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1 **Serological screening in animals combined with environmental surveys provides definite**
2 **proof of the local establishment of *Burkholderia pseudomallei* in Guadeloupe**

3

4 Short title: *Burkholderia pseudomallei* in the French West Indies

5

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23

24

25

26 **Abstract**

27 **Background**

28 Melioidosis is an emerging infectious disease caused by the soil-dwelling bacterium
29 *Burkholderia pseudomallei* that affects both humans and animals. It is endemic in South and
30 Southeast Asia, and northern Australia, causing an estimated 165,000 human cases annually
31 worldwide. Human cases have been reported in the French West Indies (Martinique and
32 Guadeloupe) since the 1990s. Conversely, no human cases have been reported in French
33 Guiana, a French territory in South America. Our study aimed to investigate whether *B.*
34 *pseudomallei* is locally established in Guadeloupe and French Guiana. We assessed animal
35 exposure by serology and examined the presence of *B. pseudomallei* in the environment of
36 seropositive animals.

37 **Methodology/Principal findings**

38 Blood samples were collected from domestic animals in two goat farms in Les Saintes,
39 Guadeloupe (n=31), and in 56 farms in French Guiana (n=670) and tested by ELISA.
40 Serological follow up was performed on selected farms. Soil, water and goat rectal swabs
41 were collected and analysed by culture and PCR. In French Guiana, the highest prevalence
42 rates were observed in equids (24%) and cattle (16%), while in Les Saintes, a prevalence of
43 39% was observed in goats. The longitudinal study in Les Saintes revealed consistent high
44 seropositivity in goats. A *B. pseudomallei* strain was isolated from the soil from one of the
45 farms and detected in goat rectal swabs from the other farm.

46 **Conclusions/Significance**

47 Our environmental investigation prompted by the serologic data confirms the presence of *B.*
48 *pseudomallei* in Les Saintes, consistent with documented human cases of melioidosis on this
49 island. In French Guiana, our serologic results call for environmental surveys and a re-

50 evaluation of human infections with melioidosis-like symptoms. The approach developed in
51 this study may help to identify high-risk areas that warrant further investigation.

52

53 **Keywords:** serology, veterinary science, ELISA, environment, melioidosis, *Burkholderia*
54 *pseudomallei*

55

56 **Author summary**

57 *Burkholderia pseudomallei*, an environmental bacterium, is the causative agent of melioidosis
58 in humans and animals. If the disease has been historically reported to be endemic in South
59 Asia and northern Australia, recent studies reveal its presence outside of these territories, both
60 in the environment and among patients who have not travelled to endemic areas. Furthermore,
61 the projected increase in extreme climatic events in the near future could increase the
62 prevalence of the disease as well as cause its emergence in new territories. For these reasons,
63 it is important to identify new areas at risk.

64 Our study aimed to investigate the presence of the pathogen in French West Indies. We
65 combined surveys in domestic animals (cattle, goats, horses, sheep, and pigs) and in the
66 environment. The identification of seropositive animals without clinical signs, together with
67 the isolation of *B. pseudomallei* in the environment of a goat farm in Guadeloupe, underscores
68 the importance of including melioidosis in animal surveillance programs. The use of serologic
69 methods can help identify animal exposure to the pathogen, thereby helping to identify areas
70 where the pathogen may be present in the environment.

71 **Introduction**

72 Melioidosis is an opportunistic infectious disease with a significant prevalence in South Asia,
73 Southeast Asia, and northern Australia, primarily due to environmental exposure to
74 *Burkholderia (B.) pseudomallei*. This telluric bacterium can affect both humans and animals
75 through inhalation, ingestion, or skin contact [1]. It is listed as a Class 1 pathogen by the U.S.
76 Centers for Disease Control and Prevention (CDC) and as a selected agent under the
77 Microorganisms and Toxins (MOT) regulations by the French National Agency for the Safety
78 of Medicines and Health Products (ANSM).

79 The global melioidosis burden was predicted to be 165,000 human cases in 2015 (2). Moreover,
80 reports of human and, occasionally, animal cases are increasing along with environmental
81 evidence of the bacteria worldwide, highlighting the probable presence of the bacterium in all
82 continents, including Africa and the Americas [3–5].

83 The clinical presentation of the disease is highly variable between individuals and species,
84 ranging from chronic forms to acute septicaemia [1,6]. Diagnosis and treatment of human
85 melioidosis are complicated by the lack of pathognomonic clinical features and a long
86 treatment duration. Several risk factors, such as diabetes, and chronic diseases, contribute to
87 increased susceptibility [7]. In animals, melioidosis affects a variety of species with multiple
88 cases documented in domestic animals such as cats, cattle, deer, dogs, goats, equids, sheep, and
89 sporadic cases in other species, including marine mammals, birds and even crocodiles in zoos
90 or wildlife parks [6].

91 Human melioidosis is a neglected disease and, as such, lacks accurate epidemiological data and
92 diagnostic tools. These are even more limited for animal melioidosis, and the diagnostic
93 approaches for animal melioidosis are generally the same as those in humans [6,8,9]. Although
94 diagnostic methods such as indirect hemagglutination (IHA), complement fixation test (CFT)
95 and enzyme-linked immunosorbent assay (ELISA) have been developed for commonly tested

96 species such as small ruminants, pigs, and equids [10–12], standardised tests for the diagnosis
97 of melioidosis in all susceptible species are currently lacking. A recent study highlighted the
98 effectiveness of serological tests developed for glanders (a disease caused by *B. mallei*, a
99 bacterium closely related to *B. pseudomallei*) for accurately detecting melioidosis in equids.
100 These tests include both a reference method (CFT) and alternative methods (ELISA, Western
101 blot) [13].

102 The bacterium *B. pseudomallei* is primarily found in moist clay soils and in turbid waters
103 [14,15]. Its distribution in soils is strongly influenced by climatic events, with more cases
104 observed during the rainy season and sporadic contamination peaking during extreme weather
105 events such as storms or floods [16,17]. Environmental detection of *B. pseudomallei* is
106 challenging and specific culture protocols are required to isolate the bacterium [18]. Its
107 detection could be harder in non-endemic areas where the bacterium may be present at lower
108 levels. A new culture medium, with erythritol as a carbon source, was recently developed to
109 improve culture isolation from soil samples [19]. It was successfully used to isolate *B.*
110 *pseudomallei* from a rice farm in South-Central Ghana, Western Africa [20].

111 Cases of melioidosis have been reported in both South, Central and North America. This
112 includes the French West Indies, i.e., Guadeloupe and Martinique, where 21 human cases have
113 been recorded since 1993 [21–25], some of whom had no history of travel to endemic areas. In
114 contrast, no human cases have been reported in French Guiana (Guiana Shield, South America),
115 while human cases have been reported in neighboring Brazil [3], as well as in Venezuela and
116 Colombia [26].

117 The objective of this study was to demonstrate the local establishment of *B. pseudomallei* in
118 French Guiana and Guadeloupe. We aimed to document animal exposure through a serology
119 study, to document their immune response over time, and to investigate the link between
120 seropositive animals and the presence of *B. pseudomallei* in their close environment. The

121 serological analysis was performed with an ELISA kit – using a recombinant protein and
122 double antigen technology – originally developed for the diagnosis of equine glanders [11] and
123 was used in our study on different domestic species (goats, cattle, equids and sheep).

124

125 **Materials and Methods**

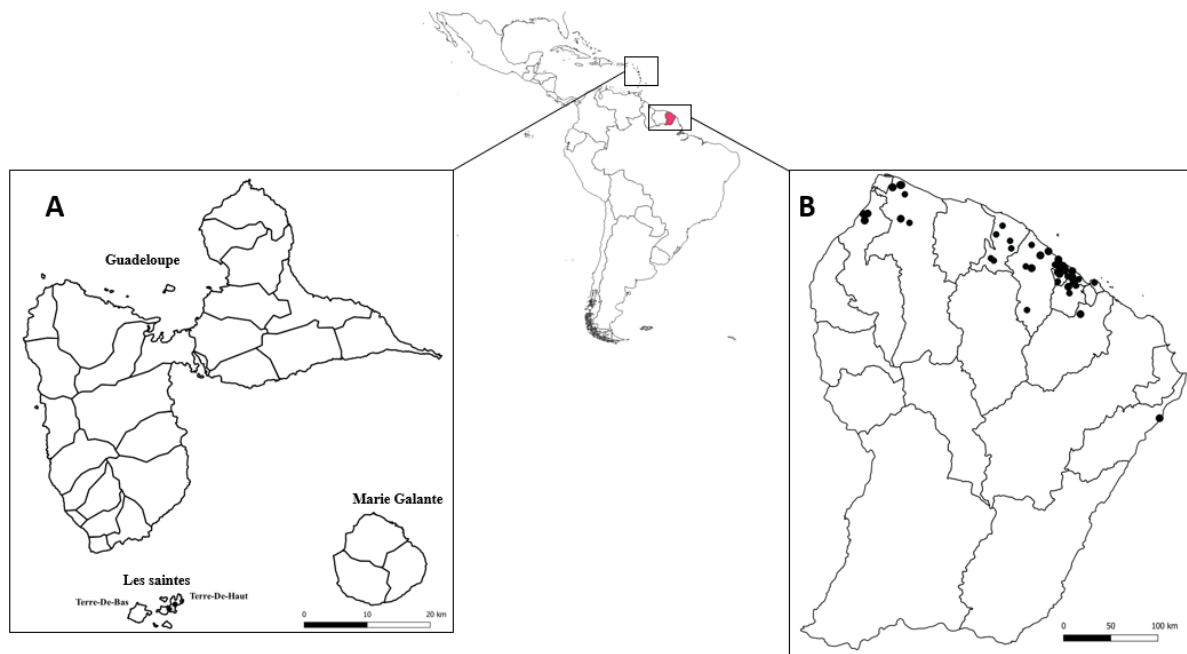
126 **Animal sampling in French Guiana and Guadeloupe**

127 In French Guiana, a total of 56 farms were surveyed, primarily along the northern coast where
128 80% of the population resides (Fig. 1A). These farms included both single-species and
129 multispecies farms, and 2 to 31 animals per farm were analysed. Among the different regions,
130 24 farms were located in Macouria, 14 in Kourou, 9 in Mana, 7 in Saint Laurent du Maroni, 5
131 in Sinnamary, 3 in Montsinery Tonnegrande, 2 in Saint George de l'Oyapack and Cayenne, and
132 1 in Roura and Iracoubo. Notably, 10 of these farms had multiple species, including cattle,
133 sheep and equids. In Les Saintes, part of the Guadeloupe archipelago, two goat farms were
134 sampled, Farm A with approximately 40 goats and Farm B with approximately 60 goats on the
135 island of Terre de Haut (Fig. 1B). On both farms, mixed-sex goats grazed freely in open
136 pastures, consuming local vegetation, and no birth control measures were used, hence the
137 approximate herd sizes reported by the farmers. The topography of the pastures was
138 characterized by steep terrain and both were susceptible to significant surface water runoff.

