A SHERLOCK toolbox for the eco-epidemiological surveillance of animal African trypanosomosis reveals a similar parasite diversity in domestic pigs in two ancient sleeping sickness foci in Western Africa.

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Short title

A SHERLOCK toolbox for AAT surveillance

Abstract

Animal African trypanosomosis (AAT), caused by protist parasites of the genus *Trypanosoma,* puts upward of a million head of livestock at risk across 37 countries in Africa. The economic impact of AAT and the presence of human-infectious trypanosomes in animals place a clear importance on improving diagnostics for animal trypanosomes to map the distribution of the veterinary parasites and identify reservoirs of human-infectious trypanosomes. We have adapted the CRISPR-based detection toolkit SHERLOCK (Specific High-sensitivity Enzymatic Reporter unLOCKing) for trypanosomatid parasites responsible for AAT (SHERLOCK4AAT) including Pan-trypanosomatid, *Trypanozoon*, *T. vivax*, *T. congolense*, *T. theileri*, *T. simiae* and *T. suis* assays. To test the applicability of this technique in the field, we analysed dried blood spots collected from 200 farm and 224 free-ranging pigs in endemic and historical human African trypanosomiasis foci in Guinea and Côte d'Ivoire, respectively. The results revealed that SHERLOCK4AAT can detect and discriminate between trypanosome species involved in multiple infections with a high sensitivity. 62.7 % [58.1, 67.3] of pigs were found infected with at least one trypanosome species. *T. brucei gambiense*, a human-infectious trypanosome, was found in one animal at both sites, highlighting the risk that these animals may act as persistent reservoirs. These data suggest that, due to their proximity to humans and their attractiveness to tsetse flies, pigs could act as sentinels to monitor *T. b. gambiense* circulation using the SHERLOCK4AAT toolbox.

Introduction

Through a combination of vector control, reactive screening, passive diagnosis, new therapeutic drugs and the support of national control programmes, donors and stakeholders, interrupting the transmission of gambiense human African trypanosomiasis (HAT) at pan-African level is in reach (***Barrett et al., 2024***). Reported annual cases have been maintained below 1,000 since 2018, making the elimination (zero transmission) of gambiense HAT (gHAT) by 2030, one of the major objectives of the World Health Organisation (WHO), achievable (***Franco et al., 2024***). Nevertheless, animal African trypanosomosis (AAT), also known as *nagana*, remains a significant

burden to sustainable agricultural development, and represents a threat to livestock and small holder economies (***Boulangé et al., 2022***) with a total estimated loss of 4.75 billion USD per year in terms of agricultural gross domestic product in tsetse-infested lands (***Budd, 2001***). Unlike HAT, that is caused by either *Trypanosoma brucei (T. b.) gambiense* in West Africa, or *T. b. rhodesiense* in East Africa, AAT encompasses a wider range of trypanosomatid parasites. In sub-Saharan Africa, animal infecting trypanosomes including *T. vivax*, *T. congolense*, *T. brucei*, *T. theileri*, *T. simiae* and *T. suis* are transmitted by blood-sucking flies, such as Glossinidae and Tabanidae, to a wide range of wild and domestic mammals. Socio-environmental factors such as land cover, humidity, temperature and livestock migration impact trypanosome transmission (***Karshima et al., 2016***). This is particularly evident in rural areas of sub-Saharan Africa, where high incidences of *T. brucei*, *T. congolense* and *T. vivax* are observed, due to conditions favourable to the proliferation of flies (***Okello et al., 2022***).

Symptoms of AAT are host and pathogen-dependent, with an infection potentially leading to spontaneous abortions, reduction in milk and meat production, delays in sexual maturity, low calving rates, loss of draught power, and ultimately to high mortality, all of which compound poverty in many Sub-Saharan countries (***Kristjanson et al., 1999***). Indirect control strategies including the combination of vector control, breeding of trypanotolerant animals, and avoiding grazing in infested areas have proved to be efficient in reduce the number of infections (***Meyer et al., 2016***). Chemotherapy and chemoprophylaxis are the main means for controlling AAT, although inadequate usage, presence of fake drugs and emergent drug resistance are hampering trypanocide efficacy (***Giordani et al., 2016; Richards et al., 2021***). Reliable and adapted diagnostic tests are crucial for parasite detection to better target infected animals for treatment, as well as for parasite identification to understand the distribution of trypanosomes in endemic areas. Microscopic, serological and molecular methods are generally used to diagnose AAT. Microscopy based methods are the most commonly used diagnostic methods, but lack sensitivity with a limit of detection (LoD) ranging from 10^4 to 10^5 parasites/mL when using fresh blood, to 250 to 5,000 parasites/mL for methods combining centrifugation (***Desquesnes et al., 2022a***). Serological methods, such as the card agglutination test (CATT) and ELISA, can be used to identify anti-*Trypanosoma* antibodies, but their reliability is often compromised by the poor antigen characterization in animal infecting trypanosomes or by the

detection of antibodies from previous infections. In contrast, molecular diagnostic techniques, including PCR and Loop-mediated isothermal amplification (LAMP), offer increased sensitivity (73-100 %) and LoD (1-1000 parasites/mL) by targeting specific regions of the parasites DNA. For the main AAT-related *Trypanosoma* species, pan-species primers (targeting ITS1 for instance) and genus/species/type-specific primers are available and currently used to detect the DNA of parasites from the subgenus *Nannomonas* (*T. congolense*), *Duttonella* (*T. vivax*) and *Trypanozoon* (*T.b.brucei, T. evansi* and *T. equiperdum*) (***Álvarez-Rodríguez et al., 2022; Desquesnes et al., 2022a***).

AAT-related trypanosome species circulate co-endemically with *T. b. gambiense* in West and Central Africa, where domestic animals such as pigs, dogs, cattle, sheep and goats are suspected reservoirs of *T. b. gambiense* (***Magang et al., 2023; Traoré et al., 2021***). In these regions, Suids (warthogs, domestic and bush pigs) and bovids (bushbucks and buffalos) are the preferred hosts for most tsetse fly species (***Serem et al., 2024***). In a historical HAT focus of Côte d'Ivoire, free-range pigs sharing the same environment as humans were identified as reservoirs for *T. b. gambiense* (***Traoré et al., 2021***). The diagnostic techniques employed in this study, involving single round (***Radwanska et al., 2002***) or nested PCR (***Morrison et al., 2008***), however tended to overestimate the prevalence of the human infective parasites in pigs (***Traoré et al., 2021***). Therefore, an active and accurate surveillance of the infection status of domestic animals using a more reliable diagnostic tool will be key to reach and sustain gHAT elimination (***Simo and Rayaisse, 2015; Vourchakbé et al., 2020***). In addition, a comprehensive map of the animal infective trypanosomes circulating in tsetse-infested areas would enable an improved control strategy and prevent or limit further economic losses.

In this context, developing new diagnostic tools that are species-specific and highly sensitive is essential to accurately diagnose infected individuals and define the circulation of trypanosomes in domestic animals. For this purpose, we have adapted the Specific High-sensitivity Enzymatic Reporter unLOCKing (SHERLOCK) detection that offers unprecedented levels of diagnostic specificity and sensitivity (***Gootenberg et al., 2017***), and has been adapted for the detection of HAT (***Sima et al., 2022***). SHERLOCK combines an isothermal amplification by recombinase polymerase (RPA) with Cas13a detection of the target RNA (***Gootenberg et al., 2017; Kellner et al.,***

127 ***2019; Piepenburg et al., 2006***). The RPA step, in combination with a reverse
128 transcriptase, allows the use of total nucleic acid (TNA) as an input, increasing the
129 flexibility of the assay. Here, we have developed a broad panel of SHERLOCK assays
130 for the detection of the six most prevalent AAT-related species. We have applied the
131 SHERLOCK4AAT panel to two populations of domestic pigs from two eco-
132 epidemiological regions of West Africa to determine their importance as reservoirs of
133 both animal and human-infective trypanosomes. A total of 424 blood samples were
134 collected (1) from free-ranging pigs in the last hypo-endemic foci of gHAT in Côte
135 d'Ivoire, and (2) from farm pigs bred in the historical gHAT focus of forested Guinea
136 where unconfirmed seropositive cases of gHAT are still sporadically detected (***Courtin***
137 ***et al., 2019***). These samples were screened using the complete SHERLOCK4AAT
138 panel to provide an in-depth profile of the trypanosome species circulating among
139 these domestic animals.

140 **Materials and Methods**

141 Ethics statement

142 The only reliable method available to maintain and amplify field-isolated animal
143 trypanosomes is to experimentally infect laboratory animals, here mice. For this study,
144 experimental infections were carried out at UMR INTERTRYP and were approved by
145 the regional ethics committee for animal experimentation as part of the project APAFIS
146 #34149-2020091118136527v5 and authorized by the French Ministry for Higher
147 Education and Research. In Guinea, ethical approval was obtained from the *Comité*
148 *National d'Ethique pour la Recherche en Santé* (CNERS) under agreement number
149 102/CNERS/19. In Côte d'Ivoire, sample collection was conducted within the
150 framework of epidemiological surveillance activities supervised by the HAT National
151 Elimination Program (HAT-NEP). No ethical statement is required by local authorities
152 for domestic animal sampling. Any veterinarian may carry out blood sampling on
153 domestic animals, with the authorization of the owner, as it is performed during
154 prophylaxis or diagnostic campaign. Breeders gave their consent for animal sampling
155 after being informed of the objectives of the study. For pig care, venous sampling was
156 performed by a veterinary of the Laboratoire National d'Appui au Développement Rural
157 (Ministry of Agriculture).

158 Mice infection and parasite purification

159 24 hours prior to infection with trypanosome parasites, 8-week-old female BALB/c mice
160 (Charles River Laboratory) were immunosuppressed by intraperitoneal injection of 0.1
161 mL Endoxan 500 mg (Baxter). *T. congolense* Savannah subgroup IL1180 strain
162 (Tanzania, Serengeti 1971, STIB212), *T. vivax* Magnan strain (Burkina Faso, Magnan
163 2011) and *T. theileri* (Nederland, Utrecht 2003, UNUT) parasites (INTERTRYP unit's
164 cryobank) were inoculated by intraperitoneal injection of 0.15 mL of the cryopreserved
165 blood containing parasites. Two mice were infected per strain and parasitaemia was
166 monitored daily by tail blood sampling. Parasite yields were estimated by counting in a
167 fast read 102 counting cells (Kova). Whole blood was collected by cardiac puncture
168 under deep isoflurane anaesthesia when parasitaemia reached 1 x 10^8 cells per mL.
169 Infected blood was passed over a diethylaminoethyl (DEAE)-cellulose column and the
170 column washed using phosphate buffer saline (PBS) supplemented with 1 % glucose
171 (PBSG). Parasites were collected in PBSG and concentrated by centrifugation (2000
172 rpm, 10 min, RT).

173 Cell culture

174 *T. b. brucei* Lister 427 bloodstream form and *T. b. gambiense* ELIANE cells were
175 cultured in HMI-11 medium (ThermoFisher scientific) supplemented with 10 % fetal
176 bovine serum (Sigma) at 37°C with 5 % CO_2. Human embryonic kidney (HEK) 293T
177 cells were cultured in complemented dMEM (Merck) at 37°C with 5 % CO_2.

178 Epidemiological surveys

179 Pig blood samples were collected during two independent surveys in Côte d'Ivoire and
180 Guinea. In Côte d'Ivoire, 224 free-ranging pigs were swampled in May, June and July
181 2021 in the Bonon and Sinfra hypoendemic HAT foci, the Bouaflé and Vavoua
182 historical HAT foci and the Brobo HAT-free area {Kaba, 2023 #8655}. In Guinea, 200
183 farm pigs were sampled in March 2023 in the N'zérékoré prefecture, an ancient gHAT
184 focus. Four drops of blood were dried on 4th grade Whatman filter paper (Cytivia Life
185 Sciences) and conserved in sealed plastic bags with silica gel until total nucleic acid
186 (TNA) extraction.

187 Nucleic acid extraction

188 RNA was extracted from bloodstream parasites (*T. b. brucei* Lister 427, *T. b.*
189 *gambiense* ELIANE, *T. congolense* Savannah subgroup IL1180, *T. vivax* Magnan and
190 *T. theileri*), HEK 293T cells and whole blood from French cattle, pig, sheep and goat

191 (Bergerie de la Combe aux Loups). Bloodstream form parasites from infected mice, as
192 well as cultured HEK 293T cells, were harvested in TRIzol (Life Technologies) and
193 Chloroform:Isoamyl alcohol (24:1, Sigma-Aldrich) was added, followed by
194 centrifugation at 13,000 rpm at 4°C for 15 min. The upper aqueous phase was
195 transferred to a fresh tube containing 0.5 mL of 75 % ethanol. After incubation for 10
196 min at room temperature, RNA was extracted using a NucleoSpin RNA column
197 (Macherey Nagel) or a RNAeasy kit (Qiagen) according to the manufacturer's
198 instructions. 500 µL of blood sampled from French cattle, pig, sheep and goats was
199 treated with 500 µL lysis buffer supplemented by 50 µL Proteinase K and incubated for
200 30 min at 56°C. The resulting mixture was transferred into a QIAshredder column
201 (Qiagen) and centrifuged at 13,000 rpm for 2 min. The flow-through was then used for
202 RNA extraction using a RNeasy Mini kit (Qiagen) according to the manufacturer
203 recommendations. After extraction, qualities and quantities of RNA were evaluated
204 using the NanoDrop™ One (Ozyme).

205 For extraction of TNA from dry blood spots (DBS), three 6 mm-punches from one spot
206 of each animal DBS were resuspended in 300 µL of lysis buffer supplemented by 30
207 µL of Proteinase K and incubated for 30 min at 56°C. The punches and liquid were
208 transferred into a QIAshredder column (Qiagen) and centrifuged at 13,000 rpm for 2
209 min. TNA was extracted from the flow-through using the Maxwell RSC Buccal Swab
210 Kit (AS1640) with the Maxwell RSC Instrument (AS4500) according to the
211 manufacturer's instructions. For each sample, all SHERLOCK assays were performed
212 from a single TNA extraction.

213 Target selection, RPA primers and crRNAs design

214 Alignments of the 18S rRNA, IFX and GAPDH genes were performed on sequences
215 from different species (Supplementary Table 1) using Clustal Omega (***Sievers and***
216 ***Higgins, 2018***). The distance matrix representing the percentage identity between
217 each sequence was represented using GraphPad Prism software (V.10).

218 BLAST analysis (***McGinnis and Madden, 2004***) of the 18S rRNA and GAPDH genes
219 selected target sequences was performed in the NCBI standard database of using the
220 following selection of organisms: Microsporidia (taxid:6029), Ciliophora (taxid:5878),
221 Haemosporida (taxid:5819), Diplomonadida (taxid:5738), Symbiontida (taxid:797087),
222 Diplonemea (taxid:191814), Euglenida (taxid:3035), Kinetoplastea (taxid:5653),
223 Metakinetosplastina (taxid:2704647), Trypanosomatida (taxid:2704949),

224 Trypanosomatidae (taxid:5654) and Euglenozoa (taxid:33682). The program selection
225 was optimized for somewhat similar sequences (blastn) and in algorithm parameters,
226 the maximum number of aligned sequences to display was set as 5000.

227 The presence of single nucleotide polymorphism (SNPs) in the target regions was
228 assessed by aligning sequence variants of the parasite species using Clustal Omega
229 (***Sievers and Higgins, 2018***). Sequence variants used are listed in Supplementary
230 Table 1. SNPs identified in the TriTrypDB (https://tritrypdb.org) database were also
231 taken into account in the design: NGS_SNP.Tb927_03: v5.1.921431, v5.1.921465,
232 v5.1.921571, v5.1.921612, v5.1.921937, v5.1.922234, v5.1.922298, v5.1.922511,
233 v5.1.922637, v5.1.922803, v5.1.923180, v5.1.959274; NGS_SNP.Tb927_07:
234 v5.1.1951525, v5.1.1952283, v5.1.1951291; NGS_SNP.Tb927_02: v5.1.371291,
235 v5.1.371584 and v5.1.370058.

236 Primers for Recombinase Polymerase Amplification (RPA) of targeted regions were
237 designed in Primer-BLAST (***Ye et al., 2012***) using the customised parameters
238 described by (***Kellner et al., 2019***). Oligonucleotides prone to generate self-dimers or
239 cross primer dimers detected by Multiple Primer Analyzer (ThermoFisher) were
240 discarded. Primers selected for each SHERLOCK assays are listed and highlighted in
241 Supplementary Table 2. The T7 promoter sequence (5'
242 GAAATTAATACGACTCACTATAGGG) was added at each 5' end of the selected
243 forward primers to enable the *in vitro* transcription necessary for Cas13a detection
244 (***Kellner et al., 2019***).

