

Molecular detection of *Mycobacterium tuberculosis sensu stricto* in the soil of Niger

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Abstract

Mycobacterium tuberculosis (MTB) complex is comprising of pathogenic mycobacteria responsible for human and animal tuberculosis, a major public health problem in Niger. Although infected individuals are paramount sources of contamination, nevertheless alternative, neglected sources may play some role in minority forms of the infection. Accordingly, we investigated the presence of *Mycobacterium tuberculosis* complex in soil samples in Niger. A total of 103 soil samples were collected in six different areas in Niger in October and November 2018 and April and May 2020 from residential areas of tuberculosis patients. Screening PCR targeting *M. tuberculosis* complex CRISPR-Csm4 and Xpert MTB/RIF Ultra assay were applied to detect the *M. tuberculosis* complex. *M. tuberculosis* DNA was positively detected in five of 103 (5/103; 4.8%) soil samples (Dosso: one sample, Zinder: one sample and Niamey: three samples) using the CRISPR-Csm4 system. CRISPR-Csm4 gene sequence identified four *M. tuberculosis sensu stricto* (may be lineages 1, 3 or 4) and one *M. tuberculosis* L2 lineage (Beijing). Moreover, the five positive samples were confirmed by Xpert MTB/RIF Ultra assay as rifampicin-susceptible *M. tuberculosis* complex strains. However, culture remained negative after 42 days. In this study, we announced for the first time the presence of *M. tuberculosis sensu stricto* in the soil of Niger. Moreover, these detected lineages were identical to the dominant *M. tuberculosis* lineages in patients. The presence of common lineages of *M. tuberculosis* between the soil and human highlight the risk of transmission from the soil to human.

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Introduction

Tuberculosis is a deadly transmissible disease, which remains a major cause of death in the world despite the availability of effective chemotherapy for more than 60 years [1]. It is caused by mycobacteria forming so-called *Mycobacterium tuberculosis* (MTB) complex that is currently comprising ten species (*Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium canettii*, *Mycobacterium caprae*, *Mycobacterium microti*,

Mycobacterium mungi, *Mycobacterium orygis*, *Mycobacterium pinnipedi*, *M. tuberculosis sensu stricto* and *Mycobacterium suricattae*) [2–4]. While *M. tuberculosis sensu stricto* is majoritarily diagnosed in human populations; nevertheless, it also could infect non-human mammals and so be implicated as a zoonotic and reverse-zoonotic pathogen [1]. Likewise, the other above-cited *M. tuberculosis* complex species exhibited a relative host specificity, with *M. bovis* responsible for zoonotic tuberculosis in the human population, being majoritarily diagnosed in cattle [5], *M. caprae* being primarily a pathogen of goats and sheep [6] also infecting human patients [7], *Mycobacterium microti* affecting voles [8,9] and rarely Human patients [10].

Zoonotic *M. bovis* tuberculosis supported shared efforts of veterinary and medical microbiologists to further investigate the natural cycle of transmission of this pathogen to animals and humans. For example, in West and Central Africa, there is an

intermittent exposure of humans to body fluids, faeces and tissues of great apes that are constantly hunted [11–13] and have been demonstrated to host *M. tuberculosis* complex species [14,15]. In Africa also, *M. tuberculosis* has been reported in elephants [16,17] and birds [18], while *M. tuberculosis* was found in milk of cows in Ethiopia [19]. Faeces elimination of *M. tuberculosis* by such infected animals questioned the possibility to further detect *M. tuberculosis* in soil, and a recent review of experimental data and field observations confirmed the possibility of prolonged conservation of *M. tuberculosis* in the soil [20]. Accordingly, experimental isolation of *M. tuberculosis* from soil samples has been reported, and it has evidence that *M. tuberculosis* indeed remains viable and infectious after one year in sterilized soil in a laboratory environment was demonstrated [21].

The geographical diversity, composition and abundance of environmental mycobacteria reservoirs are not well described in Niger, despite the need to address the risk of exposure in the context of disease. The objective of this study was to further evaluate the detection of *M. tuberculosis* in the soil to contribute to understanding the ecology of *M. tuberculosis* in Niger.

