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REVISED VERSION

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

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Keywords: Tuberculosis, *Mycobacterium tuberculosis* complex, soil, Niger, West Africa.

15 **Abstract**

16 **Background:** *Mycobacterium tuberculosis* (MTB) complex is comprising of pathogenic
17 mycobacteria responsible for human and animal tuberculosis, a major public health problem in
18 Niger. Although infected individuals are paramount sources of contamination, nevertheless
19 alternative, neglected sources may play some role in minority forms of the infection.
20 Accordingly, we investigated the presence of *Mycobacterium tuberculosis* complex in soil
21 samples in Niger.

22 **Methods:** A total of 103 soil samples were collected in six different areas in Niger, in
23 October and November 2018 and April and May 2020 from residential areas of tuberculosis
24 patients. Screening PCR targeting *M. tuberculosis* complex CRISPR-Csm4, and Xpert
25 MTB/RIF Ultra assay were applied to detect the *M. tuberculosis* complex.

26 **Results:** *M. tuberculosis* DNA was positively detected in five of 103 (5/103; 4.8%) soil
27 samples (Dosso: one sample, Zinder: one sample and Niamey: three samples) using CRISPR-
28 Csm4 system. CRISPR-Csm4 gene sequence identified four *M. tuberculosis sensu stricto*
29 (may be lineages 1, 3 or 4) and one *M. tuberculosis* L2 lineage (Beijing). Moreover, the five
30 positives' samples were confirmed by Xpert MTB/RIF Ultra assay as rifampicin-susceptible
31 *M. tuberculosis* complex strains. However, culture remained negative after 42 days.

32 **Conclusions:** In this study we announced for the first time the presence of *M. tuberculosis*
33 *sensu stricto* in the soil of Niger. Moreover, these detected lineages were identical to the
34 dominant *M. tuberculosis* lineages in patients. The presence of common lineages of *M.*
35 *tuberculosis* between the soil and human highlight the risk of transmission from the soil to
36 human.

37

38 **Introduction**

39 Tuberculosis is a deadly transmissible disease, which remains a major cause of death in the
40 world despite the availability of effective chemotherapy for more than 60 years[1]. It is
41 caused by mycobacteria forming so-called *Mycobacterium tuberculosis* (MTB) complex
42 which is currently comprising ten species (*Mycobacterium africanum*, *Mycobacterium bovis*,
43 *Mycobacterium canettii*, *Mycobacterium caprae*, *Mycobacterium microti*, *Mycobacterium*
44 *munzi*, *Mycobacterium orygis*, *Mycobacterium pinnipedii*, *M. tuberculosis sensu stricto* and
45 *Mycobacterium suricattae*) [2–4]. While *M. tuberculosis sensu stricto* is majoritary diagnosed
46 in human populations, nevertheless it also could infect non-human mammals and so be
47 implicated as a zoonotic and reverse-zoonotic pathogen [1]. Likewise, the other above-cited
48 *M. tuberculosis* complex species exhibited a relative host specificity, with *M. bovis*
49 responsible for zoonotic tuberculosis in human population, being majoritarily diagnosed in
50 cattle [5], *M. caprae* being primarily a pathogen of goats and sheep [6] also infecting human
51 patients [7], *Mycobacterium microti* affecting voles [8,9] and rarely Human patients [10].

52 Zoonotic *M. bovis* tuberculosis supported shared efforts of veterinary and medical
53 microbiologists to further investigate the natural cycle of transmission of this pathogen, to
54 animals and humans. For example in West and Central Africa, there is an intermittent
55 exposure of humans to body fluids, feces and tissues of great apes which are constantly
56 hunted [11–13] and have been demonstrated to host *M. tuberculosis* complex species [14,15].
57 In Africa also, *M. tuberculosis* has been reported in elephants [16,17] and birds [18] while *M.*
58 *tuberculosis* was found in milk of cows in Ethiopia [19]. Feces elimination of *M. tuberculosis*
59 by such infected animals, questioned the possibility to further detect *M. tuberculosis* in soil
60 and a recent review of experimental data and field observations confirmed the possibility of
61 prolonged conservation of *M. tuberculosis* in the soil [20]. Accordingly, experimental
62 isolation of *M. tuberculosis* from soil samples has been reported and it has the evidence that

63 *M. tuberculosis* indeed remains viable and infectious after one year in sterilized soil, in a
64 laboratory environment was demonstrated [21].