245 <u>Production of crRNAs and positive controls</u>

246 crRNA production was carried out as previously described (***Kellner et al., 2019; Sima***
247 ***et al., 2022***). Briefly, each crRNA DNA template, containing the spacer (specific
248 targeted region) followed by the direct repeat (5'
249 GATTTAGACTACCCCAAAAACGAAGGGGACTAAAAC) and the T7 promoter
250 sequence, were *in vitro* transcribed using the HiScribe™ T7 Quick High Yield RNA
251 Synthesis Kit (NEB) and purified using magnetic beads (Agencourt RNAClean XP).
252 DNA template sequences used for the synthesis of each crRNA are detailed in
253 Supplementary Table 2.

254 For the production of the synthetic positive controls, *in vitro* transcription of the targets
255 was performed as previously described (***Sima et al., 2022***) using RNA from the target

256 species for each specific assay. The amount of template used to produce the positive
257 control for each assay were as follows: 200 ng of RNA from *T. b. brucei* Lister 427 for
258 18S pan-trypanosomatid and 18S pan-*Trypanozoon* assays, 120 ng of RNA from *T. b.*
259 *gambiense* ELIANE strain for TgSGP assay, 120 ng of RNA from *T. vivax* Magnan
260 strain for IFX assay, 230 ng of RNA from *T. congolense* Savannah subgroup IL1180
261 strain for 18S *T. congolense*-specific assay and 5 ng of RNA from *T. theileri* for 18S *T.*
262 *theileri*-specific assay. Due to a lack of *T. simiae* and *T. suis* genetic material, the
263 positive controls were synthesized at Eurogentec using the *in-silico* predicted
264 sequence of the target region. Primers and target regions used to produce positive
265 controls are listed in the Supplementary Table 3.

266 SHERLOCK assays

267 SHERLOCK assays were performed as previously described (***Kellner et al., 2019;***
268 ***Sima et al., 2022***). RPA was performed using the TwistAmp Basic kit (TwistDx)
269 following manufacturer's instructions with slight modifications. To enable reverse
270 transcription of the RPA (RT-RPA), 2.2 U of transcriptor reverse transcriptase (Roche)
271 were added to the reaction. Specific RPA primer pairs were used for each target
272 amplification at a concentration of 480 nM per primer, and the amplification reaction
273 was carried out in a heating block at 42°C for 45 minutes (***Sima et al., 2022***).
274 *Leptotrichia wadeii* Cas13a (*Lw*Cas13a) enzyme was produced as previously
275 described (***Kellner et al., 2019; Sima et al., 2022***). Briefly, pC013-Twinstrep-SUMO-
276 huLwCas13a plasmid (Addgene plasmid #90097) was inserted into *Escherichia coli*
277 RosettaTM 2(DE3) pLysS competent cells for *Lw*Cas13a expression and purification
278 (Gootenberg et al. 2017). Purified *Lw*Cas13a was conserved in Storage buffer (SB:
279 Tris-HCl 1M, NaCl 5M, DTT 1M and glycerol) (***Kellner et al., 2019***) in single-use
280 aliquots at -80°C to avoid freeze-thaw cycles.

281 The Cas13a-crRNA mediated target detection reaction was performed simultaneously
282 with the *in vitro* transcription of the target amplified by RT-RPA. *In vitro* transcription of
283 the amplified target was performed using T7 RNA polymerase (Biosearch technology).
284 Each Cas13a detection reaction was run in triplicate on a TECAN plate reader
285 (INFINITE F200 PRO M PLEX) and fluorescence readouts were collected every 10
286 minutes for 3 hours. All reactions were carried out with the inclusion of a negative
287 control template (NCT) adding nuclease-free water as input, and a positive control
288 template (PCT), here the *in vitro* produced positive control for each specific

289 SHERLOCK. For screening samples from epidemiological surveys, the same protocol
290 as described above was used, except a TECAN plate reader (INFINITE F200 PRO
291 Option Infinite F Nano +) was used and the assay was performed without replicates.
292 Optimizations were carried out either at Institut Pasteur in Paris (France) or at
293 INTERTRYP unit in Montpellier (France), while analysis of samples from
294 epidemiological surveys was carried out at Institut Pasteur of Guinea.

295 Data representation and statistical analyses

296 Fold-change over background fluorescence was used to represent fluorescence
297 readouts for all SHERLOCK assays. Background fluorescence is given by the NCT
298 evaluation, where water is used as input material for the RPA reaction. For each test,
299 sample fluorescence obtained after 3 hours of reading was divided by NCT
300 fluorescence at the same time.

301 $$FC = \frac{Fsample\ t = 3h}{Fnct\ mean\ t = 3h}$$

302 Where *FC* represents Fold-Change over background fluorescence, *Fsample t=3h*
303 represents sample fluorescence obtained after 3 hours and *Fnct mean t=3h* represents
304 NCT fluorescence after 3 hours of reactions.

305 Analytical characteristics including threshold, sensitivity and specificity of each
306 SHERLOCK assay were evaluated using Receiver Operating Characteristic (ROC)
307 curves calculation comparing NCT versus PCT at controlled dilutions of parasites or
308 hosts genetic materials. Results obtained from SHERLOCK specific assays on
309 targeted parasites were gathered in a positive sample column, while results obtained
310 from NCT, hosts and non-targeted parasites analysed with the assay, were placed in
311 a negative sample column for ROC curve calculation and threshold evaluation. ROC
312 curves were calculated using the Wilson/Brown method with a 95 % confidence interval
313 and threshold values were selected based on the Youden's index (sensitivity +
314 specificity – 1), that defines an optimal threshold as the one that maximizes the index
315 of classification accuracy (***Brown et al., 2001; Schisterman et al., 2005***). The
316 threshold of positivity for each SHERLOCK assay was selected from ROC analyses
317 as the value corresponding to the maximum specificity (100 %). The limit of detection
318 (LoD) of each SHERLOCK reaction was assessed using specific tests on serial
319 dilutions of target parasite RNA. Dilutions ranged from 5.10^3 pg/µL (equivalent to 10^7

320 parasites/mL) to 5.10^{-5} pg/µL (equivalent to 0.1 parasites/mL). All plots and statistical
321 analyses were performed with GraphPad Prism software (V.10). The Venn diagrams
322 were made based on the Venny model by Oliveros J.C. (BioinfoGP, CNB-CSIC).

323 Maps were generated with the QGIS software version 3.42.0 with backgrounds
324 retrieved from data.gouv.fr (OSM2IGEO - Ivory Coast - SHP - EPSG:4326 for Côte
325 d'Ivoire, and OSM2IGEO - Guinea - SHP - EPSG:4326 for Guinea).

326 In the absence of reliable infection status in the field data from pigs, the posterior
327 distributions of performance estimates, such as sensitivity (Se), were jointly estimated
328 with a Bayesian latent class model (***Joseph et al., 1995***). The model takes boolean
329 outcomes (either positive or negative) of several tests as input. This Monte Carlo
330 method operates based on a Gibbs sampling process. The model iteratively updates
331 both the test performance parameters (sensitivity, specificity, and frequency of positive
332 individuals in sample collection) and the latent infection status. Flat prior was used.
333 After visually controlling that stationarity of the chain was quickly reached, we
334 discarded the first 150 burn-in iterations and calculated posterior densities based on >
335 10,000 iterations. 95% confidence intervals were calculated for each proportion to
336 quantify the uncertainty of the estimates, providing a scientific range within which the
337 population proportions are likely to be accurate (***Brown et al., 2001***).

338 **Results**

339 Selection of targets for broad and species-specific SHERLOCK assays targeting AAT
340 species (SHERLOCK4AAT).

341 Target gene selection was based on two requirements: to develop (a) a broad
342 SHERLOCK assay for the detection of all trypanosomatid parasites and (b) for species
343 discrimination. To adapt SHERLOCK for the detection of AAT-related species, we
344 followed the criteria as described in (***Sima et al., 2022***). Briefly, candidate genes must
345 (1) be expressed in the bloodstream form of the parasites, (2) be conserved between
346 strains, (3) have few or no single nucleotide polymorphisms (SNPs), or (4) show
347 sequence divergence that would allow for species discrimination. All RPA primers and
348 crRNAs designed for each SHERLOCK assays are listed in Supplementary Table 2.

349 **Pan-trypanosomatid assay.** SHERLOCK has the capacity to discriminate between
350 two sequences differing by a single nucleotide (***Gootenberg et al., 2017***). This study
351 therefore focused on regions of the *18S rRNA* gene that show limited sequence

352 homology between species. In addition to being a multicopy gene, the *18S rRNA* gene
353 shares over 70 % identity between Kinetoplastea members (Supplementary Figure 1).
354 Using BLAST analysis, the region between 1535-1672 bp (XR_002989632.1) was
355 found to be conserved between all trypanosomatid species and was selected as the
356 pan-trypanosomatid SHERLOCK target. RPA primers were designed to amplify this
357 138 bp region using Primer-Blast (***Ye et al., 2012***) (Supplementary Figure 2A and
358 Supplementary Table 2). The level of identity of the target region was assessed
359 between the different sequences described in the Trypanosomatidae family, as well as
360 in the order, subclass and class that contain this family (Supplementary Figure 3).
361 Examination of the Euglenozoa phylum, excluding the Kinetoplastea class, showed
362 98.4 % identity within the Trypanosomatidae family and 97.3 % identity within the
363 Kinetoplastea class (Supplementary Figure 3). This was reduced to 82.9 % for the
364 Phylum Euglenozoa, excluding the Kinetoplastea sequences (Supplementary Figure
365 3). Three distinct crRNAs were designed to this region, which can be used individually
366 or multiplexed to increase the sensitivity (Supplementary Figure 4A and
367 Supplementary Table 2). The sequences described in each group were 100 %
368 conserved to the crRNA. RPA primers and crRNA targets of the pan-trypanosomatid
369 SHERLOCK assay target regions (Kinetoplastea) were then compared to the
370 homologous sequences from other classes within the Phylum Euglenozoa (Euglenida,
371 Diplonemea, Symbiontida and Kinetoplastea) (Supplementary Figure 2A and 3). In
372 addition, sequences from related protozoan parasite classes (Diplomonadida,
373 Haemosporida and Ciliophora) and from the genetically close but phylogenetically
374 distinct group Microsporidia were also included in the analysis (Supplementary Figure
375 2A).

376 **Pan-*Trypanozoon* assay**. A pan-*Trypanozoon* SHERLOCK assay was designed for
377 increased selectivity to especially detect *T. brucei s.l.*. The regions within the 18S rRNA
378 that were highly conserved among the species of the *Trypanozoon* subgenus, while
379 showing significant divergence from other trypanosome species not belonging to the
380 *Trypanozoon* subgenus, were analysed (Supplementary Figure 2B). A region ranging
381 from 1318 to 1433 bp (XR_002989632.1) (Figure 1A and Supplementary Table 2) was
382 selected as it showed high conservation among the *Trypanozoon* subgenus (99.12 -
383 100 % percentage of identity, query coverage >90 %), except for three *T. evansi*
384 sequences (91.96 - 96.49 %, OL869590.1, KT844944.1, MF142286.1). The crRNA

385 target region selected was also conserved in the subgenus *Trypanozoon* and divergent
386 in other parasites. Specific alignments with trypanosome species that could share the
387 same hosts (*T. congolense*, *T. vivax*, *Leishmania spp.*, etc) were performed to assess
388 target amplicon specificity (Supplementary Figure 2B). The pan-*Trypanozoon*
389 SHERLOCK assay can discriminate between the species belonging to the
390 *Trypanozoon* subgenus (*T. b. spp., T. evansi* types A and B, and *T. equiperdum*) from
391 other non-*Trypanozoon* trypanosomes or other co-endemic parasites.

392 **Species-specific assays**. For species discrimination within a single sample, several
393 SHERLOCK assays were developed to detect the most prevalent species causing
394 AAT. These include *T. congolense*, *T. vivax*, *T. theileri*, *T. simiae* and *T. suis*.

395 *T. congolense*. Regions conserved in the 18S rRNA gene for all described *T.*
396 *congolense* sequences, including the Savannah, Forest, Kilifi and Dzanga-Sangha
397 groups, were analysed. The target region selected from position 116 to 246 bp of the
398 *18S rRNA* gene (AJ009146.1) shows 100 % identity between the described *T.*
399 *congolense* sequences. BLAST analysis of the *T. congolense* 18S rRNA gene
400 sequences, excluding the target species (*T. congolense*, (taxid: 5692)) was also
401 performed to determine the percentage of identity of closely related species. *T. simiae*
402 (86.72 to 90.62 %), *T. sapaensis* (88.28 %), *T. godfreyi* (88.28 %), and *T.*
403 *otospermophili* (87.02 %) had the highest percentage identity observed
404 (Supplementary Figure 5). Species with common hosts and of veterinary relevance
405 such as the two analysed *T. vivax* strains showed 82.81% and 87.5% identity. In
406 addition, none of these sequences were fully conserved for the selected crRNA
407 (Supplementary Figure 5), ensuring the specificity of the test and the absence of cross-
408 detection with other species.

409 *T. vivax*. The *invariant flagellum antigen* (*IFX*) gene has no apparent homologue in any
410 other *Trypanosoma* species or other related parasites, and was therefore selected
411 (***Autheman et al., 2021; Romero-Ramirez et al., 2022***). BLAST analysis, using *T.*
412 *vivax* Y486 (HE573024.1) as a reference genome, resulted in no matches (Figure 1A
413 and Supplementary Table 2).

414 *T. theileri*. A conserved region between the three *T. theileri* lineages (TthI, TthII and
415 TthIII) (***Brotánková et al., 2022***) was selected. The target region, ranging from 1014
416 to 1158 bp of the *18S rRNA* gene (AJ009163.1), has 100 % identity between the

417 described sequences of the *T. theileri* lineages, and the most closely related
418 sequences were from *T. cruzi*, *T. rangeli* and *T. dionisii*, with a 97.89 % identity (query
419 coverage =65 %). The segment 1052-1081 bp corresponding to the crRNA sequence,
420 is highly conserved between *T. theileri* variants (Figure 1A and Supplementary Table
421 2), however it contains a SNP (1060 C/T) in a number of the sequences analysed.
422 Hence, one RPA primer pair and two multiplexed crRNAs were designed to target both
423 version of the SNP for the *T. theileri* SHERLOCK assay (Supplementary Figure 4E).

424 *T. simiae.* The region from 1246-1346 bp of the *18S rRNA* gene (AJ404608.1) was
425 selected as the targeted region for RPA amplification of the *T. simiae*-specific
426 SHERLOCK assay. The segment 1273-1299 bp that corresponds to the crRNA
427 sequence, has 100 % identity between the described sequences of *T. simiae* (Figure
428 1A and Supplementary Table 2).

429 *T. suis.* The multicopy *GAPDH* gene was used, as it can be phylogenetically separated
430 into *T. suis* and *T. suis*-like strains from other African trypanosomes (***Rodrigues et al.,***
431 ***2020***). The region from 389 to 528 bp of the *GAPDH* gene (MK962702.1) has been
432 selected as the targeted region for RPA amplification of the *T. suis*-specific
433 SHERLOCK assay (Figure 1A and Supplementary Table 2). This region is 100 %
434 conserved between the *T. suis* species described, and outside this clade, the most
435 closely related sequences were found in *T. congolense*, with a 90 % of identity (query
436 coverage >90 %). The segment 410-437 bp of the *gGAPDH* gene (MK962702.1), that
437 corresponds to the crRNA target region, is highly conserved between *T. suis* species,
438 with 100 % identity, and the BLAST analysis showed that this target fragment can only
439 align to *T. suis* sequences but not to that of other Kinetoplastea species (taxid:5653).

440 <u>SHERLOCK can discriminate between closely related AAT-causing trypanosomatid</u>
441 <u>species.</u>

442 All RPA primers and crRNAs for each targeted species were screened to select the
443 optimal combinations, with the highest specificity and selectivity. For the pan-
444 trypanosomatid assay, RPA primer pair A was used to screen three crRNAs
445 (A:crRNA1, A:crRNA2 and A:crRNA3; Supplementary Figure 4A). From this, crRNA 3
446 was first selected for the simple pan-trypanosomatid assay. For the pan-*Trypanozoon*
447 assay, three RPA primer pairs (A, B and C) were screened with two crRNA candidates
448 each (A:crRNA1, A:crRNA2, B:crRNA1, B:crRNA2, C:crRNA1, C:crRNA2,
449 Supplementary Figure 4B). The A:crRNA1 combination was selected for the pan-

450 *Trypanozoon* assay, being the one that obtained a higher fold change for the positive
451 control and a lower fold change for the negative control. For the IFX *T. vivax* species-
452 specific SHERLOCK assay, two RPA primer pairs were assessed (A and B) in
453 combination with six crRNA candidates (A:crRNA1, A:crRNA2, A:crRNA3, B:crRNA4,
454 B:crRNA5, B:crRNA6). RPA primer pair B and crRNA 6 showed the lowest variability
455 between replicates and without any cross-reaction with other trypanosome species
456 (Supplementary Figure 4C). For the *T. congolense*-specific assay, one pair of RPA
457 primer (A) was combined with ten crRNA candidates (A:crRNA1, A:crRNA2,
458 A:crRNA3, A:crRNA4, A:crRNA5, A:crRNA6, A:crRNA7, A:crRNA8, A:crRNA9,
459 A:crRNA10). The crRNA1 that exhibited the lowest inter-replicate deviation and no
460 cross-reactivity when exposed to the genetic material of non-target trypanosomatid
461 parasites was selected (Supplementary Figure 4D). For the *T. theileri*-specific
462 SHERLOCK assay, the combination of the RPA primer pair A with the two designed
463 crRNAs (1 and 2) was used to cover the specific detection of all the *T. theileri* described
464 sequences (Supplementary Figure 4E). For *T. simiae* and *T. suis*, single combinations
465 of RPA primer and crRNA were tested. The specificity of each of these combinations
466 was also verified by comparing the specific recognition of each RNA target to RNA
467 from human embryonic kidney cell (HEK) (for pan-trypanosomatid and pan-
468 *Trypanozoon* tests) or RNA from *T. brucei* (for *T. vivax*, *T. congolense* and *T. theileri*
469 tests) (Supplementary Figure 4). In total, a RPA primer-crRNA combination was
470 selected for each assay, based on the highest fold change over background
471 fluorescence value, the highest specificity, and the minimum standard deviation
472 (Figure 1A).

