New molecular approach for the detection of Kinetoplastida parasites of medical and veterinary interest

Hacène Medkour
IHU Mediterranee Infection
Marie Varloud
CEVA Sante Animale
Bernard Davoust
IHU Mediterranee Infection
Oleg Mediannikov (olegusss1@gmail.com)
IHU Mediterranee Infection

Research

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Abstract

Background Kinetoplastids are a remarkable group of protists, containing a range of ubiquitous free-living species–pathogens of invertebrates, vertebrates and even some plants. Trypanosoma species cause sleeping sickness and Chagas disease, whereas the leishmaniases kill and debilitate hundreds of thousands of people worldwide. The diagnosis of a series of pathogenic kinetoplastids is based on clinical manifestations, epidemiological and laboratory data and, for laboratory methods, a gold standard is often missing for human or animal patients. The aim of this study was to implement a molecular approach for the diagnosis and study of Kinetoplastida.

Methods The TaqMan qPCR assays targeting: the 24Sa LSU for Kinetoplastida, 28S LSU for Leishmania/ Trypanosoma spp., 5.8S rRNA for Trypanosoma spp., 18S SSU for Leishmania spp., Kinetoplast minicircle DNA (kDNA) L. donovani complex and the L. infantum specific qPCR were designed, validated for their sensitivity and specificity in silico and then in vitro using a panel of known DNAs and after that they were used in the screening of 369 blood samples (358 dogs, 2 equids, 9 monkeys) . In addition, the new 28S LSU primer sets are presented for use in Kinetoplastida's identification by PCR and sequencing.

Results All qPCR assays showed high analytical sensitivities without failures. They detect about 0.01 parasite/ mL blood for the kDNA based qPCR and at least a single cell-equivalent of rDNA for the other systems. Based on the sequencing results, after screening, sensitivity (Se) and specificity (Sp) were: 0. 919 and 0.971, 0.853 and 0.979, 1.00 and 0.987, 0.826 and 0.995 for all of Kinetoplastida, Leishmania/ Trypanosoma, Trypanosoma, Leishmania spp. specific qPCRs, respectively. kDNA based qPCRs were more sensitive and specific (Se: 1.00; Sp: 0.997). PCR and sequencing allowed the detection of Kinetoplastids in animal blood samples such as: L. infantum, L. guyanensis, T. congolense, T. evansi and Bodo spp..

Conclusion The molecular approach proposed in this study is useful in epidemiological studies, in fundamental research such as screening for new Kinetoplastida species, in diagnosis and therapeutic follow-up. In addition, researchers are free to choose the molecular tools adapted to their studies.

Introduction

Kinetoplastida (or Kinetoplastea, as a class) is a group of flagellated protists belonging to the phylum Euglenozoa, characterized by the presence of a large massed DNA called kinetoplast (hence the name), an organelle that stains like the nucleus, but is actually a specific part of the mitochondrion in which large amounts of extranuclear DNA are accumulated [1][2]. Kinetoplastida contain a range of ubiquitous free-living species –pathogens of invertebrates, vertebrates and even some plants [3].

The order Trypanosomatida, whose members are exclusively parasites and which is mainly found in insects, is the most documented [4][5]. Trypanosomatidae is the only one Trypanosomatida family. Most monoxenic trypanosomatides (with only one host) parasitize insects. Dixenous (with two hosts in their life cycle) parasites employ an invertebrate (arthropod or leech) vector to shuttle between the vertebrate (genera *Endotrypanum, Leishmania, Paraleishmania, Trypanosoma)* or plant (genus *Phytomonas)* hosts. However, the trypanosomatids were brought to prominence by two genera, *Trypanosoma* and *Leishmania,* attributable to their role as human and animal pathogens. *Trypanosoma* and *Leishmania* are obligatorily dixenous, possess zoonotic or anthroponotic life-cycles and are transmitted by hematophagous insects [6][7]. These parasites cause devastating human diseases including human African trypanosomiasis due to *Trypanosoma brucei,* Chagas disease caused by *Trypanosoma cruzi* and the leishmaniases, which are attributable to about 20 species of *Leishmania* [8][9][10][11]. With the exception of strictly anthroponotic *gambiense* form of human trypanosomiasis and infections caused by *Leishmania tropica* and *L. donovani,* for which humans are considered the main reservoir, diseases associated with trypanosomiasis are mainly zoonotic, with animal reservoirs playing a key role in maintaining endemicity [12][13][14]. This includes *L. infantum* causative agent of the most common vector-borne protozoa disease worldwide [15], *T. brucei brucei* and *T. congolense,* the causative agents of Nagana or a similar disease in Africa and Asia, as well as *T. evansi,* the etiological agent of surra and the so-called “mal de cadeiras” outside Africa. Animals are also important reservoirs of *T. cruzi* in the Americas [16]

Much of the cellular biology of the different kinetoplastids is very similar. Of the 8000 genes that constitute kinetoplastids, more than 6000 are common orthologists, and the diseases they cause are very characteristic. Understanding the differences between these disease–causing pathogens at genetic, molecular and cellular levels might provide new approaches to the development of diagnostics, vaccines and tools needed to control them [17].

The diagnosis of a range of pathogenic kinetoplastids relies on clinical manifestations, epidemiological and laboratory data. With regard to laboratory methods, a gold standard for human or animal patients is often missing [18], which hinders the collection of accurate epidemiological data and thus limits disease control. In addition, false-negative results could delay treatment, thus contributing to reservoirs maintenance. Recently, several immunological and molecular diagnostic tools have been developed for diagnosis [7][19][20][17]. In particular, the use of molecular techniques has become increasingly relevant due to their high sensitivity, specificity and possible application to a variety of clinical samples. Among them, the real-time (quantitative) PCR, (qPCR), has become increasingly popular recently since it is fast, has broad dynamic range and cross-contamination is drastically reduced because there is no need to open reaction tubes for post-PCR analyses.

In this paper, we propose a new molecular approach for the diagnosis and study of Kinetoplastida. New TaqMan qPCR assays with specificity at different levels and targeting different genes have been developed and validated by screening samples of animal and human panel blood. The approach was confirmed and completed using PCR/ sequencing technology.