Materials and methods

Sample collection and preparation

This study implying transborder transportation of environmental soil specimens collected with local authorization of Secrétariat Exécutif du Conseil National de l'Environnement pour un Développement Durable N°0153/SE/CNEDD, in Niger, towards France where further research investigations were conducted, fell order rules of the Nagoya treaty consigned by Niger and France. Accordingly, a total of 103 soil samples were collected from six regions in Niger: Agadez $n = 9$, Diffa $n = 2$, Dosso $n = 7$, Niamey $n = 69$, Tillabéri $n = 10$, Zinder $n = 6$ from October to November 2018 and April to May 2020. More precisely, 99 soil samples were collected in sites exposed to microbiologically documented pulmonary tuberculosis patients (patient living environment $n = 95$ and Centre National de Lutte contre la Tuberculose et des Maladies Respiratoires CNLT/MR in Niamey $n = 4$), and four additional samples were collected in Niamey from the piles of sand used for the building of houses to serve as negative controls. Every sample consisted of 3–10 g of soil collected at 0–5 cm depth in a sterile 50-mL tube. The samples were taken in dry places, exposed to the sun for some and for others sheltered from sunlight under trees, under a shed and in boxes. These samples were collected during the

supervision visit of the health centres for the diagnosis and treatment of tuberculosis. All samples were stored at 4 °C and shipped to MEPHI, IHU Méditerranée Infection, Marseille, France, for laboratory investigations.

DNA extraction and purification

DNA extraction from soil samples was adapted from a previously described protocol [22]: 0.5 g of soil were mixed with 1 mL of lysis buffer (900 µL of 0.5 M EDTA, 10 µL of 25 mg/mL proteinase K, 90 µL nuclease-free water) into a 1.5 mL tube (Sarstedt, Nümbrecht, Germany) while water was used as a negative control. Preparations were incubated for 18 h at 37 °C on a rotative wheel, and the mixture was centrifuged for two min at 16,000 g. The clear supernatant was mixed with 10 mL of binding buffer (3.6 mL of nuclease-free water, 7.16 g of guanidine hydrochloride powder, 6 mL of isopropanol, 50 µL of freshly prepared 5% Tween-20, 450 µL of 3M sodium acetate (pH5.2)) into a DNA-free 50 mL Falcon tube and DNA was purified using the MinElute Silica Spin column previously placed on a the Qia-gen Vacuum (QIAGEN, Courtaboeuf, France). The MinElute column was washed with 700 µL of buffer PE and centrifuged for one min at 16,000 g then for the elution of DNA, 12.5 µL of pre-warmed nuclease-free water were added directly on the membrane of the column and incubate for three min at room temperature and centrifuged for one min at 16,000 g. This step using 12.5 µL of pre-warmed water was eventually repeated once, and the eluate contained 25 µL of the purified DNA.

PCR-based screening

All polymerase chain reaction (PCR) experiments incorporated sterile phosphate-buffered saline (PBS) as a negative control. A home (IHU Méditerranée Infection, Marseille) designed PCR incorporating primers targeting the *M. tuberculosis* complex CRISPR-Csm4 gene (CRISPR type III-associated RAMP protein Csm4; gene size = 909 bp; Rv2820c) with the following sequences CRISPR-Csm4-PCRs standard (CRISPR-Csm4-ST): CRISPR-Csm4-ST1 forward, 5'-AGTCGTCCACGAT-TAGCTGC-3'; CRISPR-Csm4-ST1 reverse, 5'-GTAATCGGGCCCCACATAGG-3'; in order to amplify a 150-bp fragment of the *M. tuberculosis* complex and CRISPR-Csm4-ST2 forward, 5'-GACGCTCACGACATCCCTAC-3'; CRISPR-Csm4-ST2 reverse, 5'-GCGTAGCTGTAGACCGGATG-3'; in order to amplify a 250-bp fragment of the *M. tuberculosis* complex (Unpublished data). In detail, using the CRISPR-Csm4 gene allowed to differentiate *M. tuberculosis* (lineage 1, lineage 3 and lineage 4), *M. tuberculosis* Beijing lineage (L2), *Mycobacterium canetti* and *Mycobacterium pinnipedii* against all other Animal-Adapted *Mycobacterium* strains (Fig. 1).