139 Blood samples were collected between November 2021 and December 2022 from i) cattle (361
140 animals from 37 farms), sheep (131 animals from 15 farms), goats (100 animals from 10 farms),
141 equids (63 animals from six farms), and pigs (15 animals from one farm) in French Guiana,
142 and ii) goats (31 animals from two farms) in Les Saintes.

143

144 **Fig 1. Map of sampling sites.** Farms where animals were tested in (A) Guadeloupe and (B)
145 French Guiana are represented as black dots.



146

147 After blood clotting and centrifugation at 900 rpm for 10 min, sera were collected, heat-
148 inactivated at 60°C for 30 min and stored at -20°C until analysis.

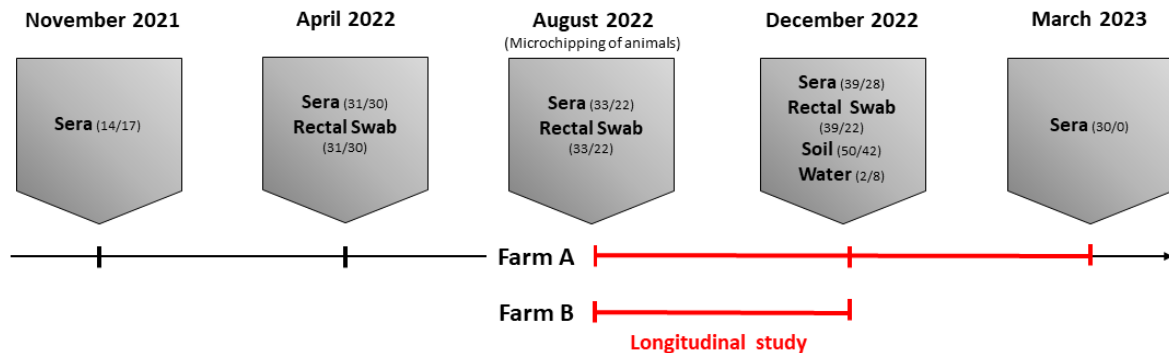
149 In some farms with GLANDA-ELISA positive animals (see § Serological analysis hereafter),
150 serological longitudinal monitoring was conducted. In French Guiana, additional serological
151 tests were conducted in one equid farm between October 2021 and January 2022. In Les
152 Saintes, a more detailed longitudinal study was conducted in the two goat farms. Blood samples
153 were collected between November 2021 and March 2023 (farm A) or December 2022 (farm
154 B) (Fig 2). Rectal swabs were collected in duplicate for each animal between April 2022 and
155 December 2022 (Fig 2). One swab was stored dry at -20°C until PCR analysis, while the second
156 was stored in Luria-Bertani (LB)-20% glycerol at -20°C for culture.

157 Animal sampling protocols were approved by the IRD Ethics Committee (21 April 2022).

158

159 **Fig 2. Sampling timeline in two goat farms in Les Saintes, Guadeloupe.** Sampling occurred
160 between November 2021 and March 2023. The longitudinal serological study (indicated in red)
161 took place between August 2022 and March 2023 for farm A, and between August 2022 and

162 December 2022 for farm B. The numbers in parentheses correspond to the number of samples
163 collected from farms A and B, respectively.



164

165 Environmental sampling in Guadeloupe

166 Soil and water samples were collected from both goat pastures (farms A and B) in December
167 2022 during the rainy season (Fig 2). Soil samples (n = 50 in farm A, n = 42 in farm B) were
168 collected every 5 m along a series of transects perpendicular to the main slope of the pasture.
169 Each transect was distant by at least 6 m from the previous transect. Samples were collected
170 30 cm deep with a standard soil auger that was disinfected with absolute ethanol after each
171 sampling. Approximately 200 g of soil was collected and placed in closed Ziploc bags to
172 maintain moisture. Samples were stored at room temperature (approximately 25°C) and
173 protected from light until shipped to France. Samples were analysed within two weeks of
174 collection (see § Environmental sample processing). Water samples were collected in farm A
175 (n = 2) and B (n = 8) in goat drinking troughs.

176 All samples (both animal and environmental) were sent to Anses, Maisons-Alfort, France, to
177 be processed.

178

179 Serological analysis

180 The ID Screen[®]Glanders Double Antigen Multi-species ELISA test (Innovative Diagnostics,
181 Grabels, France) (hereafter referred to as GLANDA-ELISA) was used according to the
182 manufacturer's instructions. This test does not rely on the use of a specific conjugate and can

183 therefore be used for multiple animal species. The relative amounts of antibodies in serum
184 samples were calculated by reference to the positive and the negative controls provided in the
185 kit. The serological titer (expressed as the Sample to Positive ratio or S/P%) was calculated
186 using the optical density (OD) reading at 450 nm following the formula: $S/P\% = ((OD \text{ sample}$
187 $- OD \text{ negative control}) / (OD \text{ positive control} - OD \text{ negative control})) \times 100$. Serum samples
188 with $S/P\% < 70\%$ were considered negative and $\geq 70\%$ were considered positive.

189

190 **Additional serological tests on equine sera, French Guiana**

191 The complement fixation test (CFT), the reference method for glanders in equids, was
192 performed as previously described using the cold method with the Malleus CFT antigen
193 (Bioveta, Czech Republic) [27]. Serum samples were initially tested at 1/5 dilution. Samples
194 with 100% hemolysis were considered negative, those with 25–75% hemolysis were
195 considered doubtful, and those with 100% inhibition of hemolysis were considered positive.
196 All samples primarily identified as suspect or positive were retested over a range of dilutions.
197 The “titer” is a six-digit barcode corresponding to the intensity of hemolysis inhibition (0 =
198 0%; 1 = 25%; 2 = 50%; 3 = 75%; 4 = 100%) at the reciprocal dilutions (1/5, 1/10, 1/20, 1/40,
199 1/80, 1/160) for each sample. Anti-complementary activity (due to incomplete elimination of
200 complement proteins during the serum heat inactivation) was checked for each serum.
201 Additionally, the Luminex assay, originally developed for the diagnosis of glanders in equids,
202 was performed as previously described using the heat shock protein (GroEL) (BPSL2697) and
203 hemolysin-coregulated protein (Hcp1) (BPSS1498) antigens [28]. MRI#1 was used as positive
204 control to determine S/P%, which was calculated for each antigen using the same formula as
205 in the GLANDA-ELISA. S/P% values greater than 45% for GroEL and 43% for Hcp1 were
206 considered positive.

207

208 **Environmental sample processing**

209 Each soil sample was homogenized, and 10 g of soil was diluted in 10 mL of sterile water.
210 After vigorous vortexing, the suspension was shaken on an orbital shaker at 160 rpm for one
211 hour and then allowed to settle for 10 min. From the supernatant, 1 mL was centrifuged at
212 8,000 rpm for 10 min and the resulting pellet was stored at -80°C until PCR analysis and 1 mL
213 was used for a two-step enrichment. First, 1 mL of supernatant was mixed with 9 mL of liquid
214 Ashdown medium [29] and incubated at 37°C for 48 h. Second, the Ashdown enrichment was
215 mixed, and 1 mL was transferred to 9 mL of erythritol medium [19] and incubated at 37°C for
216 96 h. One mL of erythritol enrichment was centrifuged at 8,000 rpm for 10 min and the
217 resulting pellet was stored at -80°C until PCR analysis. The remaining 9 mL of enriched
218 medium was stored at 4°C.

219 Within two hours of collection, 50 mL of each water sample were filtered sequentially through
220 a 3 µm filter followed by a 0.2 µm filter. The 0.2 µm filters were folded in four, preserved in
221 Lysogeny Broth (LB) (10 g peptone, 5 g yeast extract, 10 g NaCl for 1 L)-glycerol 20% and
222 stored at -80°C until analysis. For analysis, the LB-glycerol 20% was discarded and the filters
223 were aseptically cut in half. One half was placed in 10 mL of liquid Ashdown medium and
224 followed the enrichment protocol described above, while the other half was stored at -80°C for
225 PCR analysis.