473 The specificity of the RPA primer-crRNA combinations selected for each assay was
474 then cross-assessed against closely related parasites (Figure 1B-C). The single pan-
475 trypanosomatid assay detected RNA from all trypanosomatid species tested (*T. b.*
476 *brucei*, *T. b. gambiense*, *T. b. rhodesiense*, *T. evansi* (type A and B), *T. equiperdum*,
477 *T. congolense*, *T. vivax*, *T. theileri*, *T. cruzi* and *Leishmania major*) but not from HEK
478 cells, synthetic controls for *T. simiae* and *T. suis* or co-endemic parasites such as
479 *Plasmodium falciparum* (Figure 1B). The pan-*Trypanozoon* assay detected only RNA
480 from members of the *Trypanozoon* subgenus (*T. b. brucei*, *T. b. gambiense*, *T. b.*
481 *rhodesiense*, *T. evansi* (type A and B) and *T. equiperdum*) but not from HEK cells,
482 synthetic controls for *T. simiae* and *T. suis* or co-endemic parasites such as *T.*

483 *congolense*, *T. vivax*, *T. theileri* and *Plasmodium falciparum* (Figure 1B). The species-
484 specific *T. congolense*, *T. vivax*, *T. theileri*, *T. simiae* and *T. suis* assays only detected
485 their respective targeted parasite RNA or synthetic controls with no cross reactivity with
486 any other RNAs (Figure 1C). Additionally, no unspecific cross-reaction was observed
487 with the host-derived genetic material, as assessed by testing RNA from pig blood
488 (SHERLOCK single pan-trypanosomatid and pan-*Trypanozoon* assays in
489 Supplementary Figure 7).

490 The analytical sensitivity of each assay was quantified using serial dilutions of positive
491 control RNAs from known numbers of parasites. For the single pan-trypanosomatid
492 assay, the LoD was between 10 to 10^3 parasites per mL. The pan-*Trypanozoon* and
493 *T. theileri* assays tested 10 parasites per mL. For the *T. congolense* assay, the LoD
494 was found between 10 and 10^3 parasites per mL, and between 10^4 and 10^5 parasites
495 per mL for the *T. vivax* test (Figure 2A). The sensitivity of *T. simiae* and *T. suis* tests
496 were evaluated on synthetic positive controls, as no genetic materials were available,
497 and both showed a LoD between 10 and 100 fM (Figure 2B).

498 Then, to improve the sensitivity of the simple pan-trypanosomatid assay, crRNA1 was
499 tested in combination with crRNA3 for target recognition, as both crRNAs fall within the
500 same RPA amplicon A (Figure 3A). Comparative analyses between the two versions
501 were performed and multiplexing was shown to increase the performance of the assay,
502 especially for the lower dilutions evaluated (100, 500 and 10^3 p/mL) by increasing the
503 fold-change readouts of these positive controls by 1, 2.5 and 3-fold-change units,
504 respectively (Supplementary Figure 6). Using results obtained on the blood samples
505 from pigs in Côte d'Ivoire (see next section), we compared the two versions of the pan-
506 trypanosomatid SHERLOCK assays to determine the posterior distribution of
507 sensitivity estimates. We applied a Monte Carlo method (Gibbs sampling) proceeding
508 in an iterative chain using the infection frequency, specificity, and sensitivity of the two
509 tests to update the infection status. We determined the posterior sensitivity distribution
510 of the simple versus multiplex pan-trypanosomatid SHERLOCK assays. Multiplexing
511 crRNAs increased the median sensitivity from 66.1 % to 74.7 %, while keeping
512 specificity above 80 % (Figure 3B). Furthermore, a higher concordance was observed
513 between the results obtained on the same samples with the pan-*Trypanozoon*
514 SHERLOCK assay and the multiplexed pan-trypanosomatid SHERLOCK assay
515 (25.7-% of double positives), as compared to the simple version (7.8 % of double

516 positives) (Figure 3C). Given its improved detection efficiency, the multiplex version of
517 the 18S rRNA pan-trypanosomatid assay was subsequently used exclusively and
518 referred to as the multiplex pan-trypanosomatid assay.

519 To determine a positivity threshold value (fold-change cut-off) for each SHERLOCK
520 assay, ROC curve analyses were performed and use to evaluate each following
521 analytical sensitivity and specificity. The positivity threshold values were 6.050 for the
522 pan-trypanosomatid test, 11.70 for the pan-*Trypanozoon* test, 1.923 for the *T.*
523 *congolense* test, 1.831 for the *T. vivax* test, 1.805 for the *T. theileri* test, 1.546 for the
524 *T. simiae* test, and 2.609 for the *T. suis* test (Supplementary Figure 8). Subsequently,
525 for a given test, any sample with a fold change value above the threshold was
526 considered positive for this test. Unsurprisingly, assays designed against multiple copy
527 genes (*18S rRNA* and *GAPDH*) showed a greater sensitivity than those targeting single
528 copy genes (*IFX*). Taken together, all the validated tests were able to specifically detect
529 the RNA targets for which they were designed, making it possible to distinguish
530 between closely related trypanosomatid parasite species. We then applied this novel
531 SHERLOCK4AAT toolbox was applied to the analysis of field samples in a molecular
532 eco-epidemiological survey.

533 <u>Application of the SHERLOCK4AAT toolbox to eco-epidemiological surveys in pigs.</u>
534 In transmission foci of western Africa, domestic pigs can host multiple trypanosome
535 species. To investigate this, the SHERLOCK4AAT toolbox was used to survey
536 trypanosomes circulating in domestic pigs from hypo-endemic and historical foci in
537 Côte d'Ivoire and Guinea. In Côte d'Ivoire, blood samples were collected from 224 free-
538 ranging pigs across the rural districts of Bonon, Bouaflé, Sinfra, Vavoua, and Brobo
539 (Figure 4A). In the N'zérékoré prefecture of Forested Guinea, 200 farm pigs were
540 sampled, with a focus on organized farming communities (Figure 5A). In addition to
541 the SHERLOCK4AAT toolbox assays, all samples were also screened for *T. b.*
542 *gambiense* using the previously described TgsGP SHERLOCK assay (***Sima et al.,***
543 ***2022***).

544 In total, 74.6 % [68.9, 80.3] (167/224) of the samples tested positive in at least one
545 SHERLOCK assay in Côte d'Ivoire (Figure 4B), and 49.5 % [42.6, 56.9] (99/200) in
546 Guinea (Figure 5B) representing a high apparent prevalence of trypanosome infections
547 in these two populations of pigs. In parallel, 47.3 % [40.8, 53.8] (106/224) of the pigs
548 from Côte d'Ivoire tested positive using at least one parasitological method (buffy coat

549 examination, mAECT and /or isolation in mice; results detailed in a separate study,
550 manuscript in preparation). No systematic parasitological data was available for
551 Guinean samples. The difference in positivity rates between SHERLOCK detection
552 and parasitological approaches on Ivoirian samples was likely due to the higher
553 sensitivity of SHERLOCK as compared to classical methods. Indeed, we compared
554 three SHERLOCK assays (multiplex pan-trypanosomatid, pan-*Trypanozoon* and *T.*
555 *congolense*-specific SHERLOCK assays) to the three parasitological tests (buffy coat
556 examination, mAECT and /or isolation in mice) to determine the posterior distribution
557 of sensitivity estimates. A Monte Carlo method (Gibbs sampling) was used by
558 proceeding in an iterative chain using the infection frequency, specificity, and
559 sensitivity of the tests to update the infection status. This analysis suggested that, in
560 terms of sensitivity, the combination of SHERLOCK4AAT assays may compete with
561 combined parasitological methods (i.e., median sensitivity 91.4 % vs 84.3 %) while
562 controlling median specificity above 75 % (Supplementary Figure 9). Importantly, in
563 contrast to parasitological tests, SHERLOCK4AAT allowed for the identification of the
564 trypanosome species involved in more than half of these infections.

565 Using the multiplex pan-trypanosomatid assay, 41.1 % [34.6, 47.6] of the samples from
566 Côte d'Ivoire (90/219) and 29.5 % [23.2, 35.8] from Guinea (59/200) were positive
567 (Figure 4C-5C). The pan-*Trypanozoon* assay identified 38.0 % [31.6, 44.4] (85/224)
568 and 39.0 % [32.2, 45.8] (78/200) of samples in each country respectively (Figure 4C-
569 5C). As there is no specific test for *T. b. brucei*, samples that were positive in the pan-
570 *Trypanozoon* assay but negative in the TgsGP assay were presumed to be *T. b. brucei*
571 infections. Only one sample tested positive in the *T. b. gambiense*-specific assay in
572 each country (Figure 4B-5B). The TgsGP-positive sample from the district of Bonon,
573 Côte d'Ivoire was negative for all other SHERLOCK assays, as well as in all
574 parasitological tests. In contrast, the TgsGP-positive sample from Guinea was positive
575 in the multiplex pan-trypanosomatid SHERLOCK assay, as well as in the CATT and
576 mAECT tests, but it negative in the pan-*Trypanozoon* SHERLOCK assay. To further
577 explore trypanosome diversity, species-specific SHERLOCK assays for AAT were
578 applied to the samples. In total, 23.2 % [17.7, 28.7] (52/224) of the samples in Côte
579 d'Ivoire and 7.5 % [3.8, 11.2] (15/200) in Guinea were positive for *T. congolense*.
580 Additionally, a single sample from Côte d'Ivoire tested positive in the *T. suis*-specific

581 SHERLOCK assay. Furthermore, no RNA from *T. vivax* or *T. theileri* was detected with
582 the IFX SHERLOCK assay.

583 Interestingly, a substantial number of co-infections were observed. In total, 24/219
584 individuals (11.0 ± 4.1 %) in Côte d'Ivoire and 13/200 in Guinea (6.5 ± 3.4 %) tested
585 positive in the multiplex pan-trypanosomatid, the pan-*Trypanozoon* and the *T.*
586 *congolense*-specific assays. These triple positive results indicate co-infections
587 involving both a *Trypanozoon* species (likely *T. b. brucei*) and *T. congolense* (Figure
588 4D-5D). In addition, 9/219 samples (4.1 ± 2.6 %) from Côte d'Ivoire tested positive in
589 the pan-*Trypanozoon* and the *T. congolense* assays, which also represented co-
590 infections with *T. brucei s.l.* and *T. congolense*. Overall, 15.2 % [10.5, 19.9] of the
591 samples from Côte d'Ivoire showed co-infections with a *Trypanozoon* species and *T.*
592 *congolense*. Additionally, 15 samples from Côte d'Ivoire tested positive in both the
593 multiplex pan-trypanosomatid assay and the *T. congolense*-specific assay or were
594 positive only for *T. congolense*. In Guinea, two samples tested positive in both the *T.*
595 *congolense*-specific and multiplex pan-trypanosomatid SHERLOCK assays,
596 indicating *T. congolense* mono-infections (Figure 4D-5D).

597 Furthermore, 56 samples (25.6 ± 5.8 %) in Côte d'Ivoire, and 38 samples (19.0 ± 5.4
598 %) in Guinea tested positive in both the multiplex pan-trypanosomatid and the pan-
599 *Trypanozoon* SHERLOCK assays. In contrast, 17 samples from Côte d'Ivoire and 40
600 from Guinea tested positive in the pan-*Trypanozoon* assay but negative in the multiplex
601 pan-trypanosomatid assay, likely due to the lower sensitivity of the latter. Lastly, a 19
602 samples (8.7 ± 3.7 %) in Côte d'Ivoire and 19 samples (9.5 ± 4.1 %) in Guinea tested
603 positive in the multiplex pan-trypanosomatid assay only (Figure 4D-5D). The absence
604 of positivity in the *T. congolense*, *T. simiae* and *T. suis*-specific assays may be due to
605 their lower sensitivity, or to infections with other non-targeted trypanosome species.

606 **Discussion**

607 <u>The SHERLOCK4AAT toolbox, a new panel of highly sensitive and specific high-</u>
608 <u>throughput assays for detecting animal trypanosomes.</u>

609 Species identification is essential for assessing both the distribution of prominent
610 parasites impacting veterinary health and spotting reservoirs for human-infective
611 trypanosomes. In a context where symptoms are often mild and unspecific, especially
612 in hosts like pigs, accurate species-specific detection methods are essential. Ideally,

613 diagnostic assays should be able to analyse both fresh and stored (ambient
614 temperature) samples to determine parasites circulating within a specific area.

615 In this study, conserved and species-specific targets have been used to adapt
616 SHERLOCK for the detection of most members of the Trypanosomatidae family
617 (Kinetoplastea class) (Supplementary Figure 3) of veterinary importance (pan-
618 trypanosomatid), and species-specific assays to discriminate between the six most
619 common animal trypanosomes species in the study regions: *Trypanozoon* (*T. b. brucei*,
620 *T. evansi* and *T. equiperdum*), *T. congolense* (Savanah, Forest Kilifi and Dzanga-
621 sangha), *T. vivax*, *T. theileri* (TthI, TthII and TthIII), *T. simiae* and *T. suis*. The design
622 of both broad and species-specific assays was based primarily on sequences of the
623 *18S rRNA* and *GAPDH* genes which, as well as being conserved across species
624 (Supplementary Figure 1), are highly expressed and contain regions that allowed for
625 species discrimination. However, for *T. vivax*, the single-copy *IFX* gene was selected
626 for its high specificity (***Romero-Ramirez et al., 2022***). The first simple version of our
627 pan-trypanosomatid assay employed a single crRNA guide and was previously used
628 to evaluated blood samples from gHAT seropositive individuals with a specificity of 93
629 % (95.4-98.6) (***N'Djetchi et al., 2024***). To increase the performances of this assay, we
630 added a second crRNA guide into the reaction mix to target the same RPA amplicon
631 during the SHERLOCK reaction, resulting in an increased test sensitivity (Figure 3 and
632 Supplementary Figure 6). For *Trypanozoon* species, no sequences were identified to
633 distinguish *T. b. brucei*, *T. evansi* (A and B) and *T. equiperdum* as these parasites
634 cannot be easily differentiated using the *18S rRNA* gene. In the future, other genes
635 such as *RoTat1.2 VSG* or *ESAG6/7* could be explored to discriminate *T. evansi* from
636 other *Trypanozoon* by SHERLOCK (***Claes et al., 2004; Holland et al., 2001***). Overall,
637 *in silico* and *in vitro* analyses enabled us to assess the analytical specificities of these
638 new SHERLOCK assays, confirming that the targets selected were sufficiently specific
639 to be used as diagnostic for the species and groups of species concerned.

640 Using parasite RNAs, the LoD of the SHERLOCK assays were estimated to range
641 between 10 to 10^3 parasites/mL for the pan-trypanosomatid assay, 10 parasites/mL for
642 the pan-*Trypanozoon* and *T. theileri* assays, from 10 to 10^3 parasites/mL for *T.*
643 *congolense* and 10^4-10^5 parasites/mL for *T. vivax*. Most PCR-derived molecular
644 techniques currently used for detecting animal trypanosomes have a detection limit of
645 1 to 10 parasites/mL using genus- or species-specific primers, 50 parasites/mL using

646 pan-species ITS1 primers (***Desquesnes et al., 2022a***) or 5 parasites/mL using 18S
647 rRNA-targeting primers (***Deborggraeve et al., 2006***). The ROC analyses of the *T.*
648 *brucei*, *T. congolense* and *T. vivax* SHERLOCK assays (Supplementary Figure 8C)
649 also shown sensitivity and specificity comparable to those of the recently developed
650 7SL sRNA diagnostic test for Animal trypanosomosis (***Contreras Garcia et al., 2022***).
651 Therefore, in total, the analytical sensitivities obtained by the pan-trypanosomatid, pan-
652 *Trypanozoon*, *T. theileri* and *T. congolense* SHERLOCK assays were comparable with
653 those of other existing molecular tools. Yet, a significant difference in favour of using
654 SHERLOCK4AAT is that it detects RNAs, probing for active infections. The *T. vivax*
655 SHERLOCK assay, targeting the single-copy *IFX* gene (***Autheman et al., 2021***), did
656 not meet the sensitivity requirements for the molecular diagnosis of animal
657 trypanosomes and would require the identification of another target. As no genetic
658 material from parasites was available for developing *T. simiae* and *T. suis* SHERLOCK
659 assays, LoDs were determined using synthetic RNA controls. Both assays were able
660 to detect 10 to 100 fM of the synthetic controls, far from the detection capacity of
661 SHERLOCK, which can detect synthetic targets at a concentration of 2 aM in optimal
662 conditions (***Gootenberg et al., 2017***).