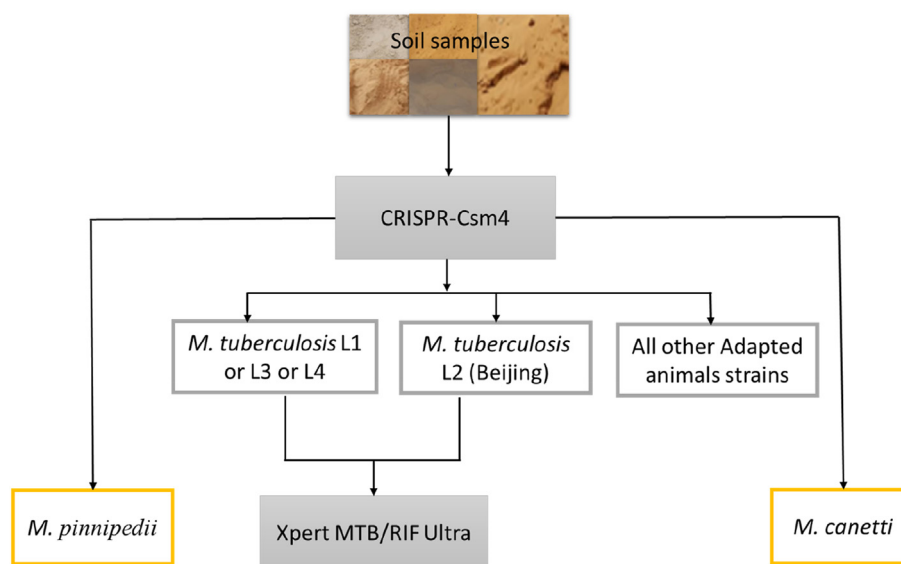


FIG. 1. CRISPR-Csm4 workflow for the detection and typing of *M. tuberculosis* in Niger soil.

PCR program included a 15-minute denaturation at 95 °C followed by 35 cycles at 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 90 seconds, followed by a final 5 min extension at 72 °C. Amplified products were visualized by 1.5% agarose gel electrophoresis. PCR products were purified and sequenced using the BigDye terminator 1.1 Cycle sequencing kit (Applied Biosystem, Courtaboeuf, France) and Genetic Analyser 3500 (Applied Bio-systems, USA).

Xpert MTB/RIF ultra assay

For that assay, 2 g of soil were mixed with 2 mL of PBS, and 1 mL of that mixture was incubated with an equal volume of magnetic beads aimed to capture the *M. tuberculosis* complex (Tb-Beads; Microsens Medtech Ltd., London, United Kingdom) at room temperature for two minutes as previously described [23]. After tubes were placed on the magnetic holder for exactly one minute, the supernatant was discarded, and beads were washed with one mL of 0.01 M NaOH (1/100 dilution of the NaOH 1 M), and the supernatant was quickly removed. The tubes were removed from the magnetic support, and acetic acid (pH 5.5, LABELIANS, Meyzieu, France) was added in the same volume as the starting samples, mixed and incubated at room temperature for two minutes. The tubes were placed on the magnetic support until the beads were on the wall of the tube, the supernatant was recovered (1 mL) and inactivated for one hour at 95 °C. Then, 1 mL supernatant was mixed with 1 mL of sample reagent buffer (supplied within the kit Xpert® MTB/RIF Ultra, Cepheid Europe, Maurens-Scopont, France), incubated for 15 min with occasional shaking and then added to the sample loading chamber of the Xpert® MTB/RIF Ultra Xpert’s cartridge for automatic processing into the GeneXpert instrument (Cepheid).