226

227 **DNA extraction and PCR analysis**

228 The swab heads were cut and placed into the lysis buffer. The half filters, used for filtering the
229 water samples, were ribolysed for three cycles of 20 s at 5,500 rpm (Lysing Matrix E two mL,
230 MPBio, Eschwege, Germany) and DNA extraction was performed on 800 µL of the
231 supernatant. Soil supernatants without pre-enrichment were extracted using the DNeasy
232 PowerSoil Pro kit (QIAGEN, Hilden, Germany), while supernatants from enriched soil/water

233 samples and rectal swabs were extracted using the High Pure PCR Template Preparation kit
234 (Roche, Mannheim, Germany) according to the manufacturer's instructions. To ensure
235 extraction efficiency, an internal inhibition extraction control (Xeno™ DNA control or
236 Diagenode IPC, Thermofisher) was added to each sample.

237 An initial PCR screening was performed using a real-time PCR assay targeting the *B.*
238 *pseudomallei* complex and targeting *aroA* [30]. DNA samples that tested positive were further
239 tested using real-time PCR assays targeting specific regions of the *B. pseudomallei* genome
240 (*orf11*, BPSS0087, BPSS0745, and/or BPTT4176-4290) [31,32] and/or *B. thailandensis* (70
241 kDa) [33]. Each PCR reaction contained 5 µL of DNA and 15 µL of reaction mix, 1X of
242 Universal Mastermix TaqMan™ Fast Advanced 2X (Applied Biosystems™, Vilnius,
243 Lithuania), 0.5 µM of each primer, 0.1 µM of probe, 1X of IPC Diagenode and 2.8 µL of sterile
244 water. The amplification procedure included a 2-min incubation at 50°C followed by a
245 denaturation step at 95°C for 20 sec, then 45 cycles at 95°C for 3 sec and 60°C for 30 sec.

246

247 **Culture and screening of suspected colonies**

248 Enrichment broths stored at 4°C were homogenized and 100 µL were plated on both
249 conventional Ashdown agar and modified *B. cepacia* CHROMagar™ agar (CHROMagar,
250 Paris, France) supplemented with 4% glycerol, 500 mg/mL gentamicin and 130 mg/mL
251 fosfomicin (CHR_GGF). Plates were incubated at 37°C and monitored daily. Suspect colonies
252 (purple on Ashdown, green on CHR_GGF) were transferred to non-selective blood agar plates
253 containing 5% horse serum (BA) and incubated at 37°C for 24 h. The bacterial suspensions
254 were resuspended in sterile water, heated at 100°C for 20 min, and then centrifuged at 13,000
255 g for 10 min. The supernatant was used for PCR analysis (*aroA* and *orf11* systems) to confirm
256 colonies identification. Isolates confirmed to be *B. pseudomallei* by PCR were further
257 characterized and their antimicrobial resistance pattern identified.

258 ***B. pseudomallei* strain characterization**

259 *Antibiogram*

260 Antibiotic susceptibility testing was performed using the disk diffusion method according to
261 European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [34]. The
262 antibiotics tested included: amoxicillin-clavulanate (20-10 µg), meropenem (10 µg),
263 trimethoprim-sulfamethoxazole (1.25-23.75 µg), chloramphenicol (30 µg), imipenem (10 µg),
264 ceftazidime (10 µg), and tetracycline (30 µg) (Bio-Rad, France). Fresh 0.5 McFarland
265 suspensions were prepared and plated on Mueller-Hinton agar (Bio-Rad, France). Antibiotic
266 discs were then placed on the agar and plates were incubated at 35°C for 18 h. EUCAST
267 breakpoints were used for interpretation. Two reference strains, *Escherichia coli* ATCC 25922
268 and *Staphylococcus aureus* ATCC 29213, were included as controls.

269

270 *Biochemical testing*

271 The API 20 NE system (Biomérieux, France) was used according to the manufacturer's
272 instructions. Bacterial suspensions were adjusted to a 0.5 McFarland standard and the API
273 cassette was then incubated at 29°C for 24 h. The internet-based APIWEB database (accessed
274 7 April 2023, Biomérieux, France, <https://apiweb.biomerieux.com/>) was used to interpret the
275 obtained profiles. Simultaneously, an oxidase test (oxidase reagent 50 x 0.75 mL, Biomérieux,
276 France) was performed on each colony.

277

278 *Multilocus sequence typing (MLST)*

279 MLST was performed as previously described [35]. Amplified fragments were sent to Eurofins
280 in Germany for sequencing. The sequences were compared to the *Burkholderia pseudomallei*
281 PubMLST database for MLST profiling

282 (<https://pubmlst.org/https://pubmlst.org/organisms/burkholderia-pseudomallei>, accessed 17
283 February 2023) [36].

284

285 **Results**

286

287 **Serological survey in French Guiana and Guadeloupe**

288 In French Guiana, seropositivity rates were 16% (58/361) in cattle, 2% (2/100) in goats, 3%
289 (4/131) in sheep, 24% (15/63) in equids and 0% (0/15) in pigs (Table 1). The highest S/P%
290 were observed in cattle, sheep and equids. In Les Saintes, 39% (12/31) of goats were
291 seropositive (Table 1) and distributed over two farms (5/14 in farm A; 7/17 in farm B (Table
292 2)), with S/P% ranging from 0 to 503%.

293

294 **Table 1. Summary of the serology (GLANDA-ELISA) results in livestock in French** 295 **Guiana (n = 670) and Les Saintes, Guadeloupe (n = 31)**

296 S/P% serological titer expressed as the Sample to Positive ratio (calculated using a formula,
297 see § Materials and Methods). Serum samples with S/P% $\geq 70\%$ were considered positive.
298 Farms where at least one animal tested GLANDA-ELISA positive were considered positive.

Sampling site	French Guiana					Guadeloupe
	Cattle	Goats	Sheep	Equids	Pigs	Goats
Number of positive animals/total (%)	58/361 (16)	2/100 (2)	4/131 (3)	15/63 (24)	0/15 (0)	12/31 (39)
[Min - Max] S/P%	[0-507]	[0-99]	[0-413]	[0-435]	[0-22]	[0-503]
Number of positive farms/total (%)*	17/37 (45)	2/10 (20)	2/15 (13)	4/6 (66)	0/1 (0)	2/2 (100)

* The number of farms in the table is higher than the total number of farms tested, as 10 farms included more than one

animal species, i.e., appear in several columns.

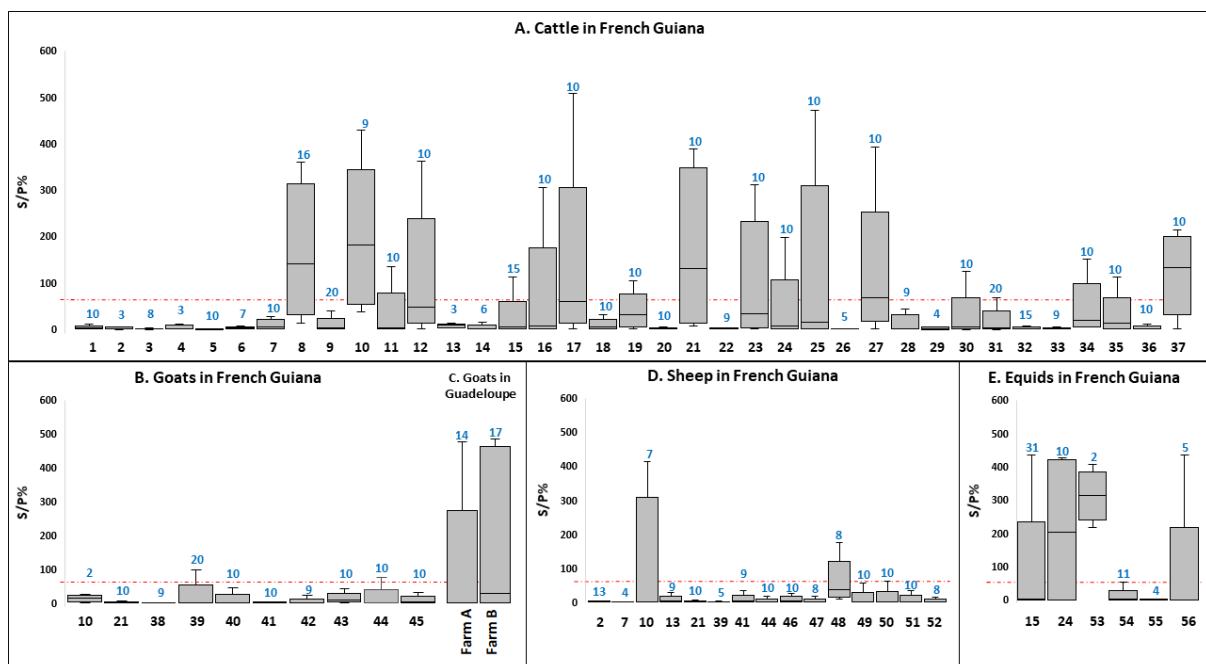
299

300 In French Guiana, 25 out of 56 farms were tested positive, of which 10 included multiple
301 species. Sixteen were in three townships: Macouria (n = 8), Kourou (n = 4), Sinnamary (n =

302 4). The serological results showed heterogeneity both between farms, differentiating positive
 303 and negative farms, as well as within positive farms, where both positive and negative animals
 304 were detected. Positive animals showed variable S/P% (**Fig 3, S1 Table**). For example, among
 305 the 25 ELISA-positive farms in French Guiana, 10 farms included animals with S/P% > 200.
 306

307 **Fig 3. Summary of GLANDA-ELISA results per farm per animal species**

308 (A) cattle (n = 37), (B) goats in French Guiana (n = 10), (C) goats in Les Saintes (n = 2), (D)
 309 sheep (n = 15), (E) equids (n = 6). S/P%: serological titer expressed as the Sample to Positive
 310 ratio. The x-axis shows the farm identification numbers. Farms are numbered from 1 to 56,
 311 when several species were on the same farm, the same number is used. The total number of
 312 animals tested per farm is shown in blue above each box plot. The red dotted line represents
 313 the S/P% threshold (70%). The bar above and below the box plot indicates the minimum and
 314 maximum S/P%. The top and bottom of the box indicate the 1st and 3rd quartiles of S/P%. The
 315 bar in the box indicates the median. Due to the small number of pig farms tested, we have
 316 excluded these data from the following figure.