Materials And Methods

Ethics statement

Dogs and equids from Algeria in this study were examined with the assistance and acceptance of their owners. Blood samples were collected by veterinarians according to the good practices of veterinary medicine. Risk assessment was submitted to and approved by the ethics committee and decision board of the

veterinary practitioners from the wilayas of the North of Algeria. These institutions are affiliated with the Algerian Ministry of Agriculture and Rural Development (Directions des Services Vétérinaires). Protocol of the study was also approved by the scientific college (Procès-Verbal du CSI N°6, 2018) at the Veterinary Science Institute, University Constantine 1, Algeria. To facilitate field work, collaborations were established with veterinary doctors and their assistants working in these establishments.

Dogs from Cote d'Ivoire were sampled after obtaining the verbal consent of their owners. The blood samples were taken from radial vein by veterinarians (French and Ivorian) with the ethical responsibility to ensure the animal care in accordance with Articles 433-434; Chapter 2 of the Ivoirian Penal Code.

Dog's blood samples from French Guiana were collected by veterinarians according to the good practices of veterinary medicine. Article R.214-88 of the French Rural Code and Sea Fishing (Decree No. 2013–118 of 1 February 2013 on the protection of animals used for scientific purposes) excludes these acts from the scope of applications for authorization granted by the Minister responsible for research. Red howler monkeys were hunted by two Amerindian hunters and intended for consumption by the family. The hunters applied the provisions of the prefectural decree regulating the quotas of species that can be taken by a person in the department of Guiana (No. 583/DEAL of April 12, 2011). Blood samples has been taken at the moment of hunting.

Primers and probe's designs

Custom protocol and *in silico* validation

First, for each PCR essay, the target gene was chosen and a fasta file was constructed from the sequences available in GenBank database. Sequences were aligned using BioEdit v 7.0.5.3 software [21] to reveal conserved areas suitable as target regions for specific primers and probes. This region was submitted in Primer3 software v. 0.4.0 (http://primer3.ut.ee/), in order to determine a valuable candidate primers and probes and the selection was based on the criteria for primer and probe design.

Settings for the PCR primers and probes were in accordance with the guidelines as described by Apte and Daniel [22] and as recommended by Invitrogen™ and Applied Biosystems™. Melting temperatures (T), secondary structures and the possibility for primer-dimers were tested using the free online software Oligo Analyzer 3.1 [23]. All primer and probe sequences were also checked for their specificity in NCBI BLAST nucleotide sequence similarity search [24]. They were also checked within the DNA databases of metazoans (taxid:33208), vertebrates (taxid:7742), bacteria (taxid:2), *Canidae* (taxid:9608), *Felidae*(taxid:9682) and humans (taxid:9605). This has been done for all possible combinations of forward-reward and probe-reward of each PCR system. Primers were synthetized by Eurogentec (Liège, Belgium) and the hydrolysis probe by Applied Biosystems™.

Specificity-based principles of oligonucleotide design

Quantitative TaqMan real-time PCR (qPCR) for Kinetoplastida parasites detection

The conserved region from the 28S rRNA gene, more exactly the 24S alpha subunit of most Kinetoplastida class, especially Bodonida and Trypanosomatida orders, has been targeted for the primers and probe design. The primers called F LSU 24a, F LSU 24a and a probe (P LSU 24a) were proposed (Table 1). The choice of this gene (28S) was based on its availability in GenBank for almost all Kinetoplastida families and its high level of conservation [28].

TaqMan qPCR targeting *Leishmania*– *Trypanosoma* spp.

Among the Kinetoplastida parasites, several members from *Leishmania* and *Trypanosoma* genera are involved in human and animal pathology [17]. Another conserved region from the 28S rRNA gene has been chosen to design primers and probe (Table1) to amplify most species belonging to *Leishmania* and *Trypanosoma* genus. Oligonucleotides were designed in order to not amplify other members of Kinetoplastida.

TaqMan qPCR assays for *Leishmania* spp. and for *Trypanosoma* spp.

As described in a previous study (Medkour et al. submitted), two qPCRs assays had been developed: i) qPCR for *Leishmania* spp. targeting the 18S rRNA gene of most *Leishmania* species affecting humans and animals: *L. donovani, L. infantum/chagasi, L. major, L. tropica, L. guyanensis, L. amazonensis, L. braziliensis, L. mexicana, L. siamensis, L. aethiopica, L. tarentolae, L. alderi, L. gymnodactyli, L. hoogstraali* and possible new species; ii) qPCR for *Trypanosoma* spp. targeting about 85 pb-long fragments from the 5.8S rRNA gene of the most species threating humans, pets, livestock and wildlife: *T. brucei brucei, T. brucei gambiense, T. brucei rhodesiense, T. congolense,T. evansi*, *T. vivax*, *T. cruzi, T.theileri, T. equiperdum, T. rangeli, T. simiae, T. godfreyi, T. minasense* and a large number of potential new species.

TaqMan qPCR for canine and human visceral leishmaniasis diagnosis (*L. donovani, L. infantum*):

The conserved region of the *Leishmania* kDNA minicircle (kDNA), recognized for its high sensitivity (several 1000's-fold repeated sequence) [29], was elected to design the specific primers targeting a 175 pb fragment from the kDNA of *L. infantum* and *L. donovani*. Two labeled probes were also designed from the same region: i) the VIC-labeled probe for the detection of both *L. infantum* and *L. donovani* kDNA; ii) the FAM-labeled probe confined only for *L. infantum* kDNA (Table 1).

Run protocols

The qPCR reactions were carried out in a final volume of 20 µl, containing 5 µl of DNA template, 10 µl of Master Mix Roche (Eurogentec). The concentration of each primer per reaction was 0.5 µl, 0.5 µl of both UDG and each probe. Finally, the volume was completed to 20 µl using ultra-purified water DNAse-RNAse free. The TaqMan cycling conditions included two hold steps at 50°C for 2 min followed by 95°C for 15 min, and 40 cycles of two steps each (f 95° C for 30 s

and 60° C for 30 s). The qPCR amplification was performed in a CFX96 Real-Time system (Bio-rad Laboratories, Foster City, CA, USA) after activating the readers of the dyes (FAM and/or VIC) used in each qPCR system.