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

70

71

72 **Materials and Methods**

73 **Sample Collection and Preparation.** This study implying transborder transportation of
74 environmental soil specimens collected with local authorization of Secrétariat Exécutif du
75 Conseil National de l'Environnement pour un Développement Durable N°0153/SE/CNEDD,
76 in Niger, towards France where further research investigations were conducted, fell order
77 rules of the Nagoya treaty consigned by Niger and France. Accordingly, a total of 103 soil
78 samples were collected from six regions in Niger: Agadez n=9, Diffa n=2, Dosso n=7,
79 Niamey n=69, Tillabéri n=10, Zinder n=6 from October to November 2018 and April to May
80 2020. More precisely, 99 soil samples were collected in sites exposed to microbiologically
81 documented pulmonary tuberculosis patients (patient living environment n=95 and Centre
82 National de Lutte contre la Tuberculose et des Maladies Respiratoires CNLT/MR in Niamey
83 n=4) and four additional samples were collected in Niamey from the piles of sand used for the
84 building of houses to serve as negative controls. Every sample consisted in 3-10 g of soil
85 collected at 0–5 cm depth in sterile 50-mL tube. The samples were taken in dry places,
86 exposed to the sun for some and for others sheltered from sunlight under trees, under a shed
87 and in boxes. These samples were collected during the supervision visit of the health centers
88 for the diagnosis and treatment of tuberculosis. All samples were stored at 4°C and shipped at
89 MEPHI, IHU Méditerranée Infection, Marseille, France for laboratory investigations.

90

91 **DNA extraction and purification.** DNA extraction from soil samples was adapted from a
92 previously described protocol [22]: 0.5 g of soil were mixed with 1mL of lysis buffer (900 µL
93 of 0.5 M EDTA, 10 µL of 25 mg/mL proteinase K, 90 µL nuclease free water) into a 1.5 mL
94 tube (Sarstedt, Nümbrecht, Germany) while water was used as negative control. Preparations
95 were incubated for 18 h at 37°C on a rotative wheel and the mixture was centrifuged for two
96 min at 16,000 g. The clear supernatant was mixed with 10 mL of binding buffer (3.6 mL of

97 nuclease free water, 7.16 g of guanidine hydrochloride powder, 6 mL of isopropanol, 50 µL
98 of freshly prepared 5% Tween-20, 450 µL of 3M sodium acetate (pH5.2) into a DNA-free 50
99 mL Falcon tube and DNA was purified using the MinElute Silica Spin column previously
100 placed on a the Qiagen Vacuum (QIAGEN, Courtaboeuf, France). The MinElute column was
101 washed with 700 µL of buffer PE and centrifuged for one min at 16,000 g then for the elution
102 of DNA, 12.5 µL of pre-warmed nuclease-free water were added directly on the membrane of
103 the column and incubate for three min at room temperature and centrifuged for one min at
104 16,000 g. This step using 12.5 µL of pre-warmed water was eventually repeated once and the
105 eluate contained 25 µL of the purified DNA.

106

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

121 PCR program included a 15-minute denaturation at 95°C followed by 35 cycles at 95°C for
122 30 seconds, 60°C for 30 seconds and 72°C for 90 seconds, followed by a final 5 min
123 extension at 72°C. Amplified products were visualized by 1.5% agarose gel electrophoresis.
124 PCR products were purified and sequenced using the BigDye terminator 1.1 Cycle sequencing
125 kit (Applied Biosystem, Courtaboeuf, France) and Genetic Analyser 3500 (Applied Bio-
126 systems, USA).

127

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

143

144 **Culture-based method.** The soil samples which have been detected positive by PCR for *M.*
145 *tuberculosis* complex DNA along with one negative control soil specimen (avoided of any

146 PCR-based piece of evidence of *M. tuberculosis* complex DNA), were further investigated by
147 culture. These soil specimens were incubated for 12 hours at 37°C in 5 mL in TransBK, a
148 liquid medium designed for the decontamination and enrichment in *M. tuberculosis* of
149 samples (Culture-Top, Marseille, France) with 2 mL of the suspension. Then 1 mL of the
150 solution was incubated in the blood culture bottle with a liquid medium designed for the
151 culture of *M. tuberculosis* complex in the BACT/ALERT® VIRTUO® an automat. The
152 detection of *M. tuberculosis* complex was performed every week. To detect contamination, 50
153 mL of liquid culture were incubated in medium Columbia agar + 5% sheep blood
154 (bioMérieux, Marcy-l'Étoile, France) for 24 hours in an aerobic atmosphere at 37°C. Then
155 colonies were identified using Bruker Biotyper Matrix-Assisted Laser Desorption Ionization–
156 Time of Flight Mass Spectrometry (MALDI-TOF) as previously described [24].