663 SHERLOCK4AAT profiling in Guinea and Côte d'Ivoire.

664 Trypanosome species identification can be used to finely map the risk of AAT parasites
665 transmission in these areas. After cattle, pigs are the main domestic hosts infected by
666 animal trypanosomes, but they can also potentially be reservoirs of *T. b. gambiense*
667 *(**Büscher et al., 2018**)*. This is all the more important than pigs are often raised in close
668 proximity to humans, increasing the risk of animal to human trypanosome
669 transmission. In blood samples from Ivorian pigs, analyses with the SHERLOCK4AAT
670 toolbox revealed 37.9 % positive samples for *Trypanozoon* RNAs and 23.2 % positive
671 samples for *T. congolense* RNAs, with 15.2 % samples positive for both tests. In
672 addition, no sample was positive for *T. vivax, T. theileri* or *T. simiae*, but one sample
673 was positive in the *T. suis* assay and another positive for *T. b. gambiense*. These
674 findings are consistent with the most recent known distribution of animal trypanosomes
675 in free-ranging pigs raised in Côte d'Ivoire's historical gHAT foci. The use of
676 conventional molecular diagnostics on 97 free-range pigs resulted in an overall
677 positivity rate of 57 % for *Trypanozoon* DNA (PCR-TBR) and 24 % for *T. congolense*
678 *forest* DNA (PCR-TCF), with 14 % of pigs positive in both PCRs and none positive for

679 *T. vivax* (PCR-TVW) (***N'Djetchi et al., 2017; Traoré et al., 2021***). The epidemiological
680 status of AAT in Guinea is unclear, as no large-scale molecular studies have been
681 published so far. The blood samples from farmed pigs analysed here were collected in
682 the prefecture of N'zérékoré, a former endemic focus of gHAT. Results showed a
683 positivity rate of 39.0 % for the detection of *Trypanozoon* RNAs, like that observed in
684 Côte d'Ivoire, but a lower rate of positivity for *T. congolense* RNAs (7.5 %), with 6.5 %
685 of samples positive for both tests. In addition, one sample tested positive for *T. b.*
686 *gambiense,* but no *T. vivax*, *T. simiae, T. theileri* nor *T. suis* was detected. The lack of
687 any samples positive for *T. vivax or T. theileri* in any study areas confirms that pigs are
688 not frequent carriers of these species (***Fetene et al., 2021***). However, the blood
689 samples positive for *T. b. gambiense* in each study site provide further evidence of the
690 ability of pigs to carry human trypanosomes and to act as potential reservoir. Similarly,
691 the presence of *T. b. gambiense* genetic material in pig samples has already been
692 suspected in Côte d'Ivoire (***Traoré et al., 2021***).

693 In both sites, a high prevalence of trypanosomatid parasites was observed using the
694 multiplex pan-trypanosomatid and/or pan-*Trypanozoon* assays (42.0 % in Côte d'Ivoire
695 and 48.5 % in Guinea). Having partially deconvolved the species present in these
696 animals, there is still a small proportion (8.7 % in Côte d'Ivoire and 9.5 % in Guinea) of
697 trypanosomatid parasites that remained uncharacterised by our tests on samples
698 found only positive in the multiplex pan-trypanosomatid SHERLOCK assay. Firstly,
699 these assays were designed by *in silico* determination of the most specific and
700 conserved region to target the entire Trypanosomatidae family, and even
701 Kinetoplastea class, or to discriminate between species, yet the limited number of
702 sequences available for some species in the literature and in the online databases may
703 under-represent the real genetic diversity of these parasites *in natura*. In line with this
704 hypothesis, there may also exist uncharacterised trypanosome species circulating
705 among these reservoirs, as described in small mammals in central and East Africa
706 (***Votypka et al., 2022***), and that were only detected by the broad multiplex pan-
707 trypanosomatid assay. Altogether, these results are consistent with those reported in
708 the few available previous studies (***N'Djetchi et al., 2017; Traoré et al., 2021***) and
709 confirm the effectiveness of the SHERLOCK4AAT toolbox in analysing the distribution
710 of trypanosomes in hypo-endemic gHAT foci. During natural infections, trypanosome
711 nucleic acids can be detected in the skin, adipose tissue and various organs even if

712 the blood remains negative (***Amisigo et al., 2024***). Hence, applying SHERLOCK
713 detection on skin samples in the future would be useful for monitoring both blood and
714 skin-dwelling parasites. The future development of multiplexed assays combined with
715 adapted lateral flow tests would also be highly desirable for reaching field-applicable
716 point-of-care molecular diagnostic versions of the tests useable in the field. The
717 absence of point of care diagnostic test for AAT is currently a major hurdle for its
718 surveillance and control (***Desquesnes et al., 2022b***).

719 *T. b. gambiense* is suspected to circulate among domestic animals raised in close
720 contact to humans, such as pigs. Here, *T. b. gambiense* has been detected in one
721 animal in each study site. Considering the resulting low prevalence of *T. b. gambiense*
722 infections in these two populations of domestic pigs (0.45 ± 0.9 % in Côte d'Ivoire to
723 0.50 ± 1.0 % in Guinea) and the short life expectancy of domestic pigs reared for
724 commercial purposes in these two rural areas (6 to 8 months), these animals do likely
725 not represent a significant risk to the elimination of gHAT. This is apparently confirmed
726 by the absence of new human cases as well as by the low abundance of tsetse flies in
727 the two sites (article in preparation). However, because of their proximity to humans
728 and their easy access for frequent sampling, pigs could be used as sentinels to monitor
729 *T. b. gambiense* circulation, possibly with the SHERLOCK4AAT toolbox.

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Author contributions

RE, EPA, AC, LG and BR conceived and designed the experiments. RE, EPA, AC, SB, ADH, MND, MT, YL, BD, ADC, MK, VJ and JMB performed the experiments. RE, EPA, AC, PC, LG and BR analysed the data. MK, DK, MC, VJ, ST, JMB, BR and LG contributed reagents, materials and analysis tools. RE, EPA, AC, LG and BR wrote the manuscript. MND, MK, VJ, ST, and JMB reviewed the manuscript.

Figure legends

Figure 1. Pan and species-specific SHERLOCK assays for animal African trypanosomoses. (A) *T. congolense*, *T. theileri* (multiplex), *T. simiae*, Pan-*Trypanozoon* and Pan-trypanosomatid SHERLOCK assays were designed using the *18S rRNA* gene, *T. vivax* SHERLOCK assay using the *IFX* gene and *T. suis* test using the *gGAPDH* gene. Gene specific accession number in brackets below the assay name. Position of the RPA amplicon in bp. RPA primers: Black arrow, extension on the forward primer represents the T7 polymerase promoter sequence. The schematic representation is not scaled to the length of each sequence. (B) Evaluation of the specificity of single pan-trypanosomatid and pan-*Trypanozoon* SHERLOCK tests on RNA from *T. b. brucei* (*Tbb*), *T. b. gambiense* (*Tbg*), *T. b. rhodesiense (Tbr*), *T. evansi* A (*Tev*A) and B (*Tev*B), *T. equiperdum* (*Teq*) *T. congolense* (*Tcg*), *T. vivax* (*Tvx*), *T. theileri* (*Tth*), *Leishmania major* (*Lm*), *Plasmodium falciparum* (*Pfl*) and HEK cells. Synthetic controls for *T. simiae* (*Tsm*) and *T. suis* (*Tsu*) were used as templates for these tests. In box plots, means are represented by single lines, and error bars correspond to minimum and maximum values. N=3 for the RPA amplifications and three Cas-13a detections for each RPA amplification, totalling 9 replicates for each assay. *T. theileri* was used in fewer replicates as an input due to the limited genetic material available. (C) Evaluation of the specificity of *T. congolense*, *T. vivax*, *T. theileri*, *T. simiae* and *T. suis* tests on RNA from *Tbb*, *Tbg*, *Tcg*, *Tvx*, *Tth* and synthetic controls for *Tsm* and *Tsu*. N=1 RPA amplification and three Cas-13a detections totalling 3 replicates for each assay. Error bars represent standard deviations from the mean of the replicates.

775 **Figure 2.** Evaluation of the sensitivity of SHERLOCK assays targeting trypanosome
776 species causing AAT. (A) Limit of detection (LoD) assays for the single pan-
777 trypanosomatid, pan-*Trypanozoon*, *T. vivax, T. congolense and T. theileri* assays were
778 performed on RNAs from bloodstream parasites diluted to obtain equivalent
779 concentrations of parasites per mL. (B) LoD of *T. simiae* and *T. suis* assays were
780 performed on consecutive dilutions of the synthetic controls. N=24 for pan-
781 trypanosomatid assay, N=12 for pan-*Trypanozoon* and assay *T. theileri* assays, N=6
782 for *T. simiae* assay and N=3 for *T. congolense, T. vivax* and *T. suis* assays. Error bars
783 represent standard deviations from the mean of the replicates; open circle represents
784 each replicate.

785 **Figure 3**. Comparison of the single and multiplex 18S pan-trypanosomatid
786 SHERLOCK assays efficiency. (A) Schematic of the single and multiplex pan-
787 trypanosomatid SHERLOCK assays designed using the *18S rRNA* gene. Gene
788 specific accession number in brackets below the assay name. Position of the RPA
789 amplicon in bp. RPA primers: Black arrow, extension on the forward primer represents
790 the T7 polymerase promoter sequence. (B) Posterior sensitivity estimates of the single
791 (green) and multiplex (purple) pan-trypanosomatid SHERLOCK assays on the 224 pig
792 blood samples from Côte d'Ivoire presented in Figure 4. Estimates were obtained with
793 the Monte Carlo method (Gibbs sampling) proceeding in an iterative chain. (C) Results
794 of the pan-*Trypanozoon* SHERLOCK assay on pig samples from Côte d'Ivoire plotted
795 against results of the single (left) and multiplex (right) 18S pan-trypanosomatid
796 SHERLOCK assays. The coloured dashed lines represent the detection thresholds set
797 for each of the tests: single pan-trypanosomatid (green), multiplex pan-trypanosomatid
798 (purple), and pan-*Trypanozoon* (orange). Numbers and proportions of double positive
799 samples are indicated at the top right of each graph.

800 **Figure 4.** Diversity of blood trypanosomatids among free-range pigs from Côte d'Ivoire
801 assessed by the SHERLOCK4AAT toolbox. (A) Map of the Côte d'Ivoire, the region
802 where the samples were collected is highlighted in purple. (B) Results of the
803 SHERLOCK4AAT screening. The coloured dashed lines represent the detection
804 thresholds set for each assay. Each colour represents a specific test: multiplex pan-
805 trypanosomatid (green), pan-*Trypanozoon* (orange), *T. b. gambiense* (yellow), *T.*
806 *congolense* (light green), *T. vivax* (purple), *T. theileri* (red), *T. simiae* (blue) and *T. suis*
807 (black). (C) Table summarizing the number of positive samples as well as the

808 percentage (%) positive infections for each test, calculated on the total number of
809 samples analysed. The total number of samples analysed were 219 for the multiplex
810 pan-trypanosomatid assay, 173 for the *T. vivax*-specific assay, and 224 for all other
811 SHERLOCK assays. (D) Venn diagram representing the crossover between the
812 positive samples analysed with the three tests (multiplex pan-trypanosomatid, pan-
813 *Trypanozoon* and *T. congolense*) giving the highest prevalence. The percentage of the
814 total is shown in brackets below the number of samples constituting each fraction.

815 **Figure 5.** Diversity of blood trypanosomatids among farm pigs from Guinea assessed
816 by the SHERLOCK4AAT toolbox. (A) Map of Guinea showing the N'zérékoré
817 prefecture where samples were collected in pink. (B) Results of the SHERLOCK4AAT
818 screening. The coloured dashed lines represent the detection thresholds set for each
819 of the tests. Each colour represents a specific test: multiplex pan-trypanosomatid
820 (green), pan-*Trypanozoon* (orange), *T. b. gambiense* (yellow), *T. congolense* (light
821 green), *T. vivax* (purple), *T. theileri* (red), *T. simiae* (blue) and *T. suis* (black). (C) Table
822 summarizing the number of positive samples as well as the percentage (%) positive
823 infections for each test, calculated on the total number of samples analysed (n=200).
824 (D) Venn diagram representing the crossover between the positive samples analysed
825 with the three tests (multiplex pan-trypanosomatid, pan-*Trypanozoon* and *T.*
826 *congolense*) giving the highest prevalence. The percentage of the total is shown in
827 brackets below the number of samples constituting each fraction.

828 **Supplementary figure legends**

829 **Supplementary Figure 1.** A: Representation of the proportions of identity between
830 *18S rRNA* (A) and *GAPDH* (B) genes from Microsporidia, Ciliophora, Haemosporida,
831 Diplomonadida, Symbiontida, Diplonemea, Euglenida and Kinetoplastea.

832 **Supplementary Figure 2.** (A) Alignments for 18S Pan-trypanosomatid SHERLOCK
833 target region. The sequences used for the alignments are as follows: Microsporidia
834 (*Nosema adalia*, KC412706.1), Ciliophora (*Vorticella sp.*, DQ487201.2),
835 Haemosporida (*Plasmodium falciparum*, XR_002966679.1), Diplomonadida (*Giargia*
836 *intestinalis*, XR_005248679.1), Symbiontida (*Calkinsia aureus*, EU753419.1),
837 Diplonemea (*Diplonema ambulator*, AY425009.1), Euglenida (*Euglena sanguinea*,
838 JQ281806.1) and Kinetoplastea (*Trypanosoma* (*T*) *brucei brucei*, XR_002989632.1).
839 (B) Alignments for 18S Pan-*Trypanozoon* SHERLOCK target region. The sequences

840 used for the alignments are as follows: Human (H, pdb|5VYC|i), *Plasmodium*
841 *falciparum* (Pfl, XR_002966679.1), *Leishmania major* (Lm, GQ332361.1), *T. cruzi*
842 (Tcz, AJ009147.1), *T. vivax* (Tvx, EU477537.1), *T. congolense* (Tcg, AJ009146.1), *T.*
843 *theileri* (Tth, AJ009163.1), *T. simiae* (Tsm, AJ404608.1), *T.suis* (Tsu, MK962702.1)
844 and *T. brucei brucei* (Tbb, XR_002989632.1).

845 **Supplementary Figure 3.** Summary of BLAST analysis obtained against the 18S pan-
846 trypanosomatid SHERLOCK amplicon and evaluation of the homology of the target
847 region of each crRNA.

848 **Supplementary Figure 4.** Screening of different RPA pairs of primers and/or crRNAs
849 guides for each SHERLOCK assay optimization. RPA pairs of primers are named A, B
850 or C, while crRNAs were name from 1 to 10. # indicates the combination of RPA primers
851 and crRNA selected for the rest of the analysis.

852 **Supplementary Figure 5.** Alignments of the 18S *T. congolense* SHERLOCK target
853 region. The sequences used for the alignments are from *T. simiae*, *T. godfreyi*, *T.*
854 *sapaensis*, *T. vivax*, *T. theileri* and *T. thomasbancrofti*.

855 **Supplementary Figure 6**. Comparison of the limit of detection of the single and
856 multiplex 18S pan-trypanosomatid SHERLOCK assays. Single and multiplex 18S pan-
857 trypanosomatid SHERLOCK assays on dilutions of *T. b. brucei* RNA and on RNA
858 extracted from HEK cells. N=9 for each dilution. Error bars represent standard
859 deviations from the mean of the replicates.

860 **Supplementary Figure 7**. Background cross-reactivity of the 18S pan-trypanosomatid
861 (A) and 18S pan-*Trypanozoon* (B) SHERLOCK assays to genetic material extracted
862 from pig blood. Means are represented by single lines. Quantities of porcine and *T. b.*
863 *brucei* genetic material extracts included in each trial are as follows: "-"none, "+"
864 1ng/µL, "++" 2 ng/µL and "+++" 3ng/µL.

865 **Supplementary Figure 8.** Threshold determination for all new SHERLOCK4AAT
866 assays. (A) Compilation of positive and negative results obtained using multiplex pan-
867 trypanosomatid and pan-*Trypanozoon* SHERLOCK tests and ROC curves obtained for
868 each analysis. The red dotted line marks the selected threshold for each test. (B)
869 Compilation of positive and negative results obtained using the species-specific
870 SHERLOCK assays and ROC curve obtained for each analysis. (C) Summary of the

871 analytical features of each SHERLOCK assay. Analytical sensitivity (95 % of CI),
872 specificity (95 % of CI) and fold change cut-off.

873 **Supplementary Figure 9.** Posterior sensitivity estimates of three SHERLOCK assays
874 (multiplex pan-trypanosomatid, pan-*Trypanozoon* and *T. congolense*-specific
875 SHERLOCK assays) to the three parasitological tests (buffy coat examination, mAECT
876 and /or isolation in mice) performed in parallel on the 224 pig blood samples from Côte
877 d'Ivoire. Estimates were obtained with the Monte Carlo method (Gibbs sampling)
878 proceeding in an iterative chain.

879 **Supplementary Table 1.** Accession numbers of organisms used to evaluate the
880 presence of single nucleotide polymorphism (SNPs) in the different target regions.

881 **Supplementary Table 2.** Primers and crRNA guides used for the SHERLOCK4AAT
882 toolbox.

883 **Supplementary Table 3.** RPA primers used to produce the *in vitro* transcribed targets
884 (positive control templates).

885 **References**

886 **Álvarez-Rodríguez A**, Jin B-K, Radwanska M, Magez S. 2022. Recent progress in
887 diagnosis and treatment of Human African Trypanosomiasis has made the
888 elimination of this disease a realistic target by 2030. *Frontiers in medicine*
889 **9**:1037094. doi: 10.3389/fmed.2022.1037094.

890 **Amisigo CM**, Amegatcher G, Sunter JD, Gwira TM. 2024. Adipose and skin
891 distribution of African trypanosomes in natural animal infections. *Parasites & vectors*
892 **17**:215. doi: 10.1186/s13071-024-06277-7.

893 **Autheman D**, Crosnier C, Clare S, Goulding DA, Brandt C, Harcourt K, Tolley C,
894 Galaway F, Khushu M, Ong H, Romero-Ramirez A, Duffy CW, Jackson AP, Wright
895 GJ. 2021. An invariant Trypanosoma vivax vaccine antigen induces protective
896 immunity. *Nature* **595**:96–100. doi: 10.1038/s41586-021-03597-x.

897 **Barrett MP**, Priotto G, Franco JR, Lejon V, Lindner AK. 2024. Elimination of human
898 African trypanosomiasis: The long last mile. *PLoS neglected tropical diseases*
899 **18**:e0012091. doi: 10.1371/journal.pntd.0012091.

900 **Boulangé A**, Lejon V, Berthier D, Thévenon S, Gimonneau G, Desquesnes M, Abah
901 S, Agboho P, Chilongo K, Gebre T, Fall AG, Kaba D, Magez S, Masiga D, Matovu
902 E, Moukhtar A, Neves L, Olet PA, Pagabeleguem S, Shereni W, Sorli B, Taioe MO,

Tejedor Junco MT, Yagi R, Solano P, Cecchi G. 2022. The COMBAT project: controlling and progressively minimizing the burden of vector-borne animal trypanosomosis in Africa. *Open research Europe* **2**:67. doi: 10.12688/openreseurope.14759.2.

Brotánková A, Fialová M, Čepička I, Brzoňová J, Svobodová M. 2022. Trypanosomes of the Trypanosoma theileri Group: Phylogeny and New Potential Vectors. *Microorganisms* **10**. doi: 10.3390/microorganisms10020294.

Brown LD, Cai TT, DasGupta A. 2001. Interval Estimation for a Binomial Proportion. *Statistical Science* **16**. doi: 10.1214/ss/1009213286.

Budd LT. 2001. Trypanosomosis, Tsestse and Africa. https://assets.publishing.service.gov.uk/media/57a08d23e5274a31e0001686/ZC0135a.pdf.

Büscher P, Bart J-M, Boelaert M, Bucheton B, Cecchi G, Chitnis N, Courtin D, Figueiredo LM, Franco J-R, Grébaut P, Hasker E, Ilboudo H, Jamonneau V, Koffi M, Lejon V, MacLeod A, Masumu J, Matovu E, Mattioli R, Noyes H, Picado A, Rock KS, Rotureau B, Simo G, Thévenon S, Trindade S, Truc P, van Reet N. 2018. Do Cryptic Reservoirs Threaten Gambiense-Sleeping Sickness Elimination? *Trends in parasitology* **34**:197–207. doi: 10.1016/j.pt.2017.11.008.

Claes F, Radwanska M, Urakawa T, Majiwa PA, Goddeeris B, Büscher P. 2004. Variable Surface Glycoprotein RoTat 1.2 PCR as a specific diagnostic tool for the detection of Trypanosoma evansi infections. *Kinetoplastid biology and disease* **3**:3. doi: 10.1186/1475-9292-3-3.

Contreras Garcia M, Walshe E, Steketee PC, Paxton E, Lopez-Vidal J, Pearce MC, Matthews KR, Ezzahra-Akki F, Evans A, Fairlie-Clark K, Matthews JB, Grey F, Morrison LJ. 2022. Comparative Sensitivity and Specificity of the 7SL sRNA Diagnostic Test for Animal Trypanosomiasis. *Frontiers in Veterinary Science* **9**. doi: 10.3389/fvets.2022.868912.

Courtin F, Camara O, Camara M, Kagbadouno M, Bucheton B, Solano P, Jamonneau V. 2019. Maladie du sommeil dans le foyer historique de Guinée forestière : actualisation grâce à une méthode géographique. *Parasite (Paris, France)* **26**:61. doi: 10.1051/parasite/2019061.

Deborggraeve S, Claes F, Laurent T, Mertens P, Leclipteux T, Dujardin JC, Herdewijn P, Büscher P. 2006. Molecular dipstick test for diagnosis of sleeping sickness. *Journal of clinical microbiology* **44**:2884–2889. doi: 10.1128/JCM.02594-05.

Desquesnes M, Gonzatti M, Sazmand A, Thévenon S, Bossard G, Boulangé A, Gimonneau G, Truc P, Herder S, Ravel S, Sereno D, Jamonneau V, Jittapalapong S, Jacquiet P, Solano P, Berthier D. 2022a. A review on the diagnosis of animal trypanosomoses. *Parasites & vectors* **15**:64. doi: 10.1186/s13071-022-05190-1.

Desquesnes M, Sazmand A, Gonzatti M, Boulangé A, Bossard G, Thévenon S, Gimonneau G, Truc P, Herder S, Ravel S, Sereno D, Waleckx E, Jamonneau V, Jacquiet P, Jittapalapong S, Berthier D, Solano P, Hébert L. 2022b. Diagnosis of animal trypanosomoses: proper use of current tools and future prospects. *Parasites & vectors* **15**:235. doi: 10.1186/s13071-022-05352-1.

Fetene E, Leta S, Regassa F, Büscher P. 2021. Global distribution, host range and prevalence of Trypanosoma vivax: a systematic review and meta-analysis. *Parasites & vectors* **14**:80. doi: 10.1186/s13071-021-04584-x.

Franco JR, Priotto G, Paone M, Cecchi G, Ebeja AK, Simarro PP, Sankara D, Metwally SBA, Argaw DD. 2024. The elimination of human African trypanosomiasis: Monitoring progress towards the 2021-2030 WHO road map targets. *PLoS neglected tropical diseases* **18**:e0012111. doi: 10.1371/journal.pntd.0012111.

Giordani F, Morrison LJ, Rowan TG, Koning HP de, Barrett MP. 2016. The animal trypanosomiases and their chemotherapy: a review. *Parasitology* **143**:1862–1889. doi: 10.1017/S0031182016001268.

Gootenberg JS, Abudayyeh OO, Lee JW, Essletzbichler P, Dy AJ, Joung J, Verdine V, Donghia N, Daringer NM, Freije CA, Myhrvold C, Bhattacharyya RP, Livny J, Regev A, Koonin EV, Hung DT, Sabeti PC, Collins JJ, Zhang F. 2017. Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science (New York, N.Y.)* **356**:438–442. doi: 10.1126/science.aam9321.

Holland WG, Claes F, My LN, Thanh NG, Tam PT, Verloo D, Büscher P, Goddeeris B, Vercruysse J. 2001. A comparative evaluation of parasitological tests and a PCR for Trypanosoma evansi diagnosis in experimentally infected water buffaloes. *Veterinary parasitology* **97**:23–33. doi: 10.1016/S0304-4017(01)00381-8.

Joseph L, Gyorkos TW, Coupal L. 1995. Bayesian estimation of disease prevalence and the parameters of diagnostic tests in the absence of a gold standard. *American journal of epidemiology* **141**:263–272. doi: 10.1093/oxfordjournals.aje.a117428.

Karshima SN, Lawal IA, Bata SI, Barde IJ, Adamu PV, Salihu A, Dross PN, Obalisa A. 2016. Animal reservoirs of Trypanosoma brucei gambiense around the old Gboko sleeping sickness focus in Nigeria. *Journal of parasitology and vector biology*.

971 **Kellner MJ**, Koob JG, Gootenberg JS, Abudayyeh OO, Zhang F. 2019. SHERLOCK:
972 nucleic acid detection with CRISPR nucleases. *Nature protocols* **14**:2986–3012.
973 doi: 10.1038/s41596-019-0210-2.
974 **Kristjanson PM**, Swallow BM, Rowlands GJ, Kruska RL, Leeuw PN de. 1999.
975 Measuring the costs of African animal trypanosomosis, the potential benefits of
976 control and returns to research. *Agricultural Systems* **59**:79–98. doi: 10.1016/S0308-
977 521X(98)00086-9.
978 **Magang EMK**, Kamga RMN, Telleria J, Tichit M, Crouzols A, Kaboré J, Hardy D,
979 Bouaka CUT, Jamonneau V, Rotureau B, Kuete V, Bart J-M, Simo G. 2023.
980 Prevalence of blood and skin trypanosomes in domestic and wild fauna from two
981 sleeping sickness foci in Southern Cameroon. *PLoS neglected tropical diseases*
982 **17**:e0011528. doi: 10.1371/journal.pntd.0011528.
983 **McGinnis S**, Madden TL. 2004. BLAST: at the core of a powerful and diverse set of
984 sequence analysis tools. *Nucleic acids research* **32**:W20-5.
985 doi: 10.1093/nar/gkh435.
986 **Meyer A**, Holt HR, Selby R, Guitian J. 2016. Past and Ongoing Tsetse and Animal
987 Trypanosomiasis Control Operations in Five African Countries: A Systematic
988 Review. *PLoS neglected tropical diseases* **10**:e0005247.
989 doi: 10.1371/journal.pntd.0005247.
990 **Morrison LJ**, Tait A, McCormack G, Sweeney L, Black A, Truc P, Likeufack ACL,
991 Turner CM, MacLeod A. 2008. Trypanosoma brucei gambiense Type 1 populations
992 from human patients are clonal and display geographical genetic differentiation.
993 *Infection, genetics and evolution : journal of molecular epidemiology and*
994 *evolutionary genetics in infectious diseases* **8**:847–854.
995 doi: 10.1016/j.meegid.2008.08.005.
996 **N'Djetchi MK**, Camara O, Koffi M, Camara M, Kaba D, Kaboré J, Tall A, Rotureau B,
997 Glover L, Traoré MB, Koné M, Coulibaly B, Adingra GP, Soumah A, Gassama M,
998 Camara AD, Compaoré CFA, Camara A, Boiro S, Anton EP, Bessell P, van Reet N,
999 Bucheton B, Jamonneau V, Bart J-M, Solano P, Biéler S, Lejon V. 2024. Specificity
1000 of serological screening tests and reference laboratory tests to diagnose gambiense
1001 human African trypanosomiasis: a prospective clinical performance study. *Infectious*
1002 *diseases of poverty* **13**:53. doi: 10.1186/s40249-024-01220-5.
1003 **N'Djetchi MK**, Ilboudo H, Koffi M, Kaboré J, Kaboré JW, Kaba D, Courtin F, Coulibaly
1004 B, Fauret P, Kouakou L, Ravel S, Deborggraeve S, Solano P, Meeûs T de, Bucheton

1005 B, Jamonneau V. 2017. The study of trypanosome species circulating in domestic
1006 animals in two human African trypanosomiasis foci of Côte d'Ivoire identifies pigs
1007 and cattle as potential reservoirs of Trypanosoma brucei gambiense. *PLoS*
1008 *neglected tropical diseases* **11**:e0005993. doi: 10.1371/journal.pntd.0005993.
1009 **Okello I**, Mafie E, Eastwood G, Nzalawahe J, Mboera LEG, Onyoyo S. 2022.
1010 Prevalence and Associated Risk Factors of African Animal Trypanosomiasis in
1011 Cattle in Lambwe, Kenya. *Journal of parasitology research* **2022**:5984376.
1012 doi: 10.1155/2022/5984376.
1013 **Piepenburg O**, Williams CH, Stemple DL, Armes NA. 2006. DNA detection using
1014 recombination proteins. *PLoS biology* **4**:e204. doi: 10.1371/journal.pbio.0040204.
1015 **Radwanska M**, Claes F, Magez S, Magnus E, Perez-Morga D, Pays E, Büscher P.
1016 2002. Novel primer sequences for polymerase chain reaction-based detection of
1017 Trypanosoma brucei gambiense. *The American journal of tropical medicine and*
1018 *hygiene* **67**:289–295. doi: 10.4269/ajtmh.2002.67.289.
1019 **Richards S**, Morrison LJ, Torr SJ, Barrett MP, Manangwa O, Mramba F, Auty H. 2021.
1020 Pharma to farmer: field challenges of optimizing trypanocide use in African animal
1021 trypanosomiasis. *Trends in parasitology* **37**:831–843. doi: 10.1016/j.pt.2021.04.007.
1022 **Rodrigues CMF**, Garcia HA, Rodrigues AC, Pereira DL, Pereira CL, Viola LB, Neves
1023 L, Camargo EP, Gibson W, Teixeira MMG. 2020. Expanding our knowledge on
1024 African trypanosomes of the subgenus Pycnomonas: A novel Trypanosoma suis-like
1025 in tsetse flies, livestock and wild ruminants sympatric with Trypanosoma suis in
1026 Mozambique. *Infection, genetics and evolution : journal of molecular epidemiology*
1027 *and evolutionary genetics in infectious diseases* **78**:104143.
1028 doi: 10.1016/j.meegid.2019.104143.
1029 **Romero-Ramirez A**, Casas-Sánchez A, Autheman D, Duffy CW, Brandt C, Clare S,
1030 Harcourt K, André MR, Almeida Castilho Neto KJG de, Teixeira MMG, Machado RZ,
1031 Coombes J, Flynn RJ, Wright GJ, Jackson AP. 2022. Vivaxin genes encode highly
1032 immunogenic, non-variant antigens on the Trypanosoma vivax cell-surface. *PLoS*
1033 *neglected tropical diseases* **16**:e0010791. doi: 10.1371/journal.pntd.0010791.
1034 **Schisterman EF**, Perkins NJ, Liu A, Bondell H. 2005. Optimal cut-point and its
1035 corresponding Youden Index to discriminate individuals using pooled blood samples.
1036 *Epidemiology (Cambridge, Mass.)* **16**:73–81.
1037 doi: 10.1097/01.ede.0000147512.81966.ba.