317

318

319 **Additional analysis on equids from French Guiana and follow-up on farm #24**

320 In French Guiana, equids originating from six farms out of 56 (2 to 31 equids per farm; n=63)
 321 were further tested by two additional methods developed for the diagnosis of glanders: the
 322 reference CFT method and the newly developed Luminex method using GroEL and Hcp1
 323 antigens [28]. All equids with ELISA-positive results were confirmed positive with Hcp1 by
 324 Luminex, but negative with CFT and with GroEL by Luminex except for farm 24 (**Table 2**).
 325 In this farm, some of the horses/equids were tested positive with CFT.

326

327 **Table 2. Serology results from 63 equids from six farms in French Guiana**

328 GLANDA-ELISA: enzyme-linked immunosorbent assay with the ID Screen® Glanders
 329 Double Antigen Multi-species ELISA test (Innovative Diagnostics, Grabels, France). CFT:
 330 complement fixation test (see § Materiel and methods; Additional serological tests on equine
 331 sera, French Guiana). S/P%: serological titer expressed as the Sample to Positive ratio.
 332 Luminex: bead-based assay targeting recombinant proteins Hcp1 or GroEL. Id: animal
 333 identifier; P: positive; D: doubtful; N: negative; AC: anti-complementarity (interpretation not
 334 possible); na: not analysed; ns: not sampled.

Id	Farm (sampling date)	GLANDA-ELISA	CFT	Luminex - Hcp1	Luminex - GroEL
		(P≥70%)		(P≥43%)	(P≥45%)
		Result (S/P%)	Result ^a	Result (S/P%)	Result (S/P%)
03684		P (411%)	N	P (103%)	N (27%)
03685		N (0%)	N	N (19%)	N (5%)
03686		P (424%)	N	P (107%)	N (7%)
03687		N (0%)	N	N (1%)	N (2%)
03688		N (0%)	N	N (31%)	N (4%)
03689		N (1%)	N	N (5%)	N (9%)
03690	Farm 15	N (0%)	N	N (8%)	N (3%)
03691	(Mar-21)	N (0%)	N	N (3%)	N (5%)
03692		N (0%)	N	N (3%)	N (29%)
03693		N (1%)	N	N (4%)	N (6%)
03694		P (137%)	N	P (107%)	N (1%)
03695		N (0%)	N	N (2%)	N (4%)
03696		N (44%)	N	P (98%)	N (2%)

03697		P (144%)	N	P (66%)	N (4%)
03698		N (1%)	N	N (28%)	N (15%)
03699		N (0%)	N	N (0%)	N (2%)
03700		N (0%)	N	N (4%)	N (10%)
03701		N (5%)	N	N (17%)	N (8%)
03702		N (20%)	N	N (2%)	N (14%)
03703		P (419%)	N	P (99%)	N (11%)
03704		P (435%)	N	P (108%)	N (4%)
03705		N (0%)	N	N (5%)	N (6%)
03706		N (0%)	AC	N (5%)	N (17%)
03707		N (0%)	N	N (1%)	N (1%)
03708		N (11%)	N	N (17%)	N (3%)
03709		N (25%)	N	N (17%)	N (10%)
03710		N (5%)	N	P (76%)	N (10%)
03711		N (0%)	N	N (19%)	N (5%)
03712		N (0%)	N	N (1%)	N (0%)
03713		N (0%)	AC	N (2%)	P (58%)
03714		P (203%)	N	P (68%)	N (3%)
11786		N (0%) / N (0%)	N / N	N (2%) / N (2%)	N (16%) / N (12%)
11787		P (382%) / P (413%)	P ¹ / P ²	P (104%) / P (101%)	N (2%) / N (2%)
11788		P (296%) / P (387%)	N / N	P (90%) / P (58%)	N (2%) / N (1%)
11789		P (420%) / P (406%)	P ³ / P ⁴	P (101%) / P (111%)	N (3%) / N (4%)
11790	Farm 24 (Oct-21 / Jan-22)	N (0%) / N (0%)	P ⁵ / P ⁶	N (1%) / N (8%)	N (19%) / N (12%)
11791		N (0%) / ns	D ⁷ / ns	N (3%) / ns	N (29%) / ns
01034		ns / N (0%)	ns / P ⁸	na	na
01036		ns / N (56%)	ns / N	na	na
01038		ns / P (427%)	ns / N	ns / P (96%)	ns / N (2%)
01040		ns / P (408%)	ns / D ⁹	ns / P (106%)	ns / N (3%)
03671	Farm 53	P (407%)	N	P (96%)	N (11%)
03672	(Mar-21)	P (217%)	N	P (70%)	N (6%)
03673		N (0%)	AC	N (1%)	N (15%)
03674		N (3%)	N	N (1%)	N (3%)
03675		N (2%)	N	N (4%)	N (3%)
03676		N (1%)	N	N (11%)	N (12%)
03677		N (0%)	AC	N (1%)	N (5%)
03678	Farm 54 (Mar-21)	N (51%)	N	N (38%)	N (3%)
03679		N (1%)	N	N (14%)	N (15%)
03680		N (0%)	N	N (2%)	N (2%)
03681		N (0%)	N	N (3%)	N (5%)
03682		N (1%)	N	N (2%)	N (3%)
03683		N (3%)	AC	N (7%)	N (3%)
04240		N (2%)	N	N (8%)	N (5%)
04241	Farm 55	N (3%)	N	N (6%)	N (3%)
04242	(Mar-21)	N (0%)	AC	N (1%)	N (0%)
04243		N (0%)	N	N (0%)	N (0%)
05946	Farm 56	N (0%)	N	N (8%)	N (17%)

05947	(Apr-21)	P (435%)	N	P (99%)	N (4%)
05948		N (0%)	N	N (7%)	N (0%)
05949		N (0%)	N	N (1%)	N (6%)
05950		N (1%)	N	N (2%)	N (3%)

*CFT titer mentioned in the footnote. ¹444310, ²431000, ³444431, ⁴421000, ⁵444431, ⁶421000, ⁷222100, ⁸431000, ⁹200000.

335

336 **Focused study in two goat farms in Les Saintes, Guadeloupe**

337

338 *Serological and molecular biology study*

339 To investigate the temporal evolution of the serological response in two goat farms that initially
340 tested ELISA-positive in November 2021, additional blood samples were collected in April
341 2022, August 2022, and December 2022. Farm A was also sampled again in March 2023.

342 Seropositive animals were consistently detected in both farms at all-time points, although the
343 percentage of positive animals varied over time. It ranged from 13% to 36% in farm A and
344 from 23% to 41% in farm B. The highest number of ELISA-positive animals was observed in
345 both farms in November 2021 (Table 3).

346 Rectal swabs were collected in parallel with blood samples to test for the fecal shedding of *B.*
347 *pseudomallei*. In total, six swabs tested positive by PCR for the *B. pseudomallei* complex, of
348 which two were positive for *B. pseudomallei* species, one collected in April 2022 and one in
349 December 2022 in farm A (Table 3).

350

351 **Table 3. Serological and molecular results from two goat farms in Les Saintes,** 352 **Guadeloupe**

353 The ratio of real-time PCR-positive rectal swabs samples to the total number of samples tested
354 is provided for six gene targets. The PCR cycle threshold (Ct) is provided for positive samples.

355 ELISA: enzyme-linked immunosorbent assay with the ID Screen® Glanders Double Antigen
356 Multi-species ELISA test (Innovative Diagnostics, Grabels, France).

GLANDA-ELISA

Real-time PCR

Farm	Date of sampling	pos/tot (%)	[min - max] S/P%	<i>Bpm</i> complex		<i>Bpm</i>			<i>Bt</i>
				<i>aroA</i>	<i>orf11</i>	BPSS087	BPSS0745	BPSS4208	70 kDa
Farm A	Nov-21	5/14 (36)	[0 - 494]	ns	ns	ns	ns	ns	ns
	Apr-22	4/31 (13)	[0 - 373]	2/31 ^(Ct 43;38)	1/2 ^(Ct 37)	0/2	0/2	0/2	0/2
	Aug-22	8/33 (24)	[0 - 289]	0/33	0/33	na	na	na	na
	Dec-22	11/39 (28)	[0 - 395]	1/39 ^(Ct 41)	1/1 ^(Ct 39)	1/1 ^(Ct 38)	1/1 ^(Ct 41)	0/1	0/1
	Mar-23	7/30 (23)	[0 - 396]	0/30	na	na	na	na	na
Farm B	Nov-21	7/17 (41)	[0 - 503]	ns	ns	ns	ns	ns	ns
	Apr-22	7/30 (23)	[0 - 351]	3/30 ^(Ct 39;40;37)	0/3	na	na	na	na
	Aug-22	5/22 (23)	[0 - 343]	0/22	0/22	na	na	na	na
	Dec-22	7/25 (28)	[0 - 455]	0/25	0/25	na	na	na	na

357 *Bpm*: *Burkholderia pseudomallei*, *Bt*: *Burkholderia thailandensis*. Apr: April; Aug: August; Mar: March; Nov:
358 November; Dec: December. ns: not sampled; na: not analysed (samples for which *aroA* and *orf11* PCR analysis
359 were negative). Pos/tot (%): number of positive animals/total (percentage), S/P%: serological titer expressed as
360 the Sample to Positive ratio.
361

362 The longitudinal serological study started in August 2022, when as many goats as possible
363 were implanted with a microchip to identify individual goats. In farm A, 20 goats were sampled
364 three times: 13 were consistently ELISA-negative, five were consistently ELISA-positive and
365 two changed status during the course of the 8-month study (**Table 4**). Of note, six goats had
366 S/P% greater than 200% on at least two sampling events. One goat with the lowest S/P%
367 ($\approx 150\%$) later tested negative in March 2023. Conversely, two goats with initially low S/P%
368 later had higher values. Of the seven goats that were seropositive at some point, five were over
369 one year of age. The two identified mother-daughter pairs were consistently negative.
370 In farm B, 11 animals were sampled in August and December 2022. Eight tested ELISA-
371 negative twice, one tested ELISA-positive twice, and two changed status during the course of
372 the study (seroconversion). All three animals that were seropositive at some points were over
373 one year of age and had S/P% values greater than 200%.