157

158 ***Mycobacterium-amoeba* coculture.** The samples which have been detected positive by PCRs
159 for *M. tuberculosis* complex DNA along with four negative control soil specimens (avoided
160 of any PCR-based piece of evidence of *M. tuberculosis* complex DNA), were further
161 investigated by co-culture with amoeba. These soil specimens have been decontaminated as
162 previously described [25]. The strain *Acanthamoeba polyphaga* LINC AP1 was used for the
163 co-culture: 2.5 mL of amoebal culture were inoculated with 100 µL of a soil suspension, and
164 vancomycin 10 µg/mL, imipenem 10 µg/mL and voriconazole 40 µg/mL were added in 12.5
165 cm square flasks (SARSTEDT, Nümbrecht, Germany). The co-culture was incubated in an
166 aerobic atmosphere at 32°C. Ziehl–Neelsen (ZN) stain for the presence of acid-fast bacilli
167 (AFB) was performed weekly up to 42 days.

168

169

170 **RESULTS.**

171 **PCR-based screening.** While negative controls remained negative, 4/103 (3.8%) positives
172 samples identified by CRISPR-Csm4 sequencing as *M. tuberculosis sensu stricto* (may be
173 lineage 1 or lineage 3 or lineage 4) by specific mutation A-807 in the C-terminal of gene
174 (gene size = 909 bp) and 1/103 (0.9%) as *M. tuberculosis* Beijing lineage with 552-bp large
175 deletion fragment in the C-terminal of gene (Figure 1, Table 1).

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

179

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

188

189 **Discussion and Conclusion**

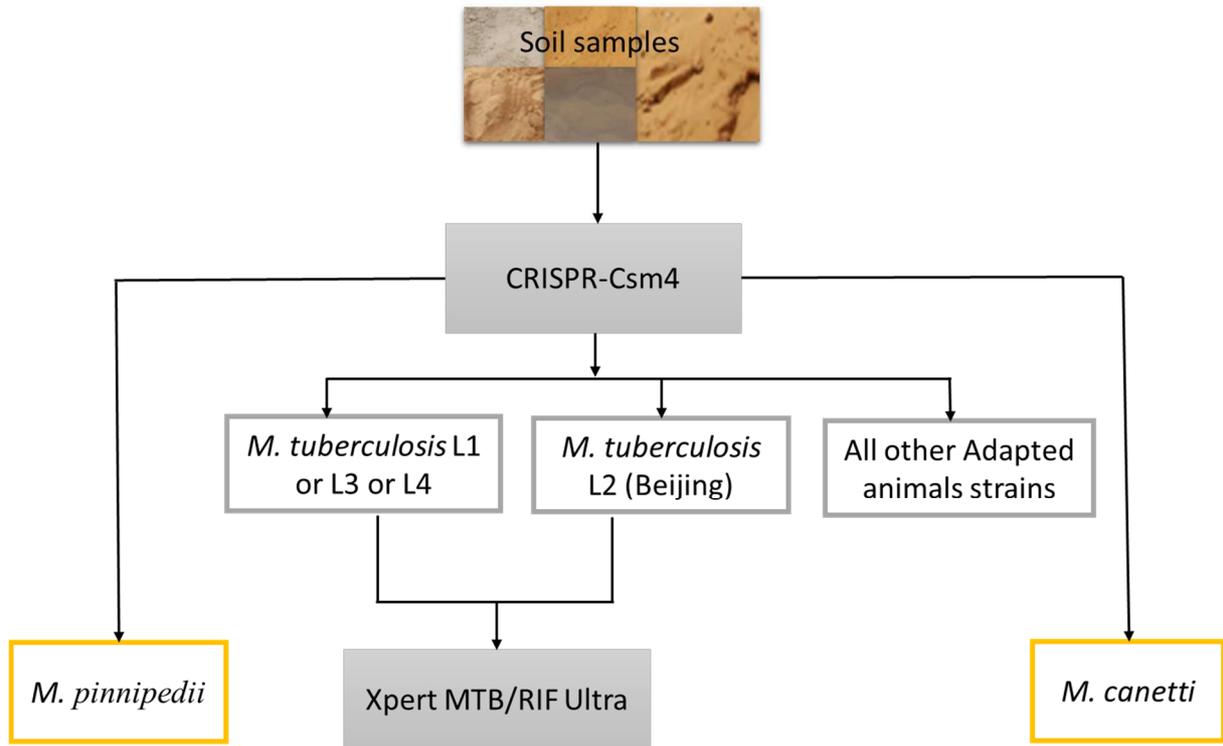
190 In this study, two PCR-based assays were used to detect the presence of *M. tuberculosis*
191 complex DNA from soil samples collected in sites exposed to tuberculosis patients in Niger.
192 Among 103 soil samples, five positive soil samples were detected. Culture attempts however
193 remained negative,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