Culture-based method

The soil samples that have been detected positive by PCR for *M. tuberculosis* complex DNA along with one negative control soil specimen (avoided of any PCR-based piece of evidence of *M. tuberculosis* complex DNA) were further investigated by culture. These soil specimens were incubated for 12 hours at 37 °C in 5 mL in TransBK, a liquid medium designed for the decontamination and enrichment in *M. tuberculosis* of samples (Culture-Top, Marseille, France) with 2 mL of the suspension. Then 1 mL of the solution was incubated in the blood culture bottle with a liquid medium designed for the culture of *M. tuberculosis* complex in the BACT/ALERT® VIRTUO® an automat. The detection of the *M. tuberculosis* complex was performed every week. For detecting contamination, 50 mL of liquid culture were incubated in medium Columbia agar +5%

TABLE I. The result of soil samples detected by CRISPR-Csm4 PCR assay

Samples	Origin	% Identification of similarity	Identification PCRs CRISPR-Csm4-standard	genes
Sample DOS5	Dosso	100%	<i>M. tuberculosis sensu stricto</i> (L1 or L3 or L4)	Mutation A-807 in the C-terminal
Sample ZD5	Zinder	100%	<i>M. tuberculosis sensu stricto</i> Beijing (L2)	Deletion fragment in the C-terminal
Sample NY20	Niamey	100%	<i>M. tuberculosis sensu stricto</i> (L1 or L3 or L4)	Mutation A-807 in the C-terminal
Sample NY36	Niamey	100%	<i>M. tuberculosis sensu stricto</i> (L1 or L3 or L4)	Mutation A-807 in the C-terminal
Sample NY52	Niamey	100%	<i>M. tuberculosis sensu stricto</i> (L1 or L3 or L4)	Mutation A-807 in the C-terminal

TABLE 2. The result of soil samples detected by Xpert MTB/RIF Ultra PCR assay

Samples	Origin	Identification	Cycle threshold (Ct)				Rifampicin
			rpoB1	rpoB2	rpoB3	rpoB4	
Negative sample 1	Niamey	<i>M. tuberculosis</i> not detected	0	0	0	0	Not Applicable
Negative sample 2	Niamey	<i>M. tuberculosis</i> not detected	0	0	0	0	Not Applicable
Negative sample 3	Niamey	<i>M. tuberculosis</i> not detected	0	0	0	0	Not Applicable
Negative sample 4	Niamey	<i>M. tuberculosis</i> not detected	0	0	0	0	Not Applicable
Sample DO5	Dosso	<i>M. tuberculosis</i> detected	32.9	31	33.8	37.4	Susceptible
Sample ZD5	Zinder	<i>M. tuberculosis</i> detected	33.9	33	35.8	39.2	Susceptible
Sample NY20	Niamey	<i>M. tuberculosis</i> detected	30.4	29.1	31.7	35.3	Susceptible
Sample NY36	Niamey	<i>M. tuberculosis</i> detected	28.5	28.1	30.1	31.5	Susceptible
Sample NY52	Niamey	<i>M. tuberculosis</i> detected	29.7	29	31.5	35	Susceptible

sheep blood (*bioMérieux*, Marcy-l'Étoile, France) for 24 hours in an aerobic atmosphere at 37 °C. Then colonies were identified using Bruker Biotyper Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF) as previously described [24].

Mycobacterium-amoeba coculture

The samples that have been detected positive by PCRs for *M. tuberculosis* complex DNA along with four negative control soil specimens (avoided of any PCR-based piece of evidence of *M. tuberculosis* complex DNA), were further investigated by coculture with the amoeba. These soil specimens have been decontaminated as previously described [25]. The strain *Acanthamoeba polyphaga* LINC API was used for the coculture: 2.5 mL of amoebal culture were inoculated with 100 µL of a soil

suspension, and vancomycin 10 µg/mL, imipenem 10 µg/mL and voriconazole 40 µg/mL were added in 12.5 cm square flasks (SARSTEDT, Nümbrecht, Germany). The coculture was incubated in an aerobic atmosphere at 32 °C. Ziehl–Neelsen (ZN) stain for the presence of acid-fast bacilli (AFB) was performed weekly for up to 42 days.