374

375 **Table 4. Longitudinal serological study in two goat farms in Les Saintes, Guadeloupe**
376 GLANDA-ELISA: enzyme-linked immunosorbent assay with the ID Screen® Glanders
377 Double Antigen Multi-species ELISA test (Innovative Diagnostics, Grabels, France). S/P%:

378 serological titer expressed as the Sample to Positive ratio, N: negative (S/P% <70), P: positive
 379 (S/P% ≥70%), F: female, M: male, m: month old, y: year-old, ns: not sampled (farm B was not
 380 sampled in March 2023).
 381 * 5483 is the mother of 5482; ** 5574 is the mother of 5465.

				GLANDA-ELISA result (S/P%)		
Farm	Id Animal	Sex	Age	Aug-22	Nov-22	Mar-23
	1625	F	4 m	N (0%)	N (0%)	N (0%)
	5456	F	4 m	N (0%)	N (0%)	N (0%)
	5482*	F	4 m	N (3%)	N (3%)	ns
	5465**	F	4 m	N (0%)	N (0%)	ns
	5578	F	4 m	N (0%)	N (0%)	N (0%)
	5486	F	6 m	P (140%)	P (377%)	P (385%)
	5554	F	6 m	N (1%)	N (1%)	N (0%)
	5464	F	1 y	N (1%)	P (389%)	P (393%)
	5626	F	1 y	N (1%)	N (1%)	N (3%)
	5469	F	2 y	P (147%)	P (164%)	N (55%)
Farm A	5473	F	3 y	P (289%)	P (395%)	P (326%)
	5472	F	4 y	P (265%)	P (385%)	P (396%)
	5478	F	4 y	P (240%)	P (109%)	P (247%)
	5571	F	4 y	N (1%)	N (3%)	N (0%)
	5483*	F	5 y	N (1%)	N (0%)	N (0%)
	5574**	F	5 y	N (0%)	N (0%)	N (0%)
	5460	F	8 y	N (2%)	N (6%)	N (2%)
	5546	M	6 m	N (1%)	N (0%)	N (0%)
	5560	M	6 m	N (2%)	N (10%)	N (1%)
	5476	M	6 m	P (263%)	P (384%)	P (384%)
	5477	M	3 y	N (1%)	N (1%)	N (0%)
	5461	M	6 y	N (7%)	N (4%)	N (1%)
	Total positive / total tested			6/20	7/20	7/20
	5496	F	1 y	N (0%)	N (7%)	
	5492	F	3 y	N (2%)	N (1%)	
	5652	F	4 y	N (0%)	N (0%)	
	5435	F	5 y	P (272%)	P (455%)	
Farm B	5646	F	6 y	N (32%)	P (398%)	
	5506	M	2 y	N (1%)	N (7%)	ns
	5562	M	2 y	N (2%)	N (67%)	
	5606	M	2 y	N (34%)	N (59%)	
	5610	M	2 y	N (14%)	P (379%)	
	5640	M	2 y	N (0%)	N (2%)	
	5690	M	2 y	N (0%)	N (3%)	

Total positive / total
tested 1/11 3/11

382 ***Environmental sampling in goat pastures***

383 In December 2022, 50 soil samples and two water samples (from the drinking troughs) were
384 collected from farm A; and 42 soil samples and eight water samples (from the drinking troughs)
385 were collected from farm B. All samples were analysed by PCR and bacteriological culture.
386 Samples from farm A were all PCR-negative, pre- or post-enrichment. In farm B, two out of
387 42 soil samples (#313 and #336) were PCR-positive for the *B. pseudomallei* complex post-
388 enrichment, but only sample #313 tested PCR-positive for *B. pseudomallei* (all four specific
389 targets) (Table 5).

390

391 **Table 5. Results from molecular testing of environmental samples from two goat farms**
392 **in Les Saintes, Guadeloupe**

393 Soil and water samples collected in December 2022 were tested by real-time PCR for the
394 presence of different *Burkholderia* spp. Results are provided as the ratio of positive samples
395 against the total number of tested samples tested for each target. For PCR-positive results, Ct
396 values are provided. na: not analysed.

Farm	Type of sample	Number of samples	<i>Bpm</i> complex	<i>Bpm</i>				<i>Bt</i>
			<i>Bpm</i> aroA	orf11	BPSS087	BPSS0745	BPSS4208	70 kDa
Farm A	soil	50	0/50*	na	na	na	na	na
	water	2	0/2	na	na	na	na	na
Farm B	soil	42	2/42* (Ct 34 ;34)	1/2*(Ct 40)	1/2*(Ct 38)	1/2*(Ct 37)	1/2*(Ct 39)	0/2*
	water	8	0/8	na	na	na	na	na

397 *Bpm*: *Burkholderia pseudomallei*, *Bt*: *Burkholderia thailandensis*. * PCR results were obtained post-enrichment
398 (see § Material and Methods).

399

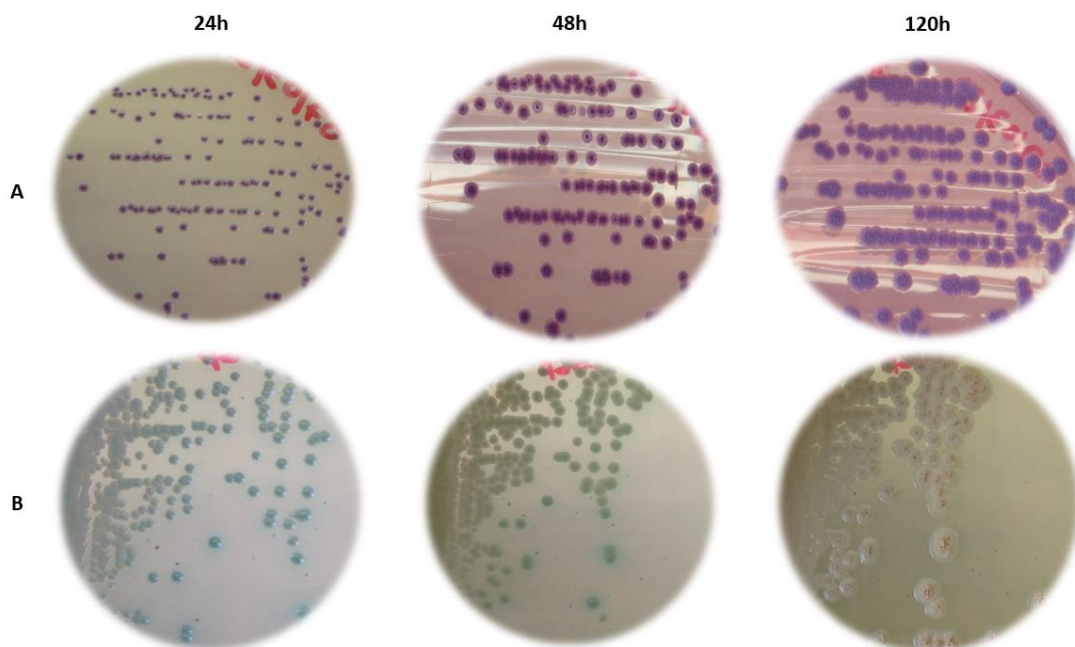
400 PCR-positive soil samples #313 and #336 were plated post-enrichment on both Ashdown and
401 CHR_GGF agar media for isolation. A total of 46 suspect colonies were isolated from soil
402 #313. Of these, 30 were PCR-positive for the *B. pseudomallei* complex and all four *B.*
403 *pseudomallei* specific targets. Fifteen suspect colonies were isolated from soil #336 and tested

404 PCR-positive for the *B. pseudomallei* complex but PCR-negative for *B. pseudomallei* and *B.*
405 *thailandensis*.

406 *B. pseudomallei* colonies on Ashdown initially appeared purple and smooth after 24 h at 37°C
407 and then changed to a rough, dry texture after 48 h (**Fig 4A**). On CHR_GGF, the same
408 morphological changes were observed: colonies were green and smooth after 24 h of culture,
409 but changed to a white, rough, and dry texture after 120 h (**Fig 4B**).

410

411 **Fig 4. *Burkholderia pseudomallei* strain D22-10884_313#20 cultures.** Photographs from
412 culture plates (A) on Ashdown and (B) on CHR_GGF (modified *B. cepacia* CHROMagar™
413 agar supplemented with 4% glycerol, 500 mg/mL gentamicin and 130 mg/mL fosfomycin)
414 after a 24, 48 and 120 h incubation at 37°C. The strain was isolated from soil #313 collected
415 from farm B in Les Saintes, Guadeloupe.