Conventional PCR primers sets design, amplification protocol and sequencing

The 28S rRNA was used to design a primer set in order to complete the molecular identification of Kinetoplastids. Primer pair combinations amplify from 550 to 1300 pb-fragments long of the 28S rRNA gene with a variable specificity among the species belonged into Kinetoplastida class (Table 1).

To identify *Leishmania* species other than *L. donovani/infantum,* a primer pair targeting 550 pb-fragments of the 18S rRNA of *Leishmania* spp. has been used (Medkour et al. submitted), as well as universal primer pairs targeting *Leishmania* spp. intergenic transcribed spacers 1 and 2 (ITS1 and ITS2) [30][31]. In addition, primers ITS1CF and ITS1 BR, previously designed to amplify the ITS1 partial gene of African trypanosomes [32] have been used in this approach.

PCR reactions were performed in a total volume of 50 µl, consisting of 25 µl of AmpliTaq Gold master mix, 18 µl of ultra-purified water DNAse-RNAse free, 1 µl of each primer and 5µl of DNA template. The thermal cycling conditions were: incubation step at 95°C for 15 minutes, 40 cycles of one minute at 95°C, 30s for the annealing at a different melting temperature for each PCR assays (Table 1), 1 min for elongation time at 72°C followed by a final extension for five minutes at 72°C (Table 1). PCR amplification was performed in a Peltier PTC-200 model thermal cycler (MJ Research Inc., Watertown, MA, USA). The results of amplification were visualized by electrophoresis on 2% agarose gel. The purification of PCR products was performed using NucleoFast 96 PCR plates (Macherey Nagel EURL, Hoerdt, France) according to the manufacturer's instructions. The amplicons were sequenced using the Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA) with an ABI automated sequencer (Applied Biosystems).

PCR systems validation

The specificity and sensitivity of all PCR assays were tested *in silico* using primer-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and validated *in vitro* using, i) DNAs of cultivated *Leishmania* species: *L. infantum* MCAN/ES/98/LLM-877 (WHO international reference strain), *L. donovani* (MHOM/IN/00/DEVI), *L. major* (MHOM/IL/81/Friedlin) and *L. guyanensis* previously detected on red howler monkey from French Guiana (MK782154) [33]; ii) for *Trypanosoma* 5.8S based qPCR system: DNA panel from cultivated *Trypanosoma* species: *T. congolense IL 3000*, *T. evansi*, *T. vivax*, *T. brucei brucei, T. brucei gambiense* and *T. brucei gambiense biyiamina* groupe II, *T. cruzi* CL Brenner, and DNA of uncultivated *T. congolense* detected on French dog died few weeks after return from 3 months mission in Cote d'Ivoire; iii) for *Leishmania-Trypanosoma* 28S based qPCR: All the DNAs cited above; iv) for Kinetoplastida 28S based PCRs: All the DNAs cited above, DNA of *Leptomonas* sp. isolated on flea *Ctenocephalides felis* from Senegal and DNA of *Bodo sp.* from blood of an Algerian dog. All PCR systems were tested for their specificity using several arthropods of laboratory-maintained colonies as well as human, monkey, donkey, horse, cattle, mouse and dog DNAs for all the PCR systems. DNA collections used to test the sensitivity and specificity of PCR systems are summarized in the Table S1.

Sensitivity and efficiency of TaqMan assays (quantification)

To assess the analytical sensitivity of the generic real-time PCR assays (28S *Leishmania-Trypanosoma* spp., 18S *Leishmania* spp. and *Leishmania donovani/ infantum* kDNA based qPCR (VIC-labeled probe)), a standard curve was established using *L. donovani* DNA; 5 ml of serial dilutions, ranging from 10000 to 0.001 parasites was introduced into reaction tubes for each qPCR system. The standard curve concentration was expressed as parasite/ml (par/ml).

To assess the sensitivity of the 28S based qPCR for Kinetoplastida and the 5.8S based qPCR for *Trypanosoma* spp., we used blood of French dog died after *T. congolense* infection, for which the number of parasites was determined by microscopical counting on blood smear (Fig.S1). The number of *T. congolense* visualized was 1.06 E^6 parasites/ mL of blood. Twelve-fold dilutions of this DNA (initial concentration 1.06 E^6 parasites/ mL) were made for standard curve analysis. Also, the standard curve concentration was expressed as parasite/mL (par/mL).

PCR tools validation by sample screening and identification of Kinetoplastida on biological samples

An already-existing collections of animal-blood samples was screened by qPCR assays assessed in this study. This includes:

1. 42 dogs from Cote d'Ivoire (April 2018) from the study of (Medkour et al. submitted);
2. 218 dogs, one horse and one donkey from Kabylia, northern Algeria (May 2018) from the previous study of Medkour et al,[34]. The samples were collected after obtaining a verbal consent from dog owners by a veterinarian specialist. Risk assessment was submitted to and approved by the ethics committee and decision board of the veterinary practitioners from the wilayas of the North of Algeria. These institutions are affiliated with the Algerian Ministry of Agriculture and Rural Development (Directions des Services Vétérinaires).
3. 98 dogs sampled in 2016 and 9 red howler monkeys sampled in 2014 from French Guiana from the previous studies [35] and [33].

The genomic DNA was extracted from 200 mL-blood of each sample after enzymatic digestion with proteinase K, using the QIAGEN DNA tissues extraction kit (QIAGEN, Hilden, Germany), following the manufacturer's recommendations. The extracted DNA was eluted in a total volume of 200 µl and stored at – 20°C until use.

Samples were screened by qPCRs: 28S Kinetoplastida, 28S *Leishmania-Trypanosoma* spp., 5.8S *Trypanosoma* spp., 18S *Leishmania* spp. and the qPCR targeting the kDNA of *L. donovani* complex. Positive samples for of *L. donovani* complex were tested to *L. infantum* using the *L. infantum* kDNA based qPCR;

All samples found positive by qPCR were subjected to conventional PCR/sequencing of ~550 pb-fragments of 28S gene of Kinetoplastida. *Leishmania*-positive samples were tested in PCR/sequencing of partial ITS2 and 18S rRNA genes. *Trypanosoma*-positive samples have also been tested by the ITS1 based-PCR and amplicons were sequenced (Table 1).