Results

PCR-based screening

While negative controls remained negative, 4/103 (3.8%) positives samples identified by CRISPR-Csm4 sequencing as *M. tuberculosis sensu stricto* (may be lineage 1 or lineage 3 or lineage 4) by specific mutation A-807 in the C-terminal of the

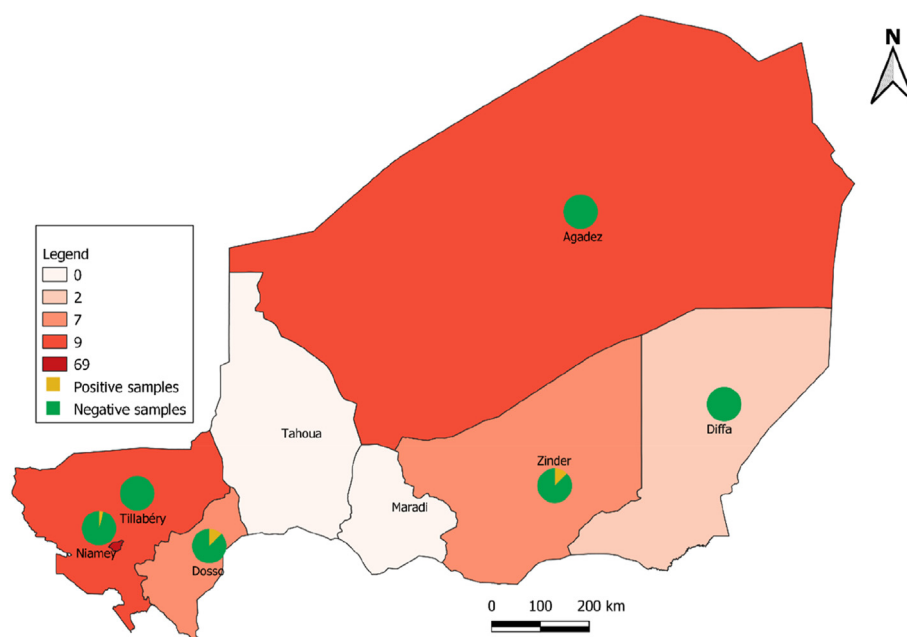


FIG. 2. Geographic spread of samples in Niger and distribution of positive and negative samples in Niger. A map of the regions in Niger is shown in which pie charts indicate negative samples in green and positive samples in yellow.

gene (gene size = 909 bp) and 1/103 (0.9%) as *M. tuberculosis* Beijing lineage with 552-bp large deletion fragment in the C-terminal of the gene (Fig. 1, Table 1).

Further, the Xpert MTB/RIF Ultra assay confirmed these five positive samples as being rifampicin susceptible (Table 2). In these two analysis steps, all negative controls remained negative (Fig. 2).

Mycobacterium culture and Mycobacterium-amoeba coculture

A positive signal was detected with the blood culture bottle after seven days of incubation in an automat BACT/ALERT® VIRTUO®. The MALDI-TOF MS was performed and identified *Enterobacter cloacae* with a score of 2.26 (one sample in Zinder), *Bacillus cereus* with a score of 2.26 (control sample and two samples in Niamey), *Bacillus megaterium* with a score of 2.19 (one sample in Dosso) and *Mycobacterium aurum* with a score of 1.85 (one sample in Niamey). In addition, using *Mycobacterium-amoeba* coculture, did not detect *M. tuberculosis* colony growth up to 42 days of incubation.

Discussion and conclusion

In this study, two PCR-based assays were used to detect the presence of *M. tuberculosis* complex DNA from soil samples collected in sites exposed to tuberculosis patients in Niger. Among 103 soil samples, five positive soil samples were detected. Culture attempts, however, remained negative, mainly due to contamination of the culture media, leaving unknown whether such finally detected *M. tuberculosis* was alive or dead.

In this study, detected lineages were known as predominant lineages in tuberculosis patients in Niger; and have been already detected in soil and water in Tehran, Iran [26]. In addition, the isolation of *M. tuberculosis* from soil samples has been reported in a Russian study [27]. Experimental observation in a laboratory environment showed an evidence that *M. tuberculosis* indeed remains viable and infectious after one year in sterilized soil [21]. On the other hand, several reports have indicated that *M. bovis* in the *M. tuberculosis* complex is able to survive in soil and has been shown to survive in, outside a host, for substantial periods of time under favourable conditions the environment [28–32].