416

417

418 One *B. pseudomallei* colony (D22-10884_S313#20) isolated from soil #313 was selected for
419 further analysis. The strain showed the API 20 NE profile 1-456-574 together with a positive
420 result for the oxidase test. When compared with the profiles listed in APIWEB, the strain had

421 the highest identification scores with *B. pseudomallei* (47.9%) and *B. cepacia* (45.3%). The
422 antibiotic susceptibility profile of strain D22-10884_S313#20 showed sensitivity to all
423 antibiotics tested (commonly used for human melioidosis treatment): amoxicillin-clavulanate,
424 ceftazidime, tetracycline, imipenem, meropenem, trimethoprim-sulfamethoxazole and
425 chloramphenicol (**S2 Table**). The MLST sequence type of strain D22-10884_S313#20 was
426 ST92.

427

428 **Discussion**

429

430 By interpreting positive ELISA results in animals as a sign of previous environmental exposure
431 to *B. pseudomallei*, our serological screening showed variable results between regions but also
432 between species. In French Guiana, the seroprevalence was 2% in sheep and goats, 15% in
433 cattle and 22% in equids; in Les Saintes, where only goats were tested, the seroprevalence was
434 39%. In comparison, studies carried out in endemic countries using different serological
435 methods showed seroprevalence of 6% and 13.6% in sheep, 0.3% and 2.6% in goats, and 2%
436 and 7.6% in cattle, in Thailand (using IHA) [37] and Malaysia (using CFT) [38], respectively.
437 Due to different levels of endemicity and methodological strategies, the data are not easily
438 comparable. Our serological screening also showed within-farm variability. The longitudinal
439 study conducted in two ELISA-positive farms in Les Saintes showed that most goats remained
440 ELISA-positive over an 8-month period. Further studies are needed to better understand the
441 long-term immune response dynamics after exposure to *B. pseudomallei*. This should help to
442 explain the within-farm heterogeneity, i.e., why animals living in farms with similar
443 environmental exposure do not all show the same serological result.

444

445 Melioidosis has been studied primarily in well-established endemic regions such as Southeast
446 Asia and Australia with limited research in Central and South America [26,39]. In the
447 Caribbean, human cases have been documented for over 30 years in Guadeloupe and
448 Martinique [21–23,25] but the local presence of *B. pseudomallei* in the environment has never
449 been established. Isolating the strain from the environment is challenging, as demonstrated by
450 a recent environmental survey in Puerto Rico where only three out of 500 soil samples from 60
451 sites tested positive for *B. pseudomallei* using PCR, with only one sample being culture positive
452 [40]. Previous exposure to *B. pseudomallei* promotes an immune response in animals that can
453 be detected serologically. Hence, one major objective of our study was to confirm the benefit
454 of an initial serological screening in animals to increase the probability of detecting *B.*
455 *pseudomallei* in the environment.

456 The GLANDA-ELISA kit, originally developed for the diagnosis of equine glanders [11], was
457 used on a goat farm in New Caledonia where one case of melioidosis was confirmed. The
458 serological survey allowed to confirm exposure in 35% of goats on this farm, while those from
459 three other farms with no clinical case were 100% seronegative (Laroucau *et al.*, en révision).
460 However, the validation of this ELISA kit in detecting clinical melioidosis and/or previous
461 exposure to *B. pseudomallei* called for further data. The application of the GLANDA-ELISA
462 kit in our multi-species study yielded heterogeneous results between individuals and between
463 farms, with some farms fully seronegative. Overall, serology results were consistent with the
464 absence (in French Guiana) or limited number (in Les Saintes) of human melioidosis cases.

465 Our study supports the idea that animals could be used as sentinels of *B. pseudomallei* presence
466 in the environment and that the GLANDA-ELISA kit is a relevant tool to do so. Further
467 research is now necessary to establish the specificity of this kit in a context different from its
468 original design and validation. The high diversity and prevalence of *Burkholderia* species in
469 the environment, including closely related species within the *B. pseudomallei* complex, raises

470 concerns about the possibility of cross-reactivity. Since the identity of the protein used in the
471 GLANDA-ELISA kit is not disclosed, it is not possible to assess its degree of conservation in
472 environmental *Burkholderia* strains, if any, and thus the risk of cross-reactivity when using this
473 kit. In addition, the cutoff used in our study was originally established for equine glanders in a
474 clinical context and not for the assessment of environmental exposure to *B. pseudomallei*. A
475 reevaluation of the cutoff for our specific goal and in different host species may help to refine
476 the data obtained with this kit.

477 While quantitative data on *B. pseudomallei* in the environment are scarce, it is plausible that in
478 non-endemic areas the bacteria are present at lower concentrations which might decrease the
479 probability of detection. In anticipation of this additional challenge, we conducted our
480 environmental sampling in Les Saintes during the rainy season (June to November), a period
481 identified as the most favorable for the presence of *B. pseudomallei* in the soil [15]. We also
482 optimized the protocol from existing guidelines [18], adding two new media to the
483 conventionally recommended Ashdown medium [29]. First, an erythritol-based medium was
484 used as the sole carbon source during the second step of a two-step enrichment to select for *B.*
485 *pseudomallei* [19]. A similar two-step enrichment protocol (TBSS-C50 + erythritol) recently
486 allowed the isolation of environmental *B. pseudomallei* strains in Ghana, West Africa [20].
487 Second, we used a chromogenic medium originally developed for the detection of *B. cepacia*
488 in clinical samples as green colonies. To increase its selectivity for *B. pseudomallei*, we
489 modified the medium by incorporating new antibiotics and confirmed that reference strains of
490 *B. pseudomallei* grew well on this medium (S1 Figure). To our knowledge, this is the first use
491 of a chromogenic medium to improve the selection of *B. pseudomallei* colonies from poly-
492 contaminated environmental matrices. Despite these protocol improvements, in our study only
493 one soil sample (out of 92 samples collected from seropositive farms) and a single *B.*
494 *pseudomallei* strain was isolated. Although the use of media CHROMagar™ *B. cepacia* seems

495 promising, further protocol optimization (e.g., sample volume, enrichment time, chromogenic
496 and/or selective medium for environmental matrices) will be needed to facilitate the detection
497 and isolation of environmental strains of *B. pseudomallei*. Of note, an additional soil sample
498 tested PCR-positive for the *B. pseudomallei* complex, but not for *B. pseudomallei* or *B.*
499 *thailandensis* species. Further characterization of all non-*B. pseudomallei* strains isolated in
500 this study will help identify all the *Burkholderia* species to which animals can be exposed and
501 to assess their cross-reactivity with the GLANDA-ELISA kit.

502 Differentiating glanders (caused by *B. mallei*) from melioidosis (caused by *B. pseudomallei*) is
503 challenging due to their high antigenic similarity [13], with *B. mallei* being a monophyletic
504 clade within *B. pseudomallei* [41]. Considering that (i) the GLANDA-ELISA kit has been
505 designed for the diagnosis of equine glanders, and that (ii) glanders is frequently reported in
506 Brazil [42], a country sharing a border with French Guiana, the detection of GLANDA-ELISA
507 positive horses in French Guiana warranted further testing. Clinically healthy horses that tested
508 ELISA-positive were all Hcp1-positive by Luminex but GroEL-negative by Luminex, both
509 proteins being key markers for glanders diagnosis [28]. When adding CFT (the reference
510 method for glanders diagnosis) as a fourth test, all ELISA-positive horses were CFT-negative,
511 except for horses on farm #24. In this farm, the detection of horses positive for three out of four
512 tests relevant to glanders diagnosis prompted close surveillance. A follow-up examination three
513 months later confirmed the initial serological results as well as the absence of clinical signs
514 typical of acute glanders (e.g., respiratory distress or abscesses). Overall, the variability in test
515 results among animals in farm #24 may indicate different exposure and/or infection dynamics
516 and/or test characteristics. Despite the absence of previously reported cases of glanders or
517 melioidosis in French Guiana, the final health status of these equids remains uncertain given
518 the chronic potential of these two diseases [43–45].

519

520 Although the serological approach is generally not favored for the epidemiology of melioidosis
521 in endemic areas, due to more frequent environmental exposure of humans and animals and the
522 resulting challenges of interpreting positive results outside of a clinical context [46,47], this
523 approach can be promising for non-endemic areas. The GLANDA-ELISA kit may be
524 particularly valuable for identifying past exposure in animals and conducting targeted
525 environmental surveys around exposed animals. This is particularly relevant for livestock that
526 are often confined to restricted areas.

527 Our serological and molecular screening on two goat farms in Les Saintes, followed by an
528 environmental survey, confirmed fecal shedding of *B. pseudomallei* in one farm and
529 successfully led to the isolation of an environmental strain of *B. pseudomallei* in the other farm.
530 These results provide the first definite confirmation of the local establishment of *B.*
531 *pseudomallei* in Les Saintes, Guadeloupe. They also support the hypothesis of a local
532 contamination of two cases of human melioidosis reported on the island in 1997 (a tourist that
533 visited Les Saintes for 3 weeks) [25] and in 2016 (a local resident) [23]. Importantly, the MLST
534 profile of the strain isolated from our study (ST92) was identical to that of the 1997 human
535 case [23]. In the *Burkholderia pseudomallei* PubMLST database, ST92 was identified in nine
536 human strains: one from a Swiss traveler to Martinique [21], one from Puerto Rico, two from
537 Mexico [48], and five from Brazil [49], illustrating the restricted spatial distribution of this
538 sequence type.

539 Even though human melioidosis has not been reported so far in French Guiana, our results call
540 for the implementation of environmental surveys around the ELISA-positive farms identified
541 in this study, as well as a retrospective re-evaluation of human infections with melioidosis-like
542 symptoms. In Guadeloupe and Martinique, where several human cases have been diagnosed in
543 recent years [23,24], we suggest adding melioidosis to existing animal surveillance programs.