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

203 In details, Nigerien patients with pulmonary tuberculosis may spit into the floor. Some
204 inhabitants emit defecation in the ground; it is done in different ways: by squatting in soil or
205 using the traditional outdoor toilets. Tuberculosis patient stools may be, in Niger a source for
206 soil contamination by *M. tuberculosis*. Indeed, studies have shown that *M. tuberculosis* can be
207 found in the stool of patients with pulmonary tuberculosis and can be used for the diagnosis of
208 tuberculosis by culture [33] and by molecular detection [34]. However, the role of soil as a
209 source of contamination of certain mammals was possible. In addition, there has been a case
210 of transmission of *M. tuberculosis* to an embalmer from the cadaver of a patient who died of
211 pulmonary tuberculosis [35] illustrating that buried corpses of tuberculosis patients could be a
212 source of soil infection [1]. A subsequent study showed that *M. tuberculosis* organisms
213 remain viable and therefore infectious for at least 24 to 48 hours after an infected cadaver has
214 been embalmed [36].

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

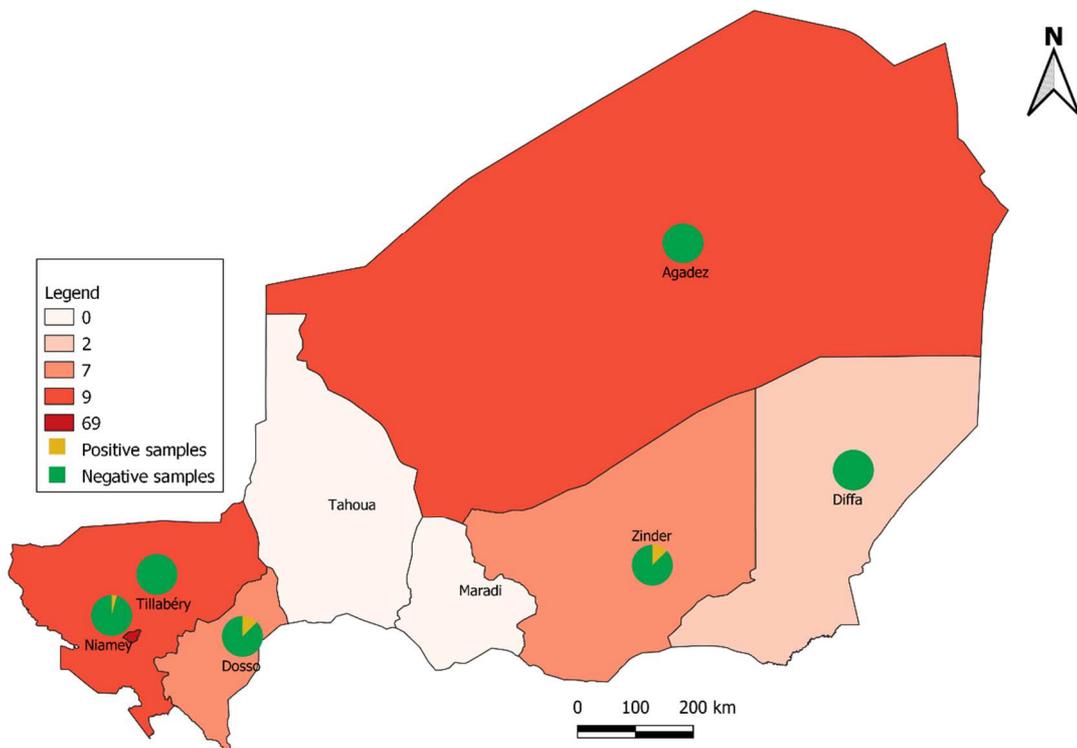
219



220

221 Figure 1: CRISPR-Csm4 workflow for the detection and typing of *M. tuberculosis* in Niger

222 soil.



223

224 Figure 2: Geographic spread of samples in Niger and distribution of positive and negative

225 samples in Niger. A map of the regions in Niger is shown in which pie charts indicate
 226 negative samples in green and positive samples in yellow.

227

Samples	Origin	% Identification of similarity	Identification PCRs CRISPR-Csm4-standard	genes
Sample DO5	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

228 **Table 1:** The result of soil samples detected by CRISPR-Csm4 PCR assay.

229

230

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

231 **Table 2:** The result of soil samples detected by Xpert MTB/RIF Ultra PCR assay.

232

233

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237 staff of the Centre National de Lutte contre la Tuberculose et des Maladies Respiratoires
238 (CNLT/MR), Niamey.

239 **Authors' Contributions:**

240 All authors contributed to this work.

241 **Conflict of Interest Statement:**

242 The authors have no conflicts of interest to declare.

243

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