1038 **Serem EK**, Mburu DM, Abdullahi OA, Bargul JL. 2024. A scoping review on tsetse fly
1039 blood meal sources and its assay methods since 1956 to 2022. *Parasites & vectors*
1040 **17**:52. doi: 10.1186/s13071-023-06114-3.
1041 **Sievers F**, Higgins DG. 2018. Clustal Omega for making accurate alignments of many
1042 protein sequences. *Protein science : a publication of the Protein Society* **27**:135–
1043 145. doi: 10.1002/pro.3290.
1044 **Sima N**, Dujeancourt-Henry A, Perlaza BL, Ungeheuer M-N, Rotureau B, Glover L.
1045 2022. SHERLOCK4HAT: A CRISPR-based tool kit for diagnosis of Human African
1046 Trypanosomiasis. *EBioMedicine* **85**:104308. doi: 10.1016/j.ebiom.2022.104308.
1047 **Simo G**, Rayaisse JB. 2015. Challenges facing the elimination of sleeping sickness in
1048 west and central Africa: sustainable control of animal trypanosomiasis as an
1049 indispensable approach to achieve the goal. *Parasites & vectors* **8**:640.
1050 doi: 10.1186/s13071-015-1254-y.
1051 **Traoré BM**, Koffi M, N'Djetchi MK, Kaba D, Kaboré J, Ilboudo H, Ahouty BA, Koné M,
1052 Coulibaly B, Konan T, Segard A, Kouakou L, Meeûs T de, Ravel S, Solano P, Bart
1053 J-M, Jamonneau V. 2021. Free-ranging pigs identified as a multi-reservoir of
1054 Trypanosoma brucei and Trypanosoma congolense in the Vavoua area, a historical
1055 sleeping sickness focus of Côte d'Ivoire. *PLoS neglected tropical diseases*
1056 **15**:e0010036. doi: 10.1371/journal.pntd.0010036.
1057 **Vourchakbé J**, Tiofack ZAA, Kante TS, Mpoame M, Simo G. 2020. L'identification
1058 moléculaire de Trypanosoma brucei gambiense chez les porcs, les chiens et les
1059 petits ruminants naturellement infectés confirme les animaux domestiques comme
1060 réservoirs potentiels de la maladie du sommeil au Tchad. *Parasite (Paris, France)*
1061 **27**:63. doi: 10.1051/parasite/2020061.
1062 **Ye J**, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. 2012. Primer-
1063 BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC*
1064 *bioinformatics* **13**:134. doi: 10.1186/1471-2105-13-134.
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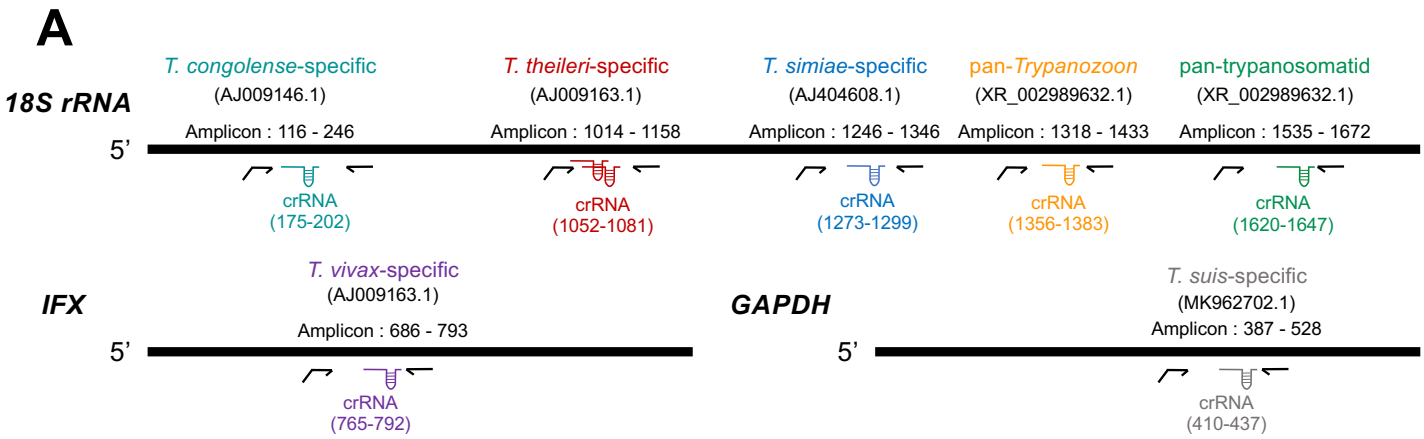
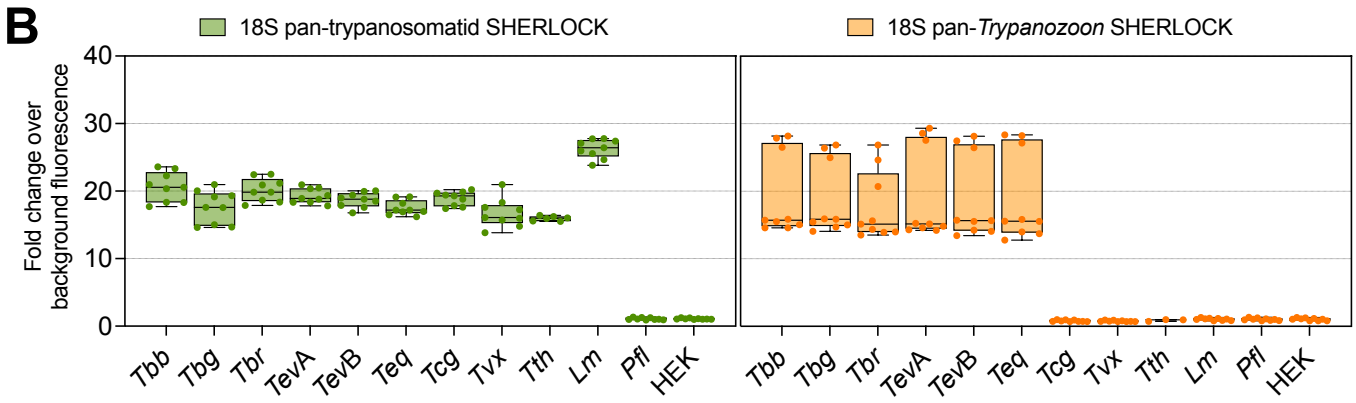
Figure 1


A
18S rRNA
T. congolense-specific
(AJ009146.1)
Amplicon : 116 - 246
T. theileri-specific
(AJ009163.1)
Amplicon : 1014 - 1158
T. simiae-specific
(AJ404608.1)
Amplicon : 1246 - 1346
pan-Trypanozoon
(XR_002989632.1)
Amplicon : 1318 - 1433
pan-trypanosomatid
(XR_002989632.1)
Amplicon : 1535 - 1672
5'
crRNA
(175-202)
crRNA
(1052-1081)
crRNA
(1273-1299)
crRNA
(1356-1383)
crRNA
(1620-1647)
IFX
T. vivax-specific
(AJ009163.1)
Amplicon : 686 - 793
5'
crRNA
(765-792)
GAPDH
T. suis-specific
(MK962702.1)
Amplicon : 387 - 528
5'
crRNA
(410-437)


B
18S pan-trypanosomatid SHERLOCK
18S pan-Trypanozoon SHERLOCK
Fold change over background fluorescence
40
30
20
10
0
Tbb
Tbg
Tbr
TevA
TevB
Teq
Tcg
Tvx
Tth
Lm
Pfl
HEK

C
T.congolense
T.vivax
T.theileri
T.simiae
T.suis
Fold change over background fluorescence
30
20
10
0
Tbb
Tbg
Tcg
Tvx
Tth
Tsm
Tsu

Figure 2

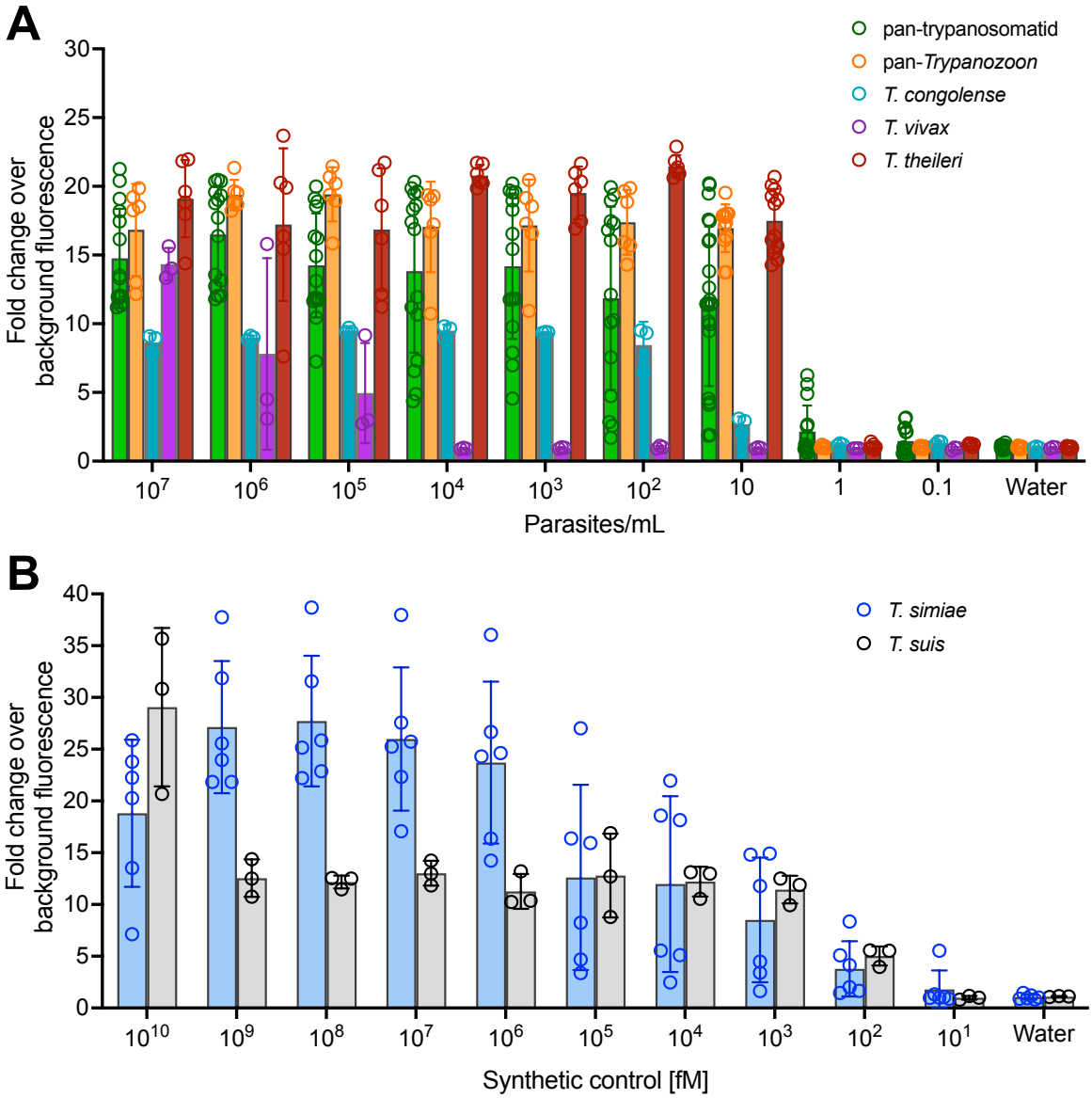

A
pan-trypanosomatid
pan-Trypanozoon
T. congolense
T. vivax
T. theileri
Fold change over background fluorescence
0
5
10
15
20
25
30
10^7
10^6
10^5
10^4
10^3
10^2
10
1
0.1
Water
Parasites/mL
B
T. simiae
T. suis
Fold change over background fluorescence
0
5
10
15
20
25
30
35
40
10^10
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
Water
Synthetic control [fM]

Figure 3

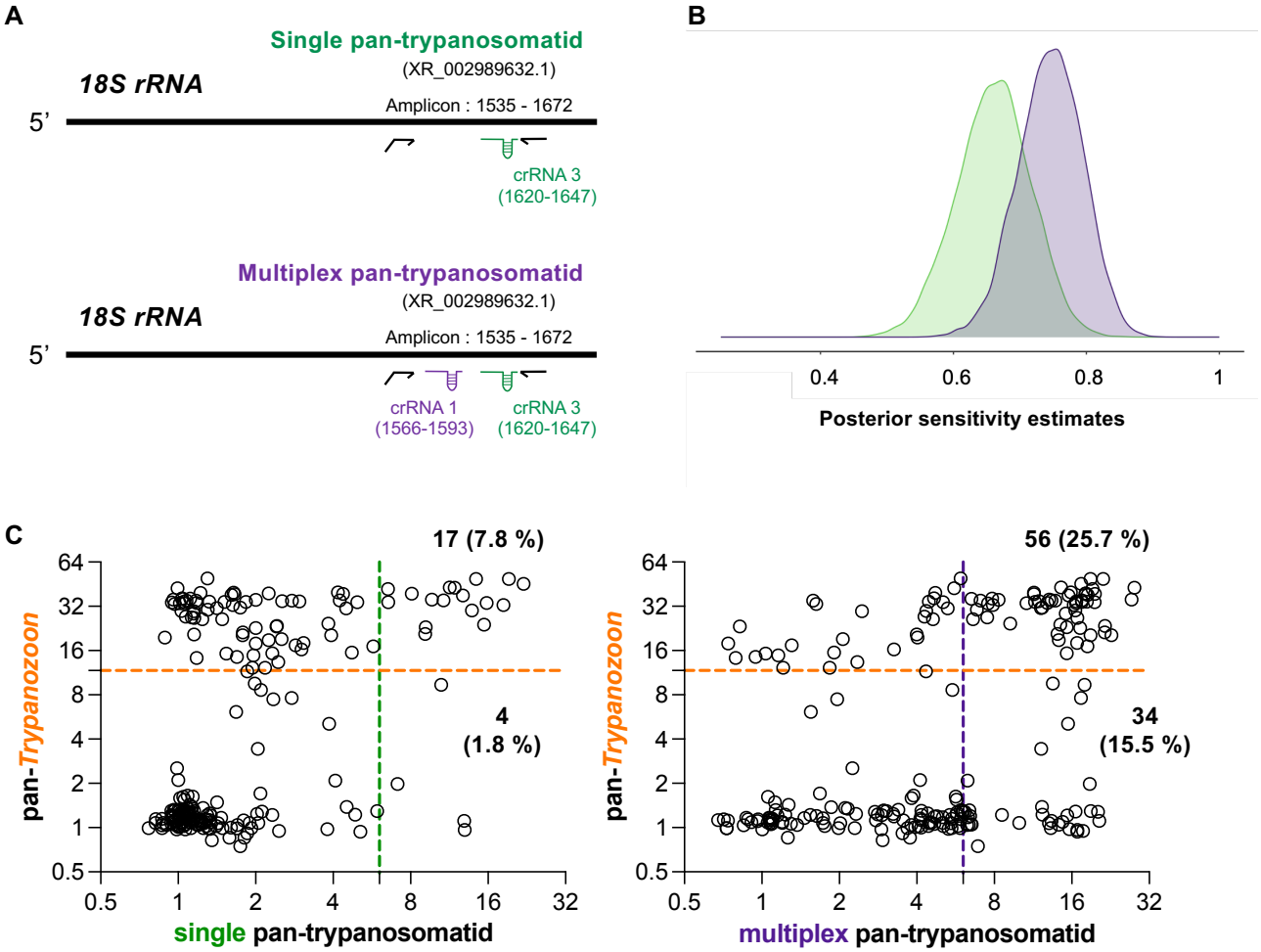
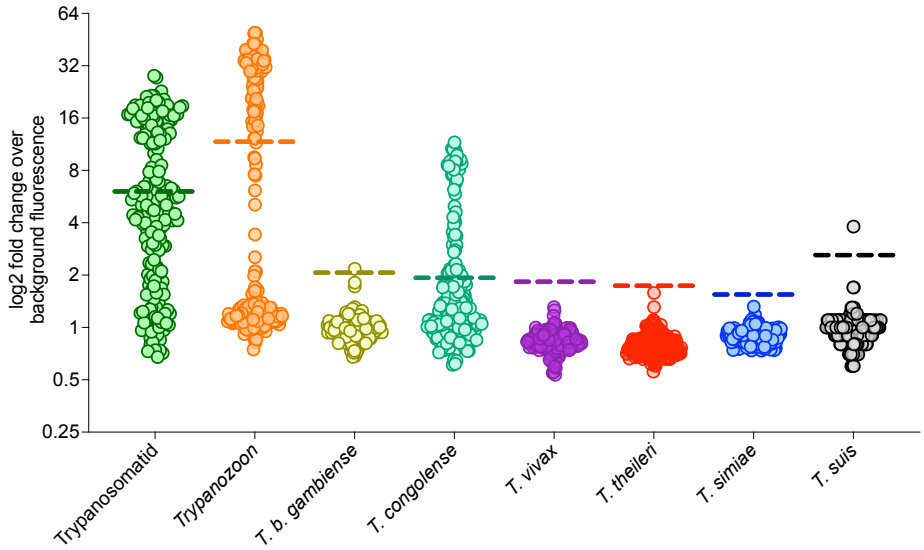

A
Single pan-trypanosomatid
(XR_002989632.1)
18S rRNA
Amplicon : 1535 - 1672
5'
crRNA 3
(1620-1647)
Multiplex pan-trypanosomatid
(XR_002989632.1)
18S rRNA
Amplicon : 1535 - 1672
5'
crRNA 1
(1566-1593)
crRNA 3
(1620-1647)
B
0.4
0.6
0.8
1
Posterior sensitivity estimates
C
17 (7.8 %)
4
(1.8 %)
pan-Trypanozoon
single pan-trypanosomatid
56 (25.7 %)
34
(15.5 %)
pan-Trypanozoon
multiplex pan-trypanosomatid

Figure 4

A

B



C

	No. positive samples	% infections (n=224)
Trypanosomatid	90	41.1 %
Trypanozoon	85	37.9 %
T. b. gambiense	1	0.45 %
T. congolense	52	23.2 %
T. vivax	0	0.0 %
T. theileri	0	0.0 %
T. simiae	0	0.0 %
T. suis	1	0.45 %