544

545 **Supporting information**

546 **S1 Table.** Serological results of animals in French Guiana and Les Saintes (Guadeloupe)

547 **S2 Table.** Antibioqram results of 22-10884_313#20 strains

548 **S1 Figure.** Evaluation of CHROMagar™ *B. cepacia* as a relevant media for other *Burkholderia*
549 strains.

550

551

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562

563 **Conflicts of Interest**

564 None of the authors have any financial or personal relationships with any individuals or
565 organizations that could inappropriately influence or bias this paper.

566

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579 Rachid Aaziz (RA), Thomas Deshayes (TD), Jules Terret (JT), Sébastien Breurec (SB)

580

581 Reference

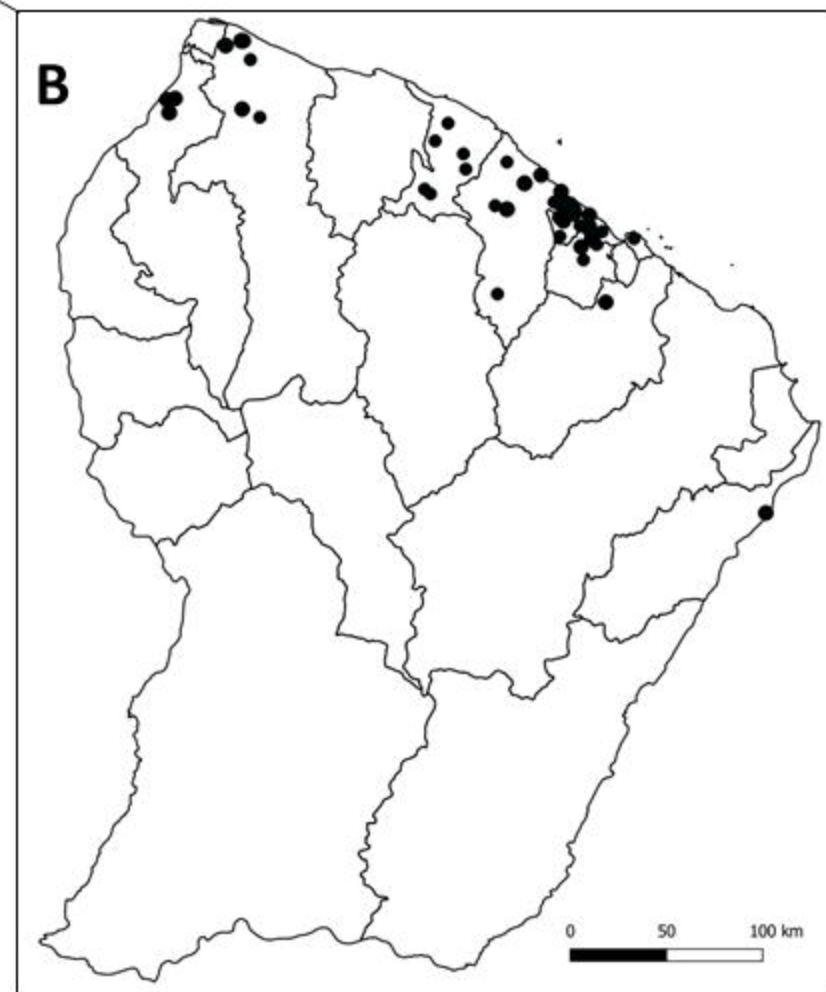
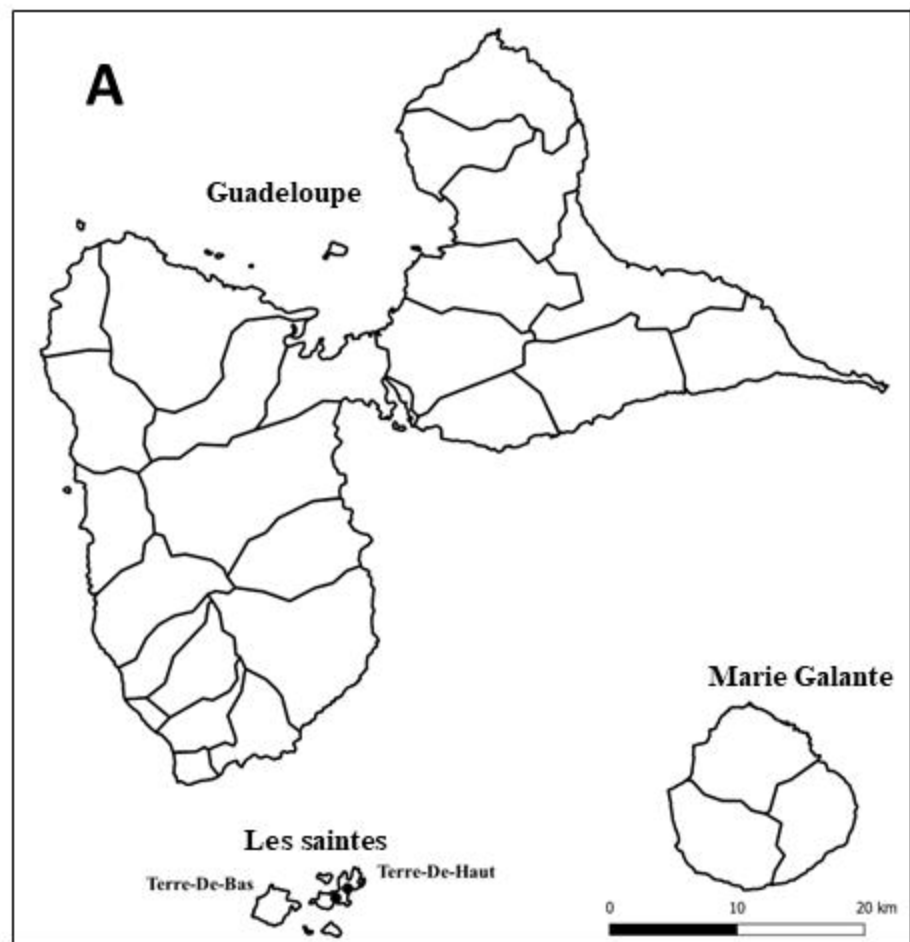
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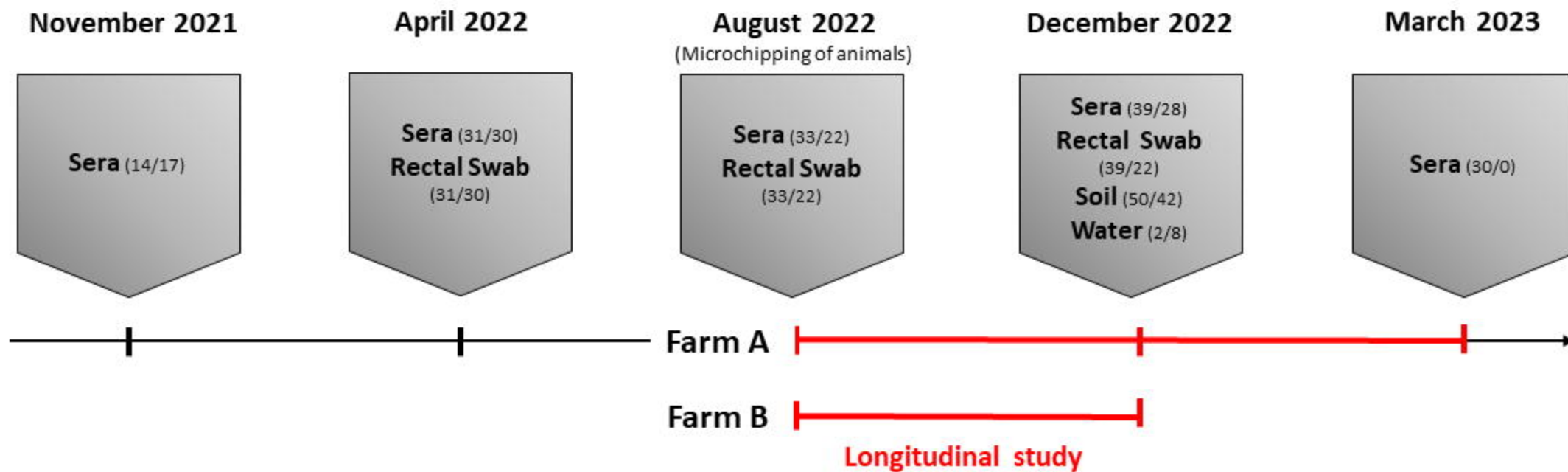
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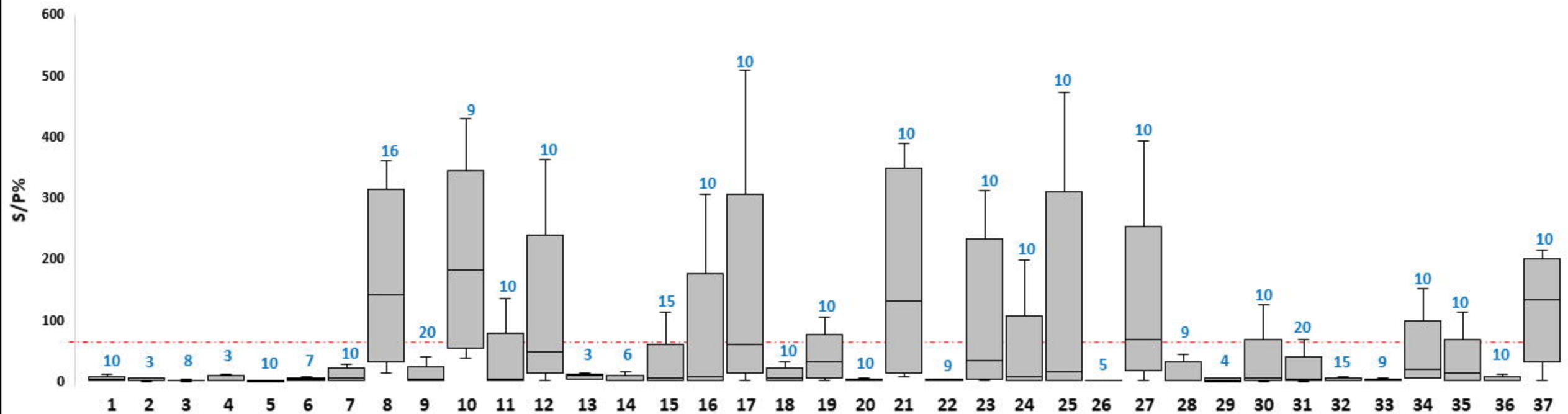
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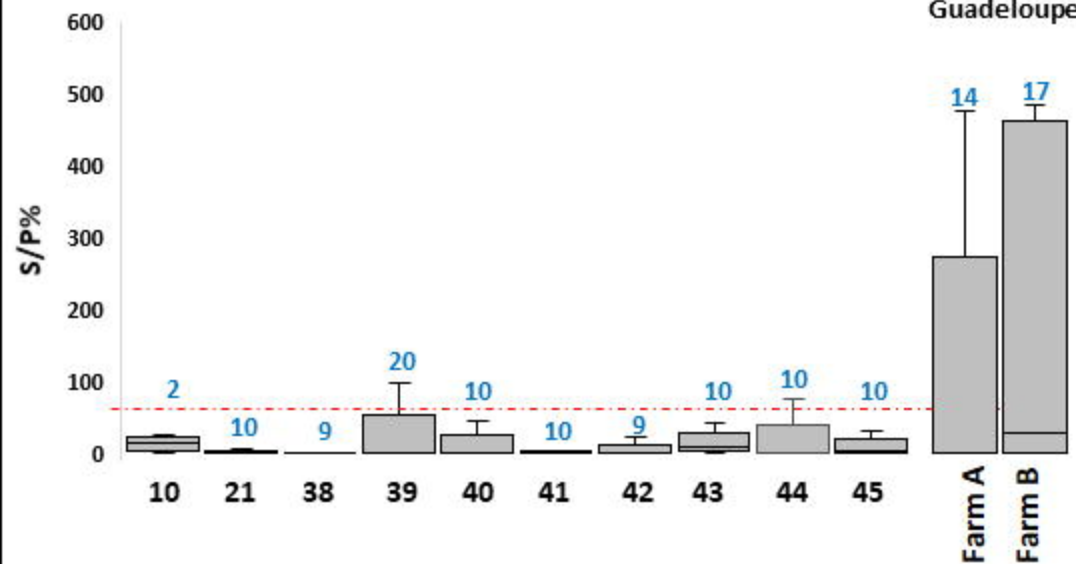