In detail, Nigerien patients with pulmonary tuberculosis may spit into the floor. Some inhabitants emit defecation in the ground; it is done in different ways: by squatting in soil or using the traditional outdoor toilets. Tuberculosis patient stools may be, in Niger, a source for soil contamination by *M. tuberculosis*. Indeed, studies have shown that *M. tuberculosis* can be found in the stool of patients with pulmonary tuberculosis and can be used for the diagnosis of tuberculosis by culture [33] and by molecular detection [34]. However, the role of soil as a source

of contamination of certain mammals was possible. In addition, there has been a case of transmission of *M. tuberculosis* to an embalmer from the cadaver of a patient who died of pulmonary tuberculosis [35], illustrating that buried corpses of tuberculosis patients could be a source of soil infection [1]. A subsequent study showed that *M. tuberculosis* organisms remain viable and therefore infectious for at least 24 to 48 hours after an infected cadaver has been embalmed [36].

As a perspective about this study, we need to isolate these positive samples in order to recover the complete genome sequence. Genome sequences were required to compare these samples with other human *M. tuberculosis* genomes strains in Niger to confirm the transmission of *M. tuberculosis* between soil and patient.

Authors' contributions

All authors contributed to this work.

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Transparency declaration

The authors have no conflicts of interest to declare.

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References

- [1] Ghodbane R, Drancourt M. Non-human sources of *Mycobacterium tuberculosis*. *Tuberculosis* 2013;93:589–95. <https://doi.org/10.1016/j.tube.2013.09.005>.
- [2] Parsons SDC, Drewe JA, Gey van Pittius NC, Warren RM, van Helden PD. Novel cause of tuberculosis in meerkats, South Africa. *Emerg Infect Dis* 2013;19:2004–7. <https://doi.org/10.3201/eid1912.130268>.
- [3] van Ingen J, Rahim Z, Mulder A, Boeree MJ, Simeone R, Brosch R, et al. Characterization of *Mycobacterium orygis* as *M. tuberculosis* complex

- subspecies. *Emerg Infect Dis* 2012;18:653–5. <https://doi.org/10.3201/eid1804.110888>.
- [4] Alexander KA, Laver PN, Michel AL, Williams M, van Helden PD, Warren RM, et al. Novel *Mycobacterium tuberculosis* complex pathogen. *M Mungi Emerg Infect Dis* 2010;16:1296–9. <https://doi.org/10.3201/eid1608.100314>.
- [5] Ocepek M, Pate M, Žolnir-Dovč M, Poljak M. Transmission of *Mycobacterium tuberculosis* from human to cattle. *J Clin Microbiol* 2005;43:3555–7. <https://doi.org/10.1128/JCM.43.7.3555-3557.2005>.
- [6] Aranaz A, Liébana E, Gómez-Mampaso E, Galán JC, Cousins D, Ortega A, et al. *Mycobacterium tuberculosis* subsp. *caprae* subsp. nov.: a taxonomic study of a new member of the *Mycobacterium tuberculosis* complex isolated from goats in Spain. *Int J Syst Evol Microbiol* 1999;49:1263–73. <https://doi.org/10.1099/00207713-49-3-1263>.
- [7] Yeboah-Manu D, Asare P, Asante-Poku A, Otchere ID, Osei-Wusu S, Danso E, et al. Spatio-temporal distribution of *Mycobacterium tuberculosis* complex strains in Ghana. *PLoS One* 2016;11:e0161892. <https://doi.org/10.1371/journal.pone.0161892>.
- [8] Wells AQ. Tuberculosis in wild voles. *Lancet* 1937;229:1221. [https://doi.org/10.1016/S0140-6736\(00\)83505-9](https://doi.org/10.1016/S0140-6736(00)83505-9).
- [9] Frota CC, Hunt DM, Buxton RS, Rickman L, Hinds J, Kremer K, et al. Genome structure in the vole bacillus, *Mycobacterium microti*, a member of the *Mycobacterium tuberculosis* complex with a low virulence for humans. *Microbiology* 2004;150:1519–27. <https://doi.org/10.1099/mic.0.26660-0>.
- [10] Maguga-Phasha NTC, Munyai NS, Mashinya F, Makgatho ME, Mbajjorgu EF. Genetic diversity and distribution of *Mycobacterium tuberculosis* genotypes in Limpopo, South Africa. *BMC Infect Dis* 2017;17:764. <https://doi.org/10.1186/s12879-017-2881-z>.
- [11] Kruse H, Kirkemo A-M, Handeland K. Wildlife as source of zoonotic infections. *Emerg Infect Dis* 2004;10:2067–72. <https://doi.org/10.3201/eid1012.040707>.
- [12] Bennett EL, Blencowe E, Brandon K, Brown D, Burn RW, Cowlishaw G, et al. Hunting for consensus: reconciling bushmeat harvest, conservation, and development policy in West and central Africa. *Conserv Biol* 2007;21:884–7. <https://doi.org/10.1111/j.1523-1739.2006.00595.x>.
- [13] Coscolla M, Lewin A, Metzger S, Maetz-Rensing K, Calvignac-Spencer S, Nitsche A, et al. Novel *Mycobacterium tuberculosis* complex isolate from a wild chimpanzee. *Emerg Infect Dis* 2013;19:969–76. <https://doi.org/10.3201/eid1906.121012>.
- [14] Une Y, Mori T. Tuberculosis as a zoonosis from a veterinary perspective. *Comp Immunol Microbiol Infect Dis* 2007;30:415–25. <https://doi.org/10.1016/j.cimid.2007.05.002>.
- [15] Shipley ST, Coksaygan T, Johnson DK, McLeod CG, DeTolla LJ. Diagnosis and prevention of dissemination of tuberculosis in a recently imported rhesus macaque (*Macaca mulatta*). *J Med Primatol* 2008;37:20–4. <https://doi.org/10.1111/j.1600-0684.2007.00266.x>.
- [16] Mikota SK, Peddie L, Peddie J, Isaza R, Dunker F, West G, et al. Epidemiology and diagnosis of *Mycobacterium tuberculosis* in captive Asian elephants (*Elephas maximus*). *J Zoo Wildl Med* 2001;32:1–16. [https://doi.org/10.1638/1042-7260\(2001\)032\[0001:EADOMT\]2.0.CO;2](https://doi.org/10.1638/1042-7260(2001)032[0001:EADOMT]2.0.CO;2).
- [17] Obanda V, Poghon J, Yongo M, Mulei I, Ngotho M, Waititu K, et al. First reported case of fatal tuberculosis in a wild African elephant with past human–wildlife contact. *Epidemiol Infect* 2013;141:1476–80. <https://doi.org/10.1017/S0950268813000022>.
- [18] Kovalev GK. Tuberculosis in wildlife (review). J... - Google Scholar n.d. https://scholar.google.com/scholar?hl=en&as_sdt=0%2C5&q=Kovalev+GK.+Tuberculosis+in+wildlife+%28review%29.+J+Hyg+Epidemiol+Microbiol+Immunol+1980%3B24%3A495e504.&btnG=. [Accessed 5 July 2021].
- [19] Fetene T, Kebede N, Alem G. Tuberculosis infection in animal and human populations in three districts of western gojam, Ethiopia: tuberculosis infection in animal and human populations in three districts. *Zoonoses Publ Health* 2011;58:47–53. <https://doi.org/10.1111/j.1863-2378.2009.01265.x>.
- [20] Martínez L, Verma R, Croda J, Horsburgh CR, Walter KS, Degner N, et al. Detection, survival and infectious potential of *Mycobacterium tuberculosis* in the environment: a review of the evidence and epidemiological implications. *Eur Respir J* 2019;53:1802302. <https://doi.org/10.1183/13993003.02302-2018>.
- [21] Ghodbane R, Medie FM, Lepidi H, Nappes C, Drancourt M. Long-term survival of tuberculosis complex mycobacteria in soil. *Microbiology* 2014;160:496–501. <https://doi.org/10.1099/mic.0.073379-0>.
- [22] Spyrou MA, Keller M, Tukhbatova RI, Scheib CL, Nelson EA, Andrades Valtueña A, et al. Phylogeography of the second plague pandemic revealed through analysis of historical *Yersinia pestis* genomes. *Nat Commun* 2019;10:4470. <https://doi.org/10.1038/s41467-019-12154-0>.
- [23] Saad J, Loukil A, Drancourt M. Bead-captured *Mycobacterium tuberculosis* for next-generation sequencing diagnosis of uncultured tuberculosis. *Eur J Clin Microbiol Infect Dis* 2020;39:205–7. <https://doi.org/10.1007/s10096-019-03700-1>.
- [24] Patel R. MALDI-TOF MS for the diagnosis of infectious diseases. *Clin Chem* 2015;61:100–11. <https://doi.org/10.1373/clinchem.2014.221770>.
- [25] Asmar S, Drancourt M. Chlorhexidine decontamination of sputum for culturing *Mycobacterium tuberculosis*. *BMC Microbiol* 2015;15:155. <https://doi.org/10.1186/s12866-015-0479-4>.
- [26] Velayati AA, Farnia P, Mozafari M, Malekshahian D, Farahbod AM, Seif S, et al. Identification and genotyping of *Mycobacterium tuberculosis* isolated from water and soil samples of a metropolitan city. *Chest* 2015;147:1094–102. <https://doi.org/10.1378/chest.14-0960>.
- [27] Kozlov VS, Rotov VI. Isolation of *Mycobacterium tuberculosis* cultures from soil. *Probl Tuberk* 1977;78–80.
- [28] Duffield BJ, Young DA. Survival of *Mycobacterium bovis* in defined environmental conditions. *Vet Microbiol* 1985;10:193–7. [https://doi.org/10.1016/0378-1135\(85\)90021-5](https://doi.org/10.1016/0378-1135(85)90021-5).
- [29] Jackson R, de Lisle GW, Morris RS. A study of the environmental survival of *Mycobacterium bovis* on a farm in New Zealand. *New Zealand Vet J* 1995;43:346–52. <https://doi.org/10.1080/00480169.1995.35918>.
- [30] Tanner M, Michel AL. Investigation of the viability of *M. bovis* under different environmental conditions in the Kruger National Park. *Onderstepoort J Vet Res* 1999;66:185–90. PMID: 10631708.
- [31] Williams RS, Hoy WA. The viability of *B. Tuberculosis* (bovinus) on pasture land, in stored faeces and in liquid manure. *Epidemiol Infect* 1930;30:413–9. <https://doi.org/10.1017/S0022172400010561>.
- [32] Maddock ECG. Studies on the survival time of the bovine tubercle Bacillus in soil, soil and dung, in dung and on grass, with experiments on the preliminary treatment of infected organic matter and the cultivation of the organism. *Epidemiol Infect* 1933;33:103–17. <https://doi.org/10.1017/S002217240001843X>.
- [33] Asmar S, Chatellier S, Mirande C, van Belkum A, Canard I, Raoult D, et al. A novel solid medium for culturing *Mycobacterium tuberculosis* isolates from clinical specimens. *J Clin Microbiol* 2015;53:2566–9. <https://doi.org/10.1128/JCM.01149-15>.
- [34] ElKhéchine A, Henry M, Raoult D, Drancourt M. Detection of *Mycobacterium tuberculosis* complex organisms in the stools of patients with pulmonary tuberculosis. *Microbiology (Reading, England)* 2009;155:2384–9. <https://doi.org/10.1099/mic.0.026484-0>.
- [35] Sterling TR, Pope DS, Bishai WR, Harrington S, Gershon RR, Chaisson RE. Transmission of *Mycobacterium tuberculosis* from a cadaver to an embalmer. *N Engl J Med* 2000;342:246–8. <https://doi.org/10.1056/NEJM200001273420404>.
- [36] Weed LA, Baggenstoss AH. The isolation of pathogens from tissues of embalmed human bodies. *Am J Clin Pathol* 1951;21:1114–20. <https://doi.org/10.1093/ajcp/21.12.1114>.