D

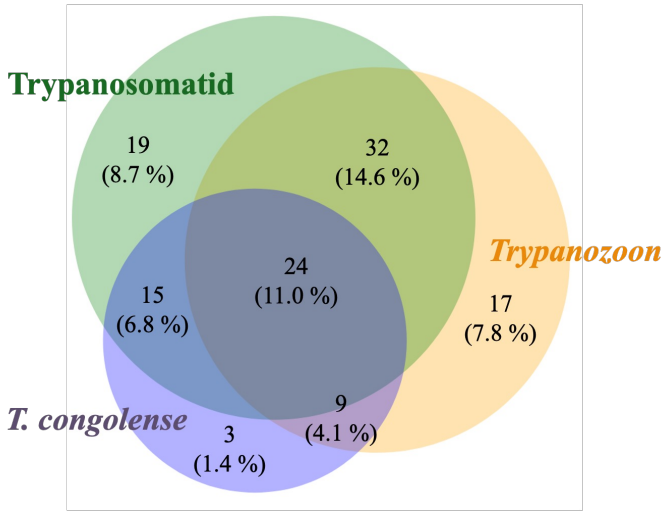
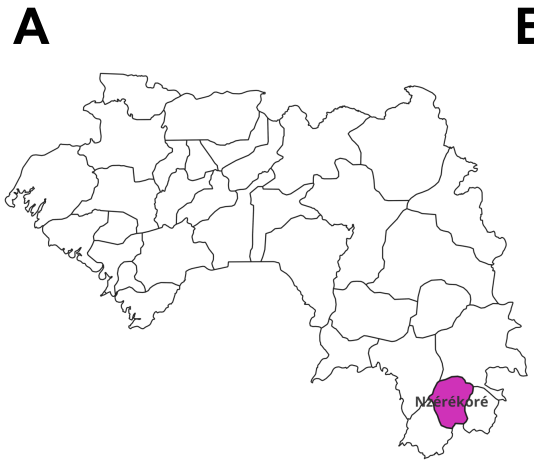
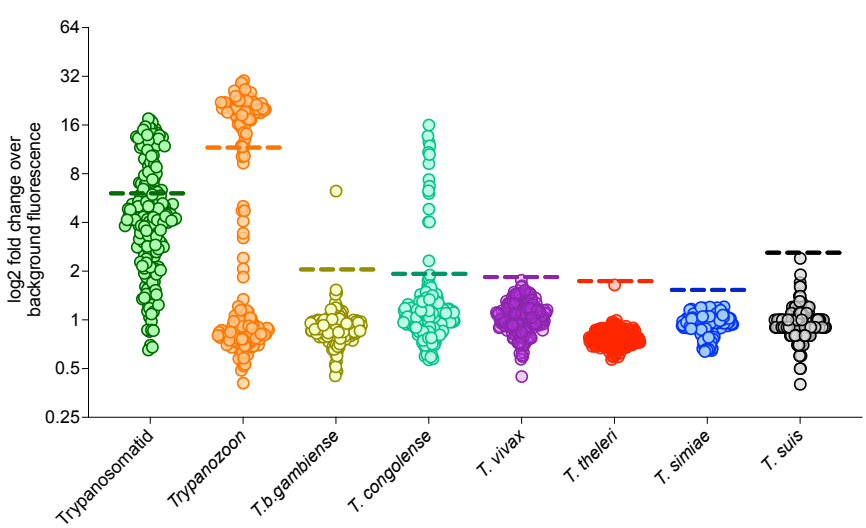


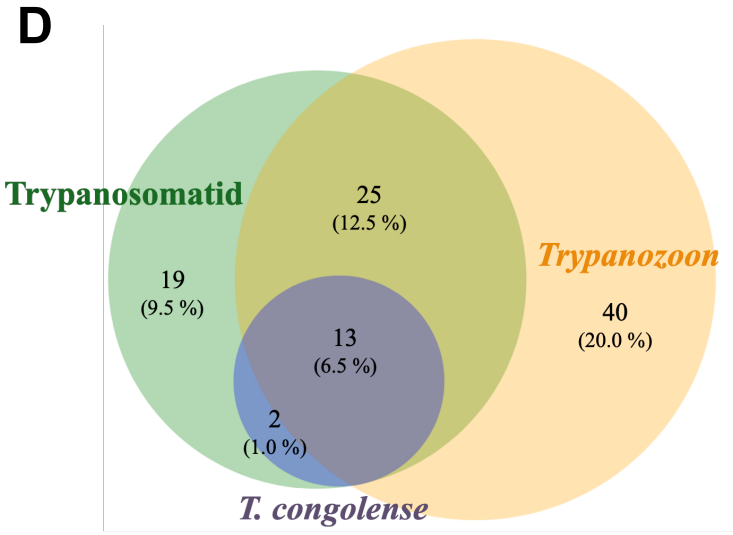
Figure 5



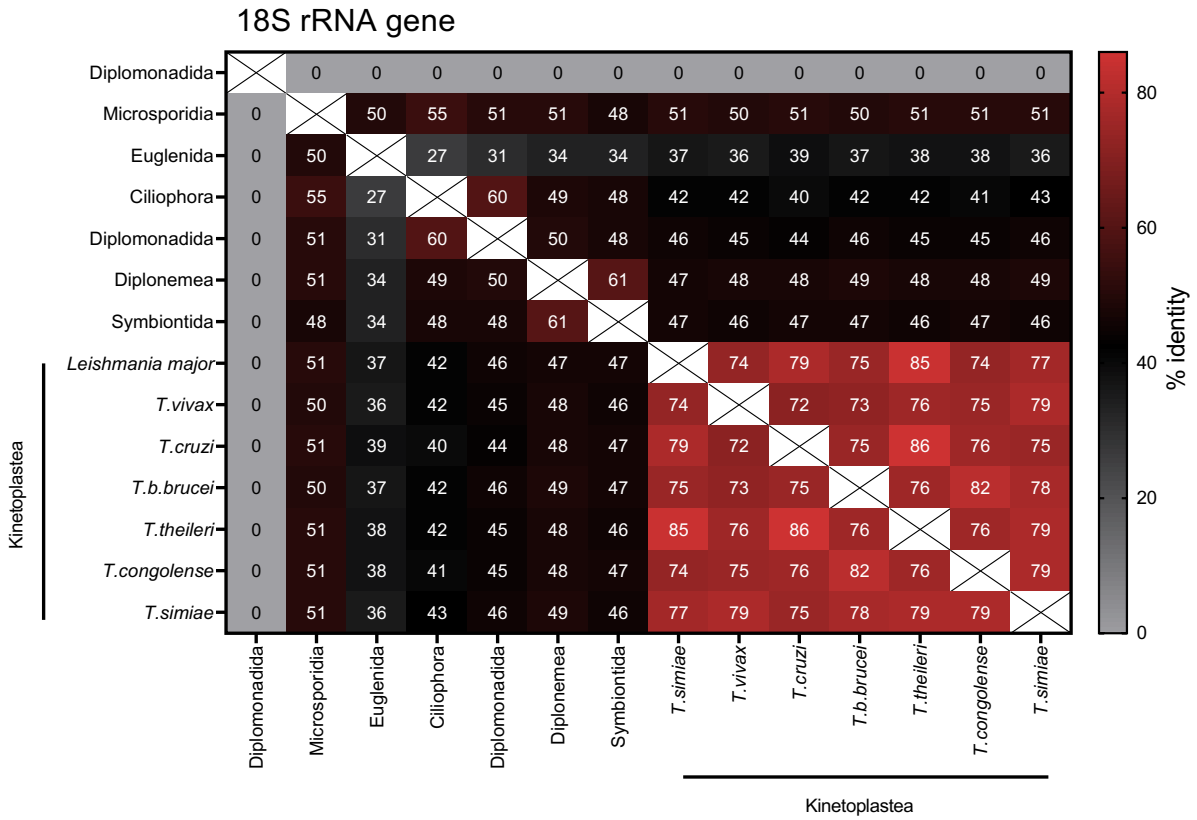


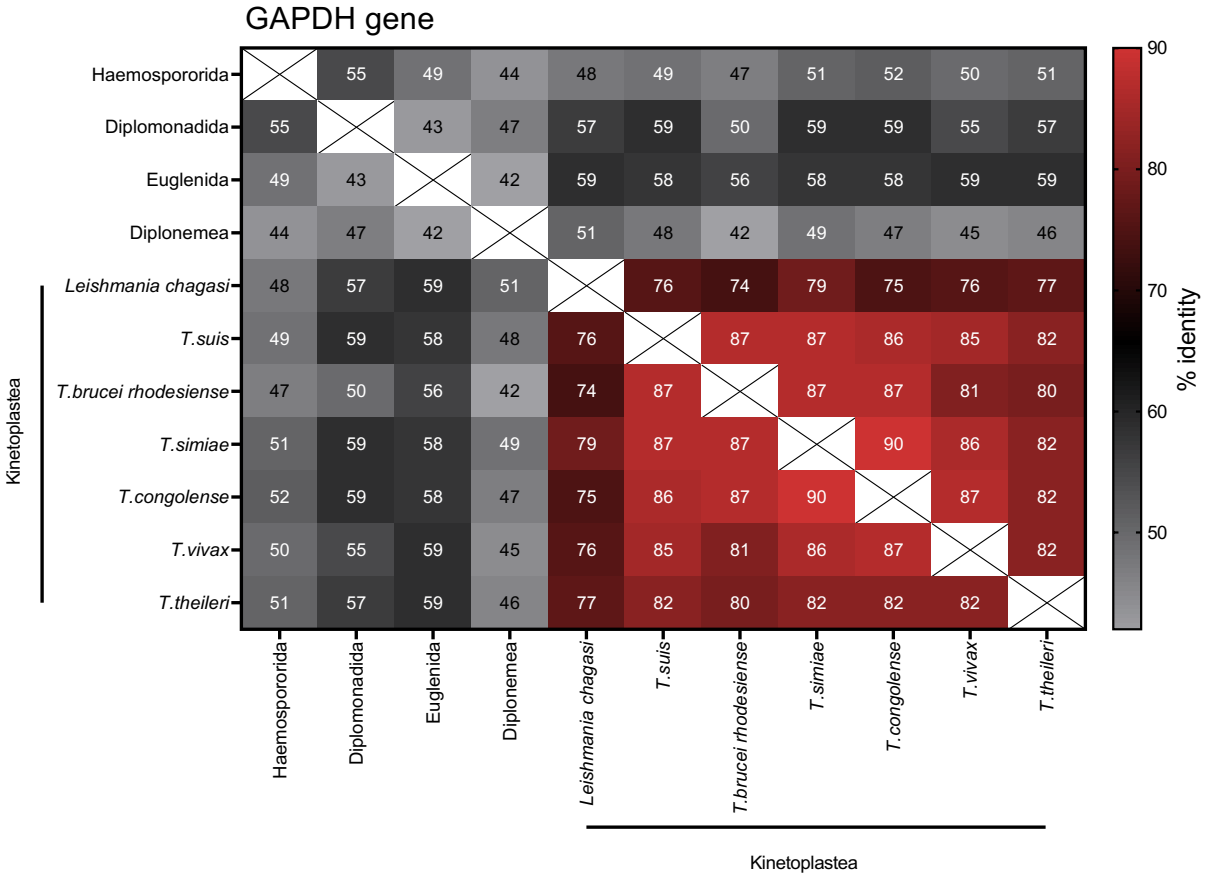
C

	No. positive samples	% infections (n=200)
Trypanosomatid	59	29.5 %
Trypanozoon	78	39.0 %
T. b. gambiense	1	0.50 %
T. congolense	15	7.5 %
T. vivax	0	0.0 %
T. theileri	0	0.0 %
T. simiae	0	0.0 %
T. suis	0	0.0 %



Supplementary Figure 1





Supplementary Figure 2

A

```
                RPA Forward Primer          target region crRNA 1       target region crRNA 3    RPA Reverse Primer

Microsporidia   CTTAATTTGACTCAACGCGAGGTAA[…]AATATTTTATTATTCAGCGAGAATGGAA---[…]ATAGTGGTGCATGGGCCGTTTTCAATGGA TGCT-GTAAAGT-TTTGATTAATTTC
Ciliophora      CTTAATTTGACTCAACACTGGGAAA[…]AGGGCAAGAA---GATTTTAGGATTGACAGA[…]GTGGTGGTGCATGGCCGTTCTTAGC-TGG TGGA-GTGAGATGTCTGGTTAATTCC
Haemosporida    CTTAATTTGACTCAACACGGGAAAA[…]AGTTTAAGAC---AAGAGTAGGATTGACAGA[…]ATGGTGATGCATGGCCGTTTTTAGT-TCG TGAATATGATTTGTCTGGTTAATTCC
Diplomonadida   CTCAATCTGACTCAACGCGCG-CAC[…]AGGCCCGGAC---GCGCGGAGGACCGACAGC[…]GCGGTGGTGCATGGCCGCTCCCAGC-CCG TGGC-GCGAGCCGTCTGCTCCATTGC
Symbiontida     CTTAATTTGACTCAACACGGGAAAT[…]AGATCAGGAC---GCCCGGTGGATTGACAGA[…]ATGGTGGTGCATGGCCGCTCCTGGT-TGG TGGA-GTGATTTGTCTGGTTGATTCC
Diplonemea      CTTAATTTGACTCAACACGGGGAAT[…]AGGTCAGGAC---AATGTAAGGATTGACAGA[…]GTGGTGGTGCATGGCCGCTCCTGGT-TGG TGGA-GTGATTTGTCTGGTTGATTCC
Euglenida       CTTAATTTGACTCAACGCGGGGAAT[…]AGGTCCGGAC---GCAGCTGGGATGGACAGA[…]ACGGTGGTGCATGGCCGCTCCTGAT-TGG TGGA-GTGATTTGTCTGGTTGATTCC
Kinetoplastea   TTTAATTTGACTCAACACGGGGAAC[…]AGATCCGGAC---AGGGTGAGGATTGACAGA[…]ATGGTGGTGCATGGCCGCTTTTGGT-CGG TGGA-GTGATTTGTTTGGTTGATTCC
```

B

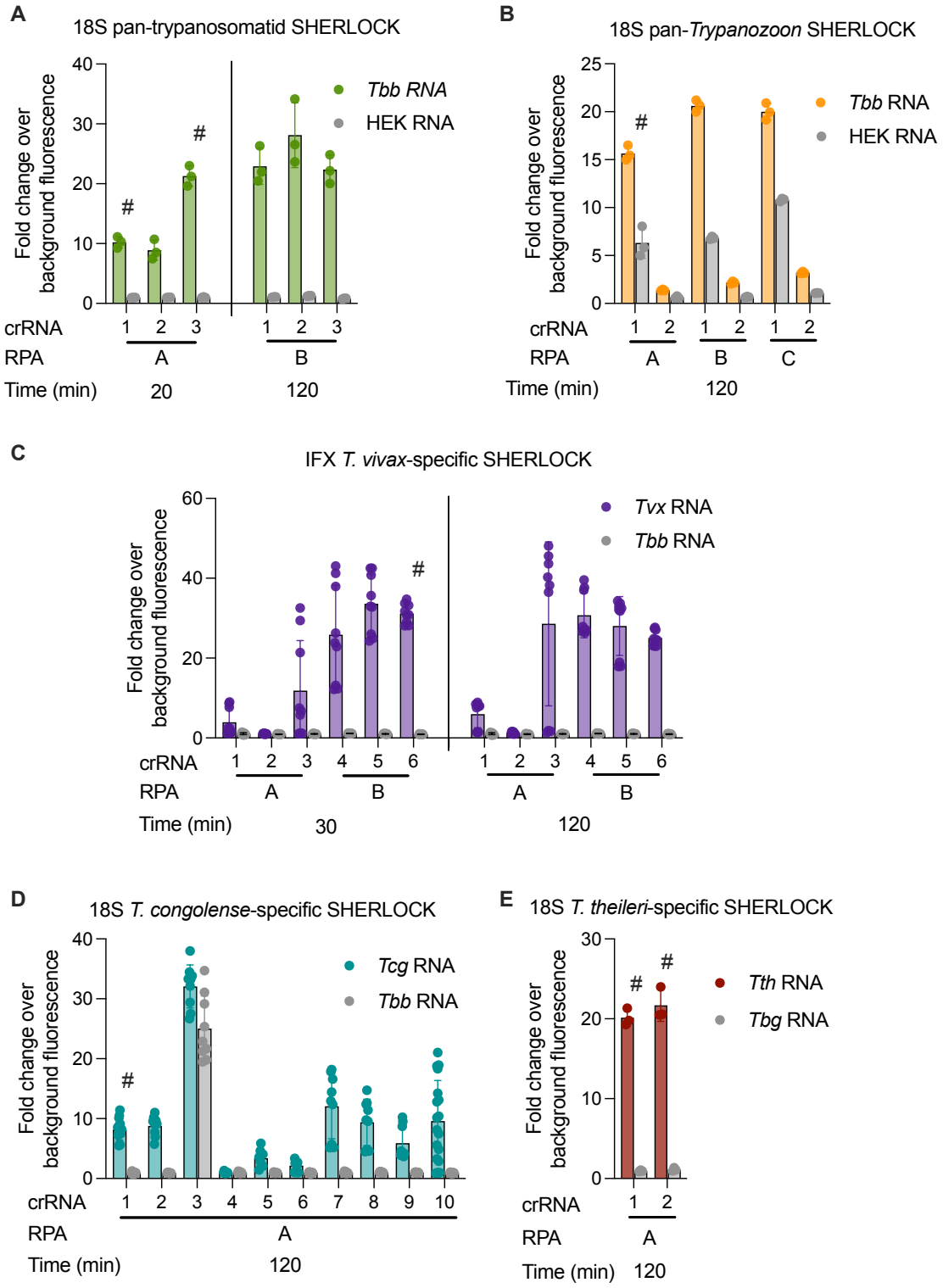
```
                    Forward RPA Primer          crRNA target region       Reverse RPA Primer

H, pdb|5VYC|i       ---------------------------[…]----------------------------[…]CCCGCCGGGCAGCTTC-------------
Pfl, XR_002966654.1 ------------------AAAATATAT[…]CATTTCTTAGGGAATGTTGATTTT----[…]TTCCTTCAGTACCTTA-------------
Lm, GQ332361.1      CCGCGCCCGCTTTTA---CCAACTTAC[…]TA----------TTCGGCCTTTAC----[…]TATCCTCAGCACGTTTTCTG--TTTTTTC
Tcz, AJ009147.1     TCTCGCCAATGAATATATTAAATTTAC[…]TT----------TTTGGTCTTCGTCTTT[…]TATCCTCAGCACGTTATCTGA-CTTCTTC
Tvx, EU477537.1     GCGCTCCACCAACCA--------TCAC[…]TTTGG-------CGCGGCCCTCGC----[…]TGTCCTCAGCACGTCC------AACCGCC
Tcg, AJ009146.1     GTGCAGTCACAACAA---ACAACTTAC[…]TGCTGCCGTACGTTCGCCCCTTATTTTT[…]TATCCTCAGCACGTTGTTTACATTTTTTC
Tth, AJ009163.1     CCTCGCCAATGGATA---TCAATTTAC[…]TT----------TTCGGTCCTCGC----[…]TATCCTCAGCACGTTATCTGA-CTTCTTC
Tsm, AJ404608.1     CCACAGCCAACTACA---CAAACTTAC[…]TGCCGCGGGTCGCC--------------[…]TATCCTCAGCACGTTGTTGA--CTCATTC
Tsu, MK954469.1     CGGTCCCAGTACTTA---ACAATTTAC[…]TTATATTCCGCCTGGCGTGCTTCGTTGT[…]TATCCTCAGCAC
Tbb, XR_002989632.1 GCCCCAATTTATTTA---TCAATTTAC[…]TATCACCCCCGGTTCCCTCTTTTGAGGT[…]TATCCTCAGCACGTTTCTTA--CTTCTTC
```

Supplementary Figure 3

BLAST exclusion	Range of amplicon percentage identity (arithmetic mean)	Number of sequences included	Percentage of sequences with conserved crRNA		Sequences with partially conserved crRNA	
			crRNA 1	crRNA 3	crRNA 1	crRNA 3
Family **Trypanosomatidae**	82.27 - 100% (98.4%)	1644	59.4 %	97.1 %	667	48
Order **Trypanosomatida**	82.27 - 100% (98.4%)	1652	59.6 %	97.1 %	667	48
Subclass **Metakinetoplastia**	81.88 – 100% (97.9%)	1931	57.6 %	94.8 %	818	100
Class **Kinetoplastea**	81.88 – 100% (97.3%)	2051	55.1 %	90.3 %	920	199
Phylum **Euglenozoa**	69.72 – 100% (91.7%)	3330	34.0 %	55.8 %	2199	1477
Phylum **Euglenozoa** (excluded Kinetoplastea)	69.72 – 98.55% (82.9%)	1287	0.6 %	0.7 %	1279	1278

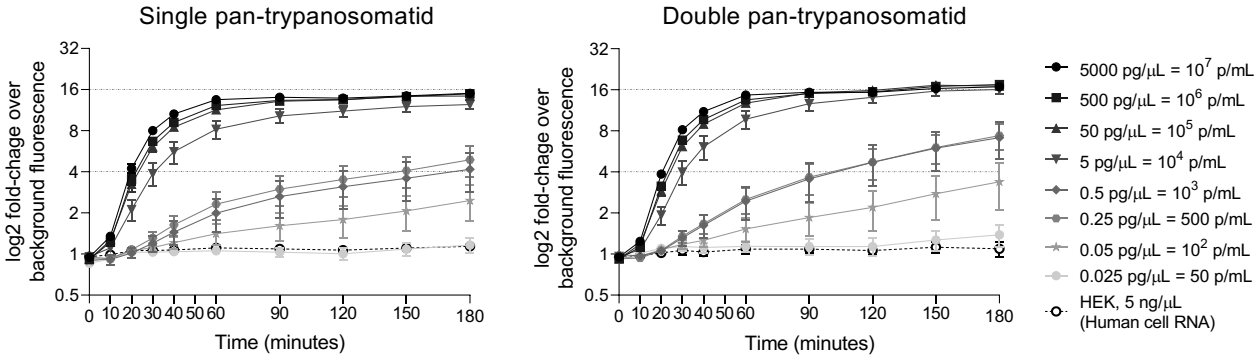
Supplementary Figure 4



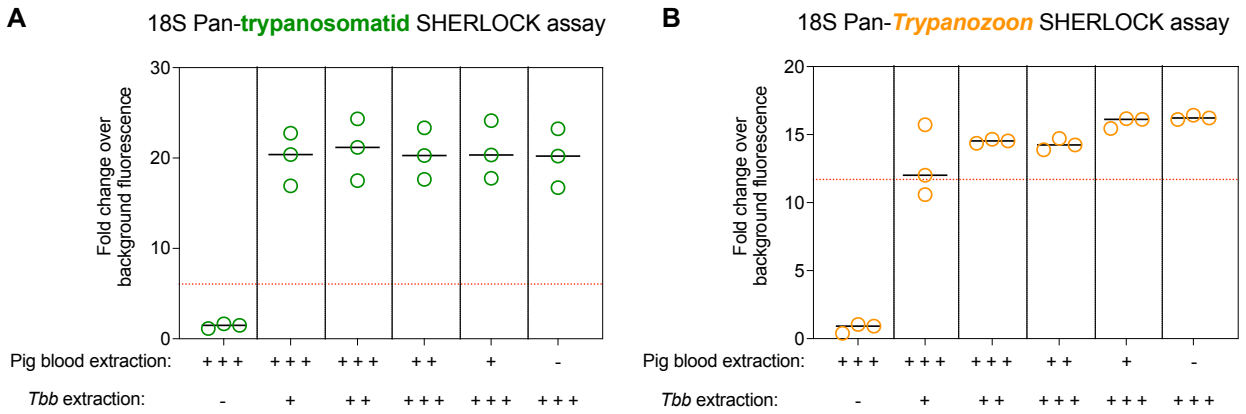
Supplementary Figure 5

	Accession	Alignment against the crRNA	Identity (%)	Query coverage
T. simiae	KR024686.1	TCCCTGCTCCGGCGGCGGGGCGACTCGC	90.62	100%
	KP307021.1	TCCCTGCTCCGGCGGCGGGGCGACTCGC	90.62	100%
	AJ009162.1	TCCCTGCTCCGGCGGCGGGGCGACTCGC	90.62	100%
	AJ223569.1	TCCCTGCTCCGGCGGCGGGGCGACTCGC	90.62	100%
	U22320.1	TCCCTGCTCCGGCGGCGGGGCGACTCGC	90.62	100%
	KP307022.1	TCCCTGCTCCGGCGGCGGGGCAACTCGC	89.06	100%
	AJ404608.1	TCCCTACCTCGGCGGCGGGGCAACTCGC	86.72	100%
	U22318.1	TCCCTACCTCGGCGGCGGGGCAACTCGC	86.72	100%
T. godfreyi	AJ009155.1	TCCCTACCCCGGCGGCGGGGCGACTCGC	88.28	100%
T. sapaensis	AB242822.1	TCTCTGTTCCGGCTGCGGGGCAACTCGT	88.28	100%
Trypanosoma vivax	EU477537.1	TCCCTGCTCCGGCGGCGGGGCAACCCGC	87.50	100%
	KM391829.1	TCCCTGCTCCGGCGACGGGGCAACCCGC	84.38	100%
	KM391828.1	TCCCTGCTCCGGCGACGGGGCAACCCGC	84.38	100%
	KM391827.1	TCCCTGCTCCGGCGACGGGGCAACCCGC	84.38	100%
	KM391826.1	TCCCTGCTCCGGCGACGGGGCAACCCGC	84.38	100%
	KM391823.1	TCCCTGCTCCGGCGACGGGGCAACCCGC	84.38	100%
	KM391822.1	TCCCTGCTCCGGCGACGGGGCAACCCGC	84.38	100%
	HE573019.1	TCCCTGCTCCGGCGACGGGGCAACCCGC	84.38	100%
	U22316.1	TCCCTGCTCCGGCGACGGGGCAACCCGC	84.13	98%
	KM391825.1	TCCCTGCTCCGGCGACGGCTTCCACCGC	83.59	100%
	KM391824.1	TCCCTGCTCCGGCGACGGCTTCCACCGC	83.59	100%
	KM391821.1	TCCCTGCTCCGGCGACGGCTTCCACCGC	83.59	100%
	KM391820.1	TCCCTGCTCCGGCGACGGCTTCCACCGC	83.59	100%
	KM391818.1	TCCCTGCTCCGGCGACGGCTTCCACCGC	83.59	100%
	KM391816.1	TCCCTGCTCCGGCGACGGCTTCCACCGC	83.59	100%
	KM391819.1	TCCCTGCTCCGGCGACGGCTTCCACCGC	82.81	100%
T. theileri	KR024688.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	86.72	100%
	LC618031.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	86.72	100%
	AJ009163.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	86.72	100%
	AB007814.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	86.72	100%
	LC618030.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	79%
	JX178191.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.29	79%
	JX178190.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	84.31	79%
	MG255951.1	TCTCTGTTACGGCGGTGGGGCAACTCAC	85.42	75%
	MG255958.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	84.38	75%
Trypanosoma thomasbancrofti	KT728394.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728393.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728392.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728391.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728390.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728389.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728388.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728387.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728386.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728385.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728384.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728383.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728382.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728381.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728380.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728379.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728378.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728377.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728376.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728375.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728374.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728373.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	OM509729.1	TCTCTGTTCCGGCGGTGGGGCAACTCAC	85.94	100%
	KT728396.1	TCTCTGTTCCGGCGATGGGGCAACTCAC	84.38	100%
	KT728395.1	TCTCTGTTCCGGCGATGGGGCAACTCAC	84.38	100%
	crRNA 18STcg1	**TCCCTGCTACGGCCGCGGGGTGACTCGC**		

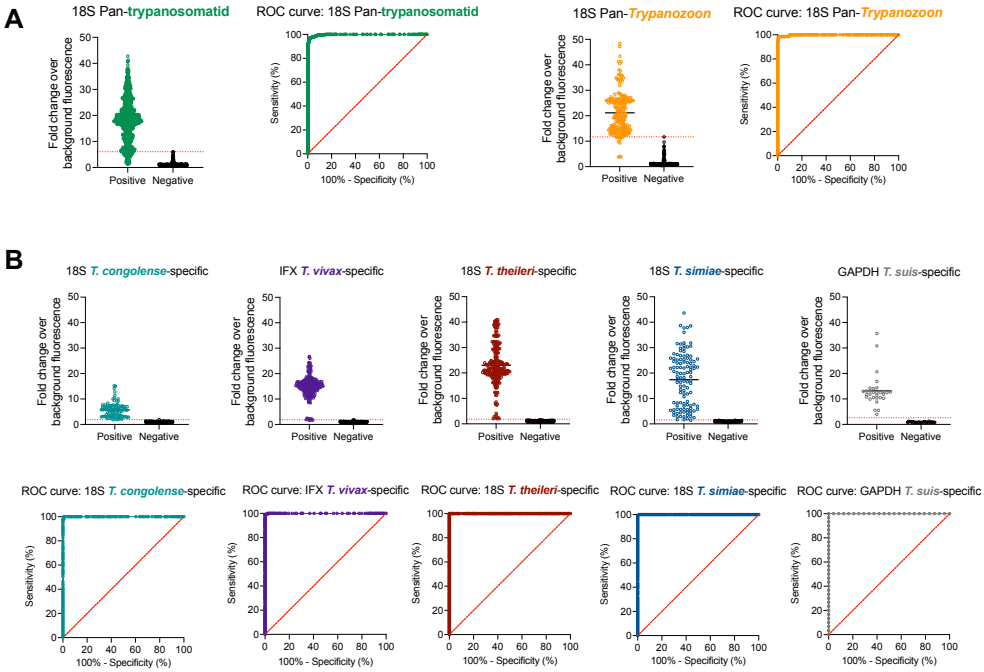
Supplementary Figure 6



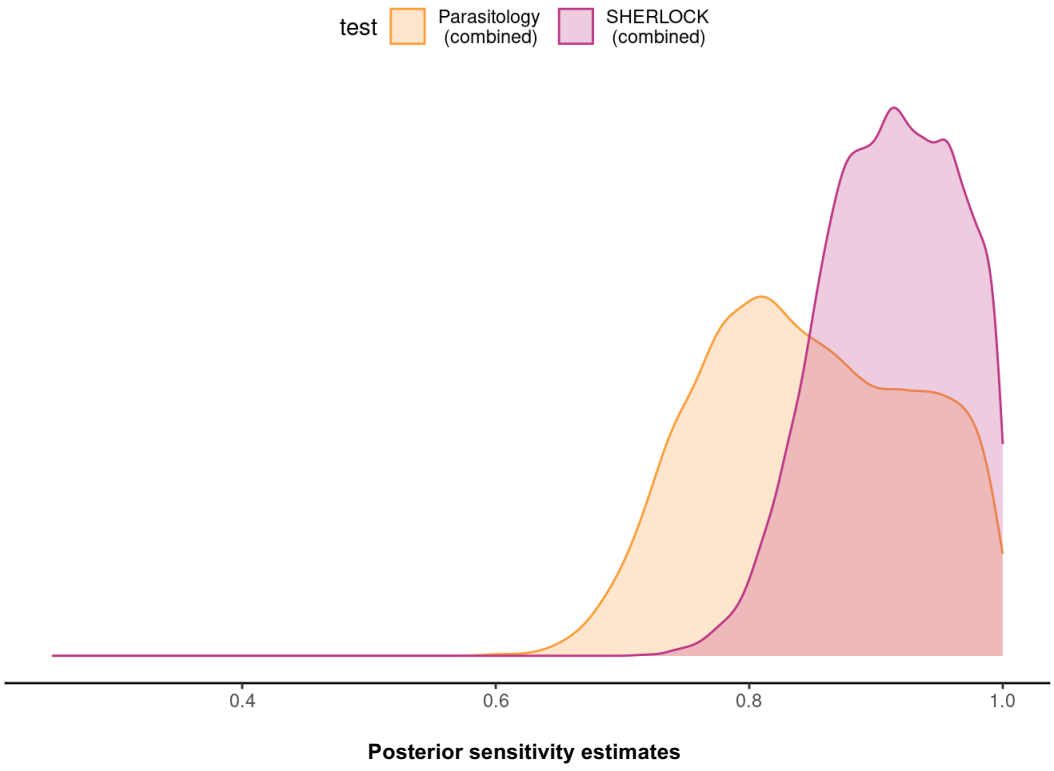
Supplementary Figure 7



Supplementary Figure 8



C

SHERLOCK Assay	Sensitivity (95 % CI)	Specificity (95 % CI)	Fold Change cut-off
Pan-trypanosomatid	**92.14 %** (90.43 to 93.57 %)	**100.0 %** (99.02 to 100.0 %)	**>6.050**
Pan-*Trypanozoon*	**95.10 %** (91.21 to 97.32 %)	**100.0 %** (97.93 to 100.0 %)	**>11.70**
T. vivax	**99.11 %** (96.82 to 99.84 %)	**100.0 %** (97.29 to 100.0 %)	**>1.831**
T. congolense	**97.98 %** (92.93 to 99.64 %)	**100.0 %** (97.04 to 100.0 %)	**>1.923**
T. theileri	**100.0 %** (98.42 to 100.0 %)	**100.0 %** (97.99 to 100.0 %)	**>1.805**
T. simiae	**99.12 %** (95.20 to 99.96 %)	**100.0 %** (96.60 to 100.0 %)	**>1.546**
T. suis	**100.0 %** (88.65 to 100.0 %)	**100.0 %** (86.20 to 100.0 %)	**>2.609**

Supplementary Figure 9