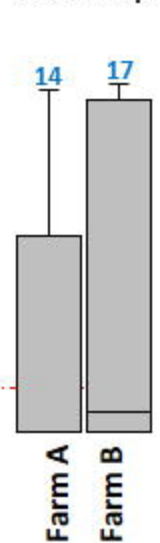
A. Cattle in French Guiana



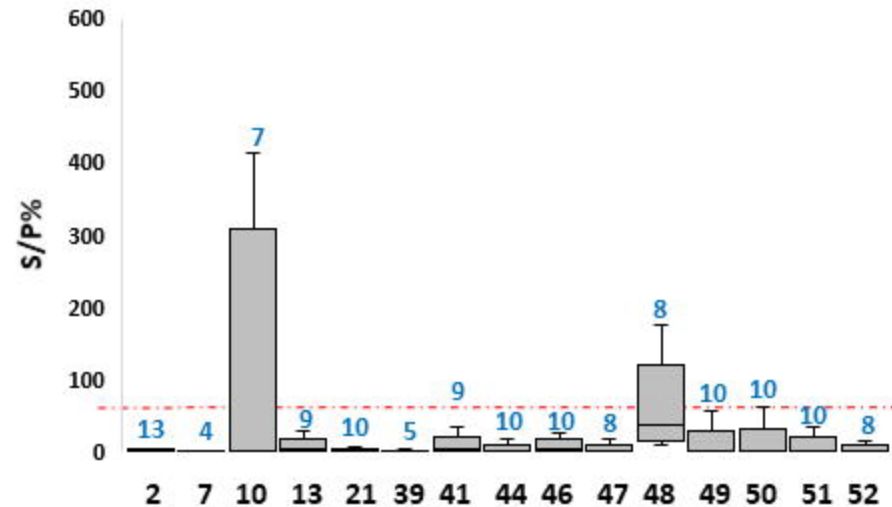
B. Goats in French Guiana



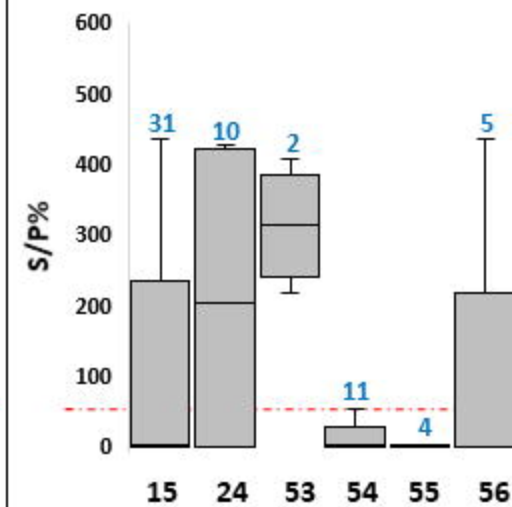
C. Goats in Guadeloupe



D. Sheep in French Guiana



E. Equids in French Guiana

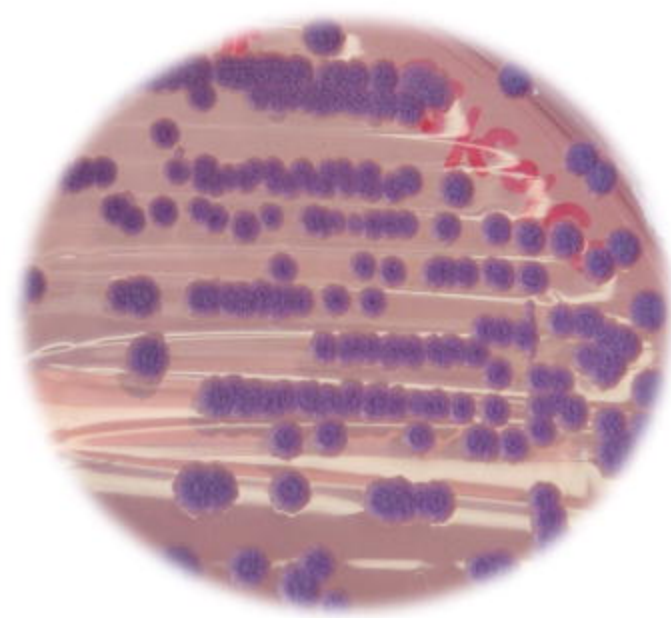
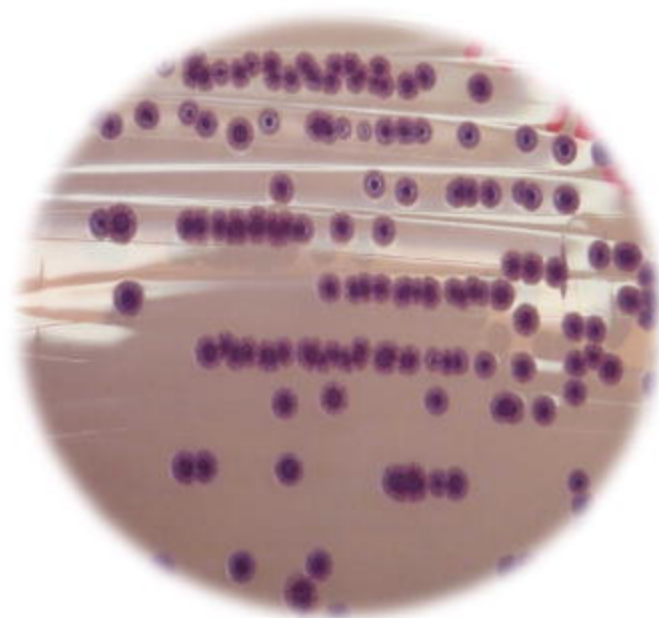
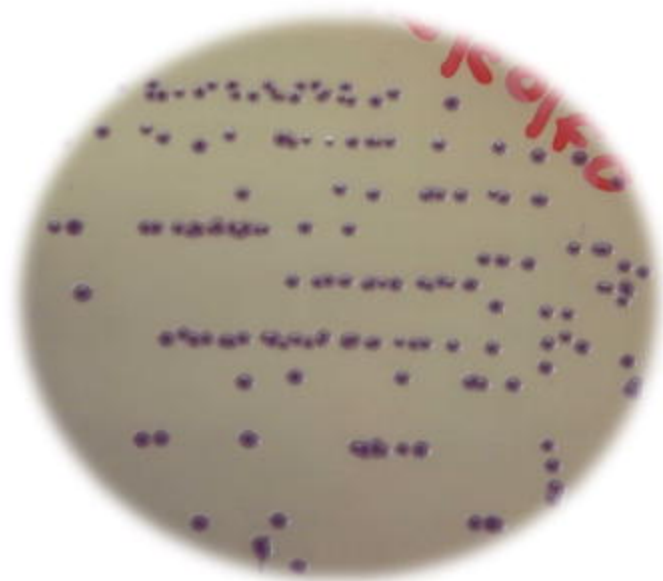


24h

48h

120h

A



B