Determination of assay performance characteristics

Here, the performances of qPCR assays were evaluated in the absence of gold standard test. The true positive samples were determined: the sample was considered true positive if at least one of the sequences was obtained by PCR/sequencing of a part of 28S rRNA for Kinetoplastida, 18S rRNA and/or ITS2 for *Leishmania* spp. and/or ITS 1 for *Trypanosoma* spp. (Table 1). By using this criterion to determine the true positive samples, we reduced the possibility of false positives and qPCR assays will be evaluated as much as possible for their specificity. One of the limitations is the difficulties to take decisions in cases found positive by qPCR and negative in standard PCR that may be linked to a sequencing defect, for X or Y reasons, e.i. low quantity of DNA in the sample, not related to failure in specificity of the qPCR.

Statistical analysis

After set of databases Microsoft Excel® program (Microsoft Corp., Redmont, USA), a descriptive study of the Kinetoplastida of infections was performed. The statistical analysis was conducted using XLSTAT Addinsoft version 2018.7 (Data Analysis and Statistical Solution for Microsoft Excel, Paris, France).

To determine assay performance characteristics for each test, Prevalence (Pr), Correct classification (Cc) and Misclassification (Mc), Se, Sp, False positive rate (FP), False negative rate (PN), Positive and negative Predictive Value (PPV & NPV), calculated for each test.

To measure the agreement of qPCR assays according to sequencing (defined as gold standard in this study), one could simply compute the percentage of cases for which both qPCR and sequencing results agree. This statistic has nevertheless an important weakness. It does not account for agreement randomly occurring. On the other hand, Cohen's Kappa measures agreement, while removing random effects, thus ensuring good reproducibility. Cohen's Kappa (k) measures agreement was used to evaluate the relevance of each qPCR assay according to the sequencing results as established by Landis and Koch (1977).

Results

In silico and *in vitro* validation

In silico validation of the customized PCR and qPCR performance characteristics were performed using primer design tools. The PCR and qPCR systems were able to specifically detect target species without failure (Table 2). In addition, *in silico* assessment of sensitivity and PCR efficiency was confirmed by the equality of melting temperature inside each set of primers, this temperature is less than that of probes. The absence of primer-dimer formation for each system has also been confirmed. The specificity was supported by an *in vitro* validation, as shown when using DNA panels in the Table S1, where the positive reaction obtained only from targeted DNA and no negative control was amplified.

An artificial *Trypanosoma* and/or *Leishmania* and/or *Leptomonas* DNA mixtures were included to mimic mixed infection controls. It comprised artificially DNA mixed in equal proportions (Table 2). The controls were processed in the same way as the qPCR samples until sequencing. The pooled DNAs were successfully detected by qPCR.

Determining assay performance characteristics

The analytic sensitivity of the 28S Kinetoplastida and the 5.8S *Trypanosoma* spp. based-qPCR was illustrated using DNA of *T. congolense* having the concentration $1.06E^{6}$ par/mL. The standard curve results spreadsheet (SCRS) showed: an efficiency (E) of 154.8% and 142.5%, slope of -2.57 and -2.60, the point at which the curve intercepts the Y-axis (Y-intercept) was 32.21 and 32.70 as well as an almost perfect correlation coefficient ($R^{\wedge 2}$) of 0.97 and 0.99 for the 28S Kinetoplastida and the 5.8S *Trypanosoma* spp. based-qPCRs, respectively. In addition, the limit of detection was fixed at $1,06E^{-2}$par/mL of blood for both PCR systems (Fig S2 and Fig. S4).

Sensitivity of the TaqMan 28S *Leishmania-Trypanosoma* spp., 18S *Leishmania* spp. and kDNA *L. donovani* based-qPCRs was calculated using a *L. donovani* DNA showing an initial concentration of $1.0E^{4}$par/mL. For the three qPCRs, efficiency ranged from 102.1% to 176.7%, the $R^{\wedge 2}$ from 0.947 to 0.999, Slope from -2.34 to -3.27 and finally Y-intercept was from 30.62 to 35.57. The limit of detection was 1par/mL for both 28S *Leishmania-Trypanosoma* spp. and 18S *Leishmania* spp. qPCRs and 0.01 par/mL for the kDNA *L. donovani complex* based-qPCR (Fig S3, Fig. S5, Fig. S6).

Performance characteristics comparison of the diagnostic tools

Among the 369 samples screened, 45 (12.2 %) were positive using the 28S Kinetoplastida qPCR, 34 samples of them had been identified by sequencing. By using the qPCR targeting *Leishmania-Trypanosoma* spp. 28S rRNA gene, 10% (37/369) were positive including 5 samples found negative by the 28S Kinetoplastida qPCR. Specific qPCR for *Trypanosoma* spp. was able to detect 18/369 (4.9%) as positive including three samples that were negative by both qPCRs cited above. Twenty-one samples (5.7%) were found positive for *Leishmania* spp. and all of them, except one, tested positive in the large screening by the 28S based qPCRs. In addition, causative agents of visceral leishmaniasis (VL), *L. infantum/ L. donovani,* were detected in 23/369 (6.2%), including 21/369 samples found positive for *L. infantum,* using the specific kDNA based qPCRs. Four of these samples were found negative using the 18S *Leishmania* spp. based-qPCR (Table S2).

Using method combining sequence typing of the 28S, 18S, ITS1 and ITS2 (Table1) targeting members of Kinetoplastida parasites; 37/369 (10%) of samples were identified to carry at least one pathogen. Taking sequencing as gold standard, our molecular tools (qPCRs) showed a sensitivity ranging from 0.826 to 1, a high specificity from 0.971 to 0.997. A substantial agreement quality with the gold standard test was observed for the 28S *Leishmania-Trypanosoma* spp.-based qPCR according to Cohen's Kappa (Cohen's Kappa = 0.8). The agreement was almost perfect for all other qPCRs (Cohen's Kappa values from 0.811 to 0.976) (Table 3). The performances were challenged by the presence of false positives (FP), especially for the 28S based qPCRs (11 and 8 FP samples for the

Kinetoplastida and *Leishmania-Trypanosoma* spp. qPCRs, respectively), and the 5.8S qPCR for *Trypanosoma* spp. (5 FP samples). Greater performances were observed for the *Leishmania* 18S and kDNA based qPCRs (Table 4).

In addition, our PCR tools were able to detect DNA of several Kinetoplastida (*Leishmania infantum*, *Trypanosoma evansi, T. congolense* and *Bodo* spp.) on different hosts (dogs, monkeys, donkeys, horses). It was also possible to detect co-infections (*L. infantum/T. congolense* con-infection) in two samples (Table S2).

Discussion

In this study, we propose an approach to explore the presence of different Kinetoplastida parasites in animal or human samples using molecular biology. We have developed and implemented new molecular tools to study these parasites, particularly those of medical and veterinary interest, focusing on visceral leishmaniasis and African trypanosomiasis. Unfortunately, we cannot propose a universal PCR system for all parasites such as 16S rRNA PCR system for bacteria. The explosion in the number of recognized taxa and discovery of new pathogenic bacteria (such: *Bartonella henselae, Anaplasma* spp., *Tropheryma whipplei* …etc.) is directly attributable to the ease in performance of 16S rRNA gene sequencing studies [26]. Our approach allows us to study Kinetoplastida parasites. This approach keeps the doors open to the discovery of pathogens that have not yet been described, especially when it is applicable at a high-level using PCR systems targeting almost all Kinetoplastida members. Furthermore, researchers are free to choose the molecular tools suitable for their studies.

The choice of the genes targeted for PCR systems design was based on two principal characteristics, (i) the conserved sequences between targeted species, as is the case with LSU (28S) and 5.8S gene[28]. Noted that the 24Sα LSU from the 28S LSU [36][37] was targeted for the Kinetoplastida class qPCR, a region from the 28S LSU was targeted for *Leishmania-Trypanosoma* spp. qPCR, 5.8S gene for *Trypanosoma* spp. qPCR and a conserved region from 18S SSU gene for the pan-*Leishmania* qPCR [38]; (ii) the discrimination between species, as is the case with kinetoplast DNA, which was targeted by TaqMan qPCRs for the detection of *L. donovani* complex and the specific detection of *L. infantum*[29]. All the customized PCR systems showed a specific detection of the targeted DNAs for which they were designed.

Currently, no universal qPCR system for Kinetoplastida class is available. A high analytical sensitivity without failure was evaluated for the pan-Kinetoplastida qPCR implemented here, with a detection limit of 1.06E−02par/mL of blood. This tool gave an almost perfect agreement (Cohen's Kappa = 0.81) with the gold standard test defined above. Besides, it allowed us, in addition to *Leishmania/Trypanosoma* spp. detection in animal and human blood samples, to identify *Bodo* spp. (free-living trypanosomes) in the blood of 3 dogs from Algeria. *Bodo* spp. are rarely detected in blood. One report showed the presence of *Parabodo caudatus (*Kinetoplastida class) in urine voided from a dog with hematuria [39], another study reported the presence of *Bodo* sp. in the blood of woylies [40]. We developed a standard PCR targeting the same gene (28S), able to distinguish between the Kinetoplastida members at the genus or subgenus level using different primers combinations. Other PCR systems pan-Kinetoplastida targeting the 28S LSU or the 18S SSU are described [28][41].

The principal Kinetoplastida pathogens are belonging to *Leishmania* and *Trypanosoma* genera [17][42]. We set up a qPCR able to detect members of these two genera. It can be used in research for leishmaniasis and trypanosomiasis. The sensitivity and specificity were 0.85 and 0.98 respectively. The pan-*Leishmania/Trypanosoma* qPCR showed a substantial agreement (Cohen's Kappa = 0.800) with the gold standard. The weakness of sensitivity could be explained by the detection limit which does not exceed 1 par/mL of blood (Fig S4). Samples found positive in qPCR with no sequence obtained after PCR/sequencing, (Tables 4 and S1) could be explained by the sequencing defect, for unknown reasons, e.i. low quantity of DNA in the sample, not related to failure in specificity of the qPCRs, especially when some samples were positive in PCR standard but no sequence was found. In addition, the two qPCR assays cited above could detect genomic DNA in Kinetoplastida's mixed- infections as single infections, i.e. we cannot detect co-infections.

Here, we developed for the first time a pan-*Trypanosoma* qPCR targeting the 5.8 S gene of almost trypanosomes of medical interest. It showed almost perfect sensitivity and specificity and perfect agreement (Cohen's Kappa = 0.83) with the gold standard. The tool presented a very high sensitivity with a limit detection of 1,060E−02 parasites/mL and two samples (identified as *T. congolense)* were detected by this system where they were negative by both qPCRs targeting the 28S gene. The cross reactivity between *Trypanosoma* and *Leishmania* spp. is one of the limits for the serological tests, such as ELISA and IFAT [43][44]. No cross-reactivity between trypanosomes and *Leishmania s*pp. was detected in system specificity tests compared to other Kinetoplastida (especially *Leishmania* spp.). In addition, the system is able to detect *Trypanosoma*-genomic DNA in mixed infections with the *Trypanosoma* members or with the other Kinetoplastida (Table 2). Co-infections should be suspected if the 5.8S qPCR *Trypanosoma* spp. is positive, while sequencing of the 28S gene for Kinetoplastida is not possible. In this case, genus specific PCR systems (ITS1 and ITS2 used for *Trypanosoma* and *Leishmania* spp., respectively, in this study) could decrypt these co-infections. In this study, *T. evansi* and *T. congolense* have been detected in dogs (Table S3).

Leishmania spp. qPCR was almost all specific (Sp = 0.994) in the detection of *Leishmania* species. The 18S SSU was one of the targets of the Leishmania PCR design and several *Leishmania*-based qPCR 18S have already been developed [45]. As mentioned for the *Trypanosoma* spp. qPCR, the *Leishmania* spp. specific qPCR palliate the problem of cross reactivity recognized for the serological tests. *Leishmania* spp. were detected in dogs, monkeys, donkey and horse in the present study. Four samples were not detectable by this qPCR system where they were detectable by the 28S based qPCRs and sequencing (Table S3), therefore the sensitivity did not exceed 0.83. This TaqMan qPCR is able to detect DNA if only there is a concentration of 1 par/mL of blood. Because visceral leishmaniasis is a major problem threating animal and public health, two qPCRs were customized to identify the major causative agents of VL [46]. One system for the detection of *L. donovani* complex (*L. donovani* and *L. infantum),,* the other system for the diagnostic of zoonotic VL due to *L. infantum (*Table 1). Both qPCR systems showed the higher Se (1.00) and Sp (0.997) compared to the other qPCR systems. The region targeted (kinetoplast DNA) for PCRs design is known for its sensitivity and allows quantification [47][48][49]. High limit of detection was observed, the TaqMan kDNA qPCRs were able to detected 0.01 parasites/mL blood. The four samples not detectable by the 18S qPCR, were detectable by both kDNA qPCRs.

One of the applications of PCR targeting *Trypanosoma* spp. and PCR essays for *Leishmania* spp., in addition to identify Kinetoplastida at the genus level, is to decrypt co-infections *Leishmania/Trypanosoma.* Another application, as our PCRs allow us to quantify parasites, allows us to define the therapeutic protocol

(molecules and doses) and to monitor.

Furthermore, the sequencing is not widely available in clinical laboratories, so that following the proposed approach; these labs could conduct studies in Kinetoplastida parasites using only TaqMan qPCR technology.

Based on the results of this study, we recommend, after analyzing our samples (Table S3), to:

- For the detection of (practically) all Kinetoplastida parasites an initial screening using at least three qPCRs targeting three different genes for Kinetoplastida e.i. screening with 28S Kinetoplastida qPCR with or without screening by the 28S pan-Leishmania/Trypanosoma, followed by a screening with the 5.8S pan-Trypanosoma and the 18S pan-Leishmania qPCR assays.
- For the species identification, the 28S based PCR was able to identify Kinetoplastida, but at the genus or subgenus level. In addition, when there were infections by more than one species, it was not possible to sequence both amplicons.
- We can resolve this problem using genus-specific PCR systems. It was the case of co-infections by T. congolense/ L. infantum in two dogs from Cote d'Ivoire (Table S3).

Finally, we invite researchers to follow the proposed molecular approach (Fig 2) according to their goals. The approach starts at the class level, samples could be screened for the presence of Kinetoplastida DNA. Positive ones will be tested for *Leishmania/* trypanosomes. Positives at this stage will be tested for the presence of *Leishmania* DNA apart and *Trypanosoma* DNA in another part. On the other hand, the negative samples by using the *Leishmania/ Trypanosoma* qPCR will be directly analyzed by the 28S PCR followed by sequencing to identify which Kinetoplastid is concerned. After that, the positive samples for *Leishmania* spp. could be tested by qPCRs for *L. donovani* complex then for *L. infantum.* Negatives at this level are necessarily *Leishmania* spp. excluding *L. infantum/ donovani,* and they could be decrypted by genotyping (28S, 18S and/or ITS2 PCRs). On the other hand, positive results for *Trypanosoma* spp. can be identified by universal primers targeting the ITS1 gene for African trypanosomes, otherwise by 28S-based PCR for all kinetoplastidae..

Conclusion

The present molecular approach developed here offers researchers the exploration of Kinetoplastida parasites in human and animal samples. It relies principally on the TaqMan qPCR and sequencing technologies. The Kinetoplastida-assays, qPCR assays for the detection of most kinetoplastids were presented and tested. All the assays showed a good sensitivity and specificity and detected at least a single cell-equivalent of rDNA. They could detect and identify Kinetoplastids at the genus level for *Trypanosoma* and *Leishmania* spp., two genera including most important pathogens threating animal and public health (African trypanosomiasis, Chagas disease and leishmaniasis). Furthermore, we have implemented a highly sensitive qPCR assay for the detection of visceral leishmaniosis (due to *L.donovani/ infantum)* and a specific qPCR for zoonotic visceral leishmaniosis (*L. infantum)*.. To identify Kinetoplastids, genotyping was performed by PCR/sequencing for *Leishmania* and *Trypanosoma* spp. A novel primer suite is presented for PCR /sequencing. It allows identification of Kinetoplastida infections detected in animal samples. Besides, this molecular approach is useful in epidemiological studies, in basic research such as probing for new Kinetoplastida species, in diagnosis and therapeutic follow-ups.

Supporting Information

Fig S1. Trypanosoma congolense in a dog from Cote d'Ivoire; Microscopy G10X100; There were 1056000 parasite/ mL of blood

Fig S2. Standard curve and the detection limit for the TaqMan qPCR 28S LSU for Kinetoplastida,

Fig S3. Standard curve and the detection limit for the TaqMan qPCR 28S LSU *Leishmania/ Trypanosoma spp.*,,

Fig S4. Standard curve and the detection limit for the TaqMan qPCR 5.8S rRNA for *Trypanosoma* spp.,

Fig S5. Standard curve and the detection limit for the TaqMan qPCR 18S SSU *Leishmania* spp.,

Fig S6. Standard curve and the detection limit for the TaqMan qPCR-kDNA for *Leishmania donovani* complex,

Table S1. Positive and negative control DNA used for sensitivity and specificity determination of designed oligonucleotides

Table S2. Individual results for samples screened by qPCR tools and typing.

Declarations

Acknowledgments

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Ethics approval and consent to participate

Dogs and equids from Algeria in this study were examined with the assistance and acceptance of their owners. Blood samples were collected by veterinarians according to the good practices of veterinary medicine. Risk assessment was submitted to and approved by the ethics committee and decision board of the veterinary practitioners from the wilayas of the North of Algeria. These institutions are affiliated with the Algerian Ministry of Agriculture and Rural

Development (Directions des Services Vétérinaires). Protocol of the study was also approved by the scientific college (Procès-Verbal du CSI N°6, 2018) at the Veterinary Science Institute, University Constantine 1, Algeria. To facilitate field work, collaborations were established with veterinary doctors and their assistants working in these establishments.

Dogs from Cote d'Ivoire were sampled after obtaining the verbal consent of their owners. The blood samples were taken from radial vein by veterinarians (French and Ivorian) with the ethical responsibility to ensure the animal care in accordance with Articles 433-434; Chapter 2 of the Ivoirian Penal Code.

Dog's blood samples from French Guiana were collected by veterinarians according to the good practices of veterinary medicine. Article R.214-88 of the French Rural Code and Sea Fishing (Decree No. 2013–118 of 1 February 2013 on the protection of animals used for scientific purposes) excludes these acts from the scope of applications for authorization granted by the Minister responsible for research. Red howler monkeys were hunted by two Amerindian hunters and intended for consumption by the family. The hunters applied the provisions of the prefectural decree regulating the quotas of species that can be taken by a person in the department of Guiana (No. 583/DEAL of April 12, 2011). Blood samples has been taken at the moment of hunting.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Availability of data and materials

The data supporting the conclusions of this article are included within the article.

Authors' contributions

HM, BD and OM designed the study. HM, BD collected the samples. HM performed the lab work. HM, MV and OM carried out the data analysis. HM, BD, MV and OM drafted the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Primers and probes, their characteristics and conditions.

PCR name	Target gene	Primers and probes Name	Primers and probes (5'-3')	Amplicon size (pb)	Tm °C	Specificity (Accordance to Fig. 1)
qPCR Pan-Kinetoplastida	28S LSU (24 alpha)	P LSU 24a; 5345-5370	FAM-TAGGAAGACCGATAGCGAACAAGTAG	200	60°C	Kinetoplastida **(1)**
		F LSU 24a; 5198-5216	AGTATTGAGCCAAAGAAGG			
		R LSU 24a; 5412-5433	TTGTCACGACTTCAGGTTCTAT			
qPCR Pan-*Leishmania/ Trypanosoma*	28S LSU	Probe S Leish tryp; 6796-6816	FAM- GGGAAGGATTTCGTGCCAACG	135	60°C	*Leishmania* spp. and *Trypanosoma* spp. **(2)**
		F lesih tryp, 6724-6741	AGATCTTGGTTGGCGTAG			
		Ra lesih tryp, 6837-6858	ATAACGTTGTGCTCAGTTTCC			
qPCR Pan-*Trypanosoma*	5. 8 S rRNA	S. 5.8 S Tryp sp 3911-3934	FAM-GTTGAAGAACGCAGCAAAGGCGAT	83	60°C	*Trypanosoma* spp. **(3)**
		F. 5.8 S Tryp sp 3874-3897	CAACGTGTCGCGATGGATGA			
		R. 5.8 S Tryp sp -Leish sp 3935-3957	ATTCTGCAATTGATACCACTTATC			
qPCR Pan-*Leishmania*	18S SSU	Probe leish S	FAM- CGGCCGTAACGCCTTTTCAACTCA	75	60°C	*Leishmania* spp. **(4)**
		F	GGTTTAGTGCGTCCGGTG			
		R	ACGCCCCAGTACGTTCTCC			
qPCR L. donovani/ L. infantum	kDNA minicircle	S. *L. infantum* 60-89	FAM-TGGGCTGGATTGGGTTTTCCTGGGCTGGA	175	60°C	-VIC: *L. donovani* complex **(5)** -FAM: *L. infantum* **(6)**
		S. *L. donovani* complex 51-78	VIC-TGGGCTCCCCTGGGCTGGATTGGGCTCC			
		F. *L.infantum/L.donovani* 4-30	GGGGTTGGTGTAAAATAGGGCCGGGTGGT			
		R. *L. inf/ L. dono/ L. maj* 151-179	CCACATCAAAGGCACCCGAACCATTAA			
PCR Pan-Kinetoplastida	28S LSU	F2 28S	ACCAAGGAGTCAAACAGACG	(F2 28S/ R2 28S) ~920	53°C	Kinetoplastida; Possibility of hemi-nested PCR **(1)**
		R1 28S	GACGCCACATATCCCTAAG			
		R2 28S	GTTGGCACGAAATCCTTCC	(F2 28S/ R1 28S) ~550	58°C	
		F1 28S	ACCTAGTAGCTGGTTCAC			
		R0 28S	TCAGCATCGCTACAGGCCTC			
PCR Pan-*Leishmania*	18S SSU	F1	CTGTGACTAAAGAAGCGTGAC	~550	52°C	*Leishmania* spp. **(4)**
		R1	AGGCCGAATAGAAAAGATACGT			
PCR Pan-*Leishmania*	ITS II	LGITSF2	GCATGCCATATTCTCAGTGTC	370 to 450	60°C	*Leishmania* spp. **(4)**
		LGITSR2	GGCCAACGCGAAGTTGAATTC			
PCR Pan-*Trypanosoma*	ITS I	ITS1-CF	CCGGAAGTTCACCGATATTG	250 to 710	58°C	African trypanosomes **(3)**
		ITS1-BR	TTGCTGCGTTCTTCAACGAA			

Tm: Annealing temperature

Table 2. In vitro validation relative specificity of the TaqMan qPCR assays

qPCR assay DNA targets	*Kinetoplastida* (28S)	*Leishmania/ Trypanosoma* spp. (28S)	*Trypanosoma* spp. (5.8S)	*Leishmania* spp. (18S)	*L. donovani/ L. infantum* (kDNA)	*L. infantum* (kDNA)
T. evansi Montecal EC8	+	+	+			
T. brucei gambiense biyiamina groupe II	+	+	+			
T. brucei brucei	+	+	+			
T. brucei gambiense (T. Féo)	+	+	+			
T. congolense (Chien Logan)	+	+	+			
T. congolense IL 3000	+	+	+			
T. congolense (Dog)	+	+	+			
T. cruzi CL Brunner	+	+	+			
T. cruzi (Dog)	+	+	+			
T. vivax	+	+	+			
L. infantum	+	+		+	+	+
L. donovani	+	+		+	+	
L. major	+	+		+		
L. guyanensis	+	+		+		
Leptomonas sp.	+					
Bodo sp.	+					
L. infantum + L. donovani	+	+				
L. infantum + L. donovani+ T. congolense IL 3000	+	+	+			
T. congolense IL 3000+ T. brucei brucei	+	+	+			
T. congolense IL 3000+T. brucei brucei+ Leptomonas sp.	+	+	+			

L.: *Leishmania*; T.: *Trypanosoma*

Table 3. Performances of the TaqMan qPCR assays developed in the present study.

Statistic	TaqMan qPCR systems					
	28S Kineto	28S Leish-Tryp	5.8S Tryp	18S Leish	kDNA L. dono cplx	kDNA L. inf
Correct classification	0,962	0,965	0,986	0,984	0,997	0,998
Misclassification	0,038	0,035	0,014	0,016	0,003	0,002
Sensitivity	**0,919**	**0,853**	**1,000**	**0,826**	**1,000**	**1,000**
Specificity	**0,967**	**0,976**	**0,986**	**0,994**	**0,997**	**0,997**
False positive rate	0,033	0,024	0,014	0,006	0,003	0,003
False negative rate	0,081	0,147	0,000	0,174	0,000	0,000
Prevalence	0,100	0,092	0,035	0,062	0,060	0,051
PPV (Positive Predictive Value)	0,756	0,784	0,722	0,905	0,957	0,955
NPV (Negative Predictive Value)	0,991	0,985	1,000	0,989	1,000	1,000
LR+ (Positive likelihood ratio)	27,735	35,717	71,200	142,913	347,000	390,000
LR- (Negative likelihood ratio)	0,084	0,151	0,000	0,175	0,000	0,000
Relative risk	81,600	52,043	-	78,714	-	-
Odds ratio	330,727	237,075	-	817,000	-	-
Cohen's Kappa	**0,81**	**0,800**	**0,832**	**0,855**	**0,976**	**0,975**
Agreement*	**almost perfect**	**substantial**	**almost perfect**	**almost perfect**	**almost perfect**	**almost perfect**

*Landis and Koch (1977) have established a scale to describe agreement quality according to Kappa values: < 0: no agreement, 0 - 0.2: small, 0.2 - 0.4: fair agreement, 0.4 - 0.6: moderate, 0.6 - 0.8: substantial, 0.8 – 1: almost perfect.

Table 4. Performances of the TaqMan qPCR assays according to the sequencing results.

TaqMan qPCR target	Detected and typed	Detected, untyped	Typed, not dectected	Not detected, untyped
28S Kinetoplastida	34	11	3	363
28S *Leishmania/ Trypanosoma*	29	8	5	369
5.8S *Trypanosoma* spp.	13	5	0	393
18S *Leishmania* spp.	19	2	4	386
kDNA *L. donovani* complex	22	1	0	388
kDNA *L. infantum*	21	1	0	389

Figures

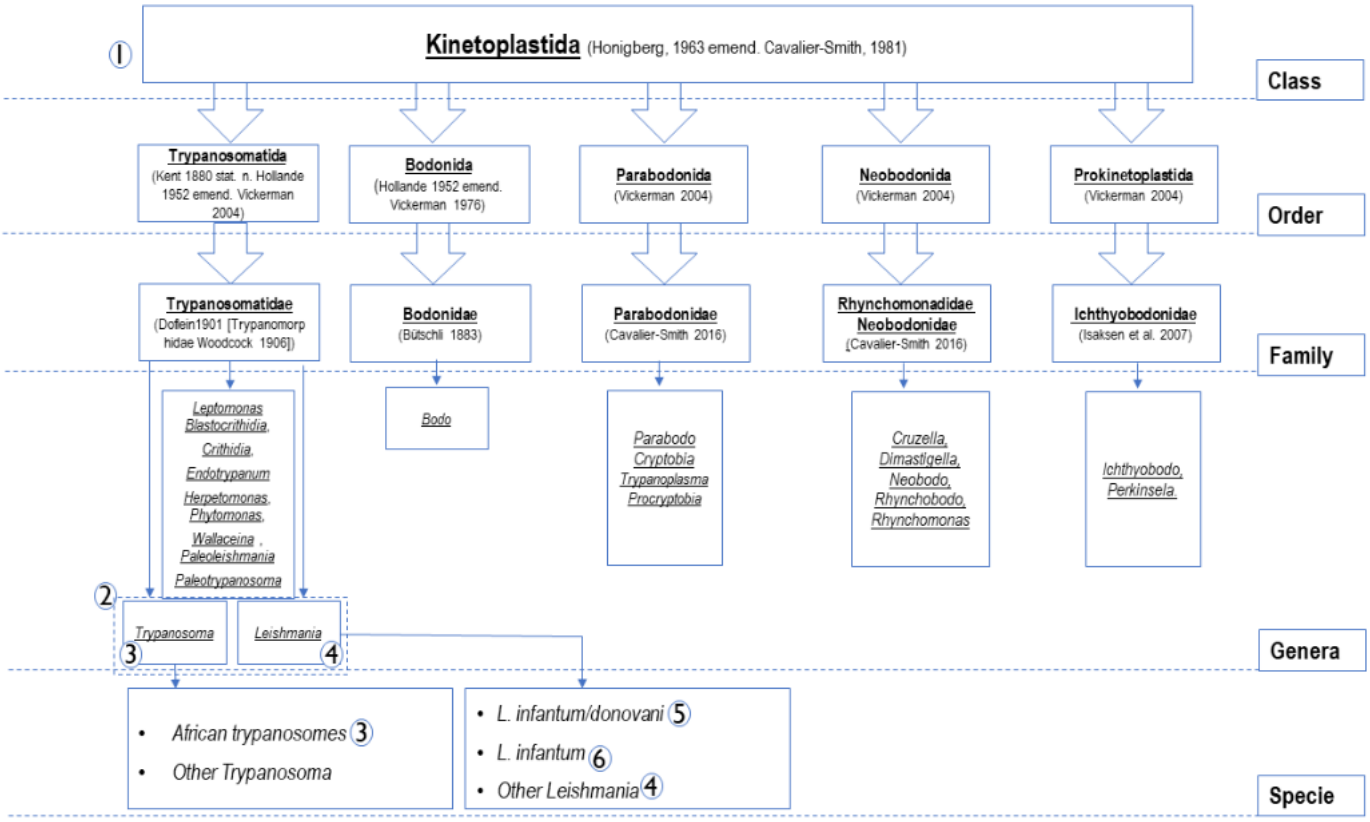

Figure 1

Members belonging to Kinetoplastida class (according to SINA M et al., 2012) and specificity-based principles of oligonucleotide design. The numbers from 1 to 6 constitute PCRs systems listed in the Table 1. As showed in this figure and in the Table 1, PCR systems were designed to amplify DNA of: PCRs (1) : all Kinetoplastