamination and culture.[7] Thus, cumulative pieces of evidence indicate the intestinal colonization of grasscutters by *M. ulcerans* complex viable mycobacteria in Côte d'Ivoire. Conversely, we did not detect *M. ulcerans* DNA in the feces of domesticated animals, such as pigs, goats, cattle, and dogs, living in the villages in close contact with the local population. These observations agree with one previous report from Togo where feces collected from chicken, goats, sheep, and cattle were negative for the presence of *M. ulcerans* DNA.[18] Among these five domesticated species, goats and cattle are also herbivores like grasscutters, suggesting that some feeding particularities expose grasscutters to contamination. This point warrants further field investigations.

Moreover, *M. ulcerans* DNA was detected in the spleens of studied animals. The fact that negative controls introduced in this study remained negative excluded laboratory contamination and the fact that spleen samples were sampled separately using sterile scalpels excluded cross-contamination from animal to animal. In some animals, the pattern of DNA sequences found in the spleen matched those found in the rectal content. These observations made in wild animals suggested that *M. ulcerans* could translocate from the digestive tract and disseminate in some wild *T. swinderianus* (grasscutters), even if the precise route of dissemination could not be determined in this study. In laboratory animals, experimental infection of *T. swinderianus* (grasscutters) skin using a clinical *M. ulcerans* strain (obtained from a Ghanaian Buruli ulcer patient) also revealed the presence of *M. ulcerans* in lymph nodes and blood vessels and smears of the small intestines and caeca in all challenged animals. However, the heart, lungs, liver, spleen, pancreas, kidneys, bladder, testis, scrotum, uterus, fallopian tubes, ovaries, and sciatic nerves

remained pathogen free.[19] These observations suggested a lymphatic and hematogenous spread dissemination of *M. ulcerans*.[19] In Buruli ulcer–endemic areas of Victoria, Australia, several possums with clinically apparent cases and possums without cutaneous lesions were found to have PCR-positive gut contents and/or feces. Some animals presented evidence of systemic disease, and in one animal, *M. ulcerans* was isolated from the skin lesions, lymph node, liver, and spleen.[12]

In the present study, we had no information regarding the healthiness of wild *T. swinderianus* (grasscutters) before they were captured and we did not perform pathological analyses of the spleens. Therefore, we do not know whether dissemination of *M. ulcerans* is associated with Buruli ulcer disease or merely testify of translocation of the pathogen from the digestive tract. These points will have to be elucidated in the next field campaign.

Wild *T. swinderianus* (grasscutters) investigated here have been captured in the Yamoussoukro area, which is a hot spot for Buruli ulcer in Côte d'Ivoire.[20] The data here reported add one more piece of evidence that wild *T. swinderianus* (grasscutters) are involved in the epidemiological chain of *M. ulcerans* in this area. The fact that some members of the rural population spend much time in close contact with the animals during hunting and subsequent evisceration of animals with unprotected hands for bushmeat preparation (authors' personal observations) increase their risk of contamination.

Received February 13, 2019. Accepted for publication May 26, 2019.

Published online July 22, 2019.

Note: Supplemental table appears at www.ajtmh.org.

Acknowledgments: We thank Emilien Drancourt for his help in sampling; the staff of Toumodi grasscutter farm, especially Toure Aboukari for providing negative control samples; Olga Cusack for manuscript editing; and Magdalen Lardière for English correction. This work was supported by IHU Méditerranée Infection Marseille, France; and Fondation Mérieux, Lyon, France; Agence Nationale de la Recherche (ANR-17-CE35-0006-01 PRIME, http://www.agencenationale-recherche.fr/); the French government under the "Investissements d'avenir" (Investments for the Future) program managed by the ANR (reference: Méditerranée Infection 10-IAHU-03); the Agence Nationale de la Recherche (ANR-17-CE35-0006-01 PRIME, http://www.agencenationale-recherche.fr/); Région Provence Alpes Côte d'Azur; and European funding FEDER IHUBIOTK.

Authors' addresses: Nassim Hammoudi, Sofiane Regoui, Bernard Davoust, Michel Drancourt, and Amar Bouam, Aix-Marseille-Univ., IRD, MEPHI, IHU Méditerranée Infection, Marseille, France, E-mails: nassimveto15@live.fr, regoui.sofiane@gmail.com, bernard.davoust@gmail.com, michel.drancourt@univ-amu.fr, and amarbouam@yahoo.fr. Agui Sylvestre Dizoe, Kongouanou Care Center, National Buruli Ulcer Control Plan, Abidjan, Côte d'Ivoire, E-mail: drdizoe@yahoo.fr.

REFERENCES

1. Kenneth DD, Kathryn EH, Janet AF, Caroline JL, Miriam E, Françoise P, Dorothy YM, Gerd P, Torsten S, Timothy PS, 2012. On the origin of *Mycobacterium ulcerans*, the causative agent of Buruli ulcer. *BMC Genomics 13:* 258.
2. Zingue D, Bouam A, Tian RBD, Drancourt M, 2018. Buruli ulcer, a prototype for ecosystem-related infection, caused by *Mycobacterium ulcerans*. *Clin Microbiol Rev 31:* 41–17.
3. Sizaire V, Nackers F, Comte E, Portaels F, 2006. *Mycobacterium ulcerans* infection: control, diagnosis, and treatment. *Lancet Infect Dis 6:* 288–296.
4. Portaels F, Chemlal K, Elsen P, Johnson PD, Hayman JA, Hibble J, Kirkwood R, Meyers WM, 2001. *Mycobacterium ulcerans* in wild animals. *Rev Sci Tech 20:* 252–264.
5. Durnez L, Suykerbuyk P, Nicolas V, Barrière P, Verheyen E, Johnson CR, Leirs H, Portaels F, 2010. Terrestrial small mammals as reservoirs of *Mycobacterium ulcerans* in Benin. *Appl Environ Microb 76:* 4574–4577.
6. Portaels F et al., 2008. First cultivation and characterization of *Mycobacterium ulcerans* from the environment. *PLoS Neglect Trop Dis 2:* e178.
7. Zingue D, Panda A, Drancourt M, 2018. A protocol for culturing environmental strains of the Buruli ulcer agent, *Mycobacterium ulcerans*. *Sci Rep 8:* 6778.
8. Aboagye SY, Emelia D, Kobina AA, Zuliehatu N, Prince A, Isaac DO, Katharina R, Dzidzo YT, Dorothy YM, 2016. Isolation of nontuberculous mycobacteria from the environment of Ghanian communities where Buruli ulcer is endemic. *Appl Environ Microbiol 82:* 4320–4329.
9. Palmer MV, Welsh MD, Hostetter JM, 2011. Mycobacterial diseases of animals. *Vet Med Int 2011:* 292469.
10. Djouaka R et al., 2018. Domestic animals infected with *Mycobacterium ulcerans* Implications for transmission to humans. *PLoS Neglect Trop Dis 12:* e0006572.
11. Mitchell PJ, Jerrett IV, Slee KJ, 1984. Skin ulcers caused by *Mycobacterium ulcerans* in koalas near Bairnsdale, Australia. *Pathology 16:* 256–260.
12. O'Brien CR et al., 2014. Clinical, microbiological and pathological findings of *Mycobacterium ulcerans* infection in three Australian possum species. *PLoS Negl Trop Dis 8:* e2666.
13. Fyfe JA et al., 2010. A major role for mammals in the ecology of *Mycobacterium ulcerans*. *PLoS Negl Trop Dis 4:* e791.
14. Tian RDB, Sébastian N, Tissot-Dupont H, Drancourt M, 2016. Detection of *Mycobacterium ulcerans* DNA in the environment, ivory coast. *PLoS One 11:* e0151567.
15. Dassi C, Mosi L, Akpatou B, Narh CA, Quaye C, Konan DO, Djaman JA, Bonfoh B, 2015. Detection of *Mycobacterium ulcerans* in *Mastomys natalensis* and potential transmission in Buruli ulcer endemic areas in Côte d'Ivoire. *Mycobact Dis 5:* 184.
16. Child MF, 2016. *Thryonomys swinderianus (errata version published in 2017)*. Cambridge, UK: The IUCN Red List of Threatened Species 2016, e.T21847A115163896.
17. Fyfe JA, Lavender CJ, Johnson PDR, Globan M, Sievers A, Azuolas J, Stinear TP, 2007. Development and application of two multiplex real-time PCR assays for the detection of *Mycobacterium ulcerans* in clinical and environmental samples. *Appl Environ Microb 73:* 4733–4740.
18. Maman I et al., 2018. Molecular detection of *Mycobacterium ulcerans* in the environment and its relationship with Buruli ulcer occurrence in Zio and Yoto districts of maritime region in Togo. *PLoS Negl Trop Dis 12:* e0006455.
19. Addo P, Adu-Addai B, Quartey M, Abbas M, Okang I, Owusu E, Ofori- Adjei D, Awumbila B, 2006. Clinical and histopathological presentation of Buruli ulcer in experimentally infected grasscutters (*Thryonomys swinderianus*). *Int J Trop Med 3:* e2.
20. Asiedu K, Sherpbier R, Raviglione M, 2000. Buruli ulcer. Mycobacterium ulcerans *Infection*. Geneva, Switzerland: World Health Organization. Available at: http://apps.who.int/iris/bitstream/10665/66164/1/WHO_CDS_CPE _GBUI_2000.1.pdf. Accessed January 31, 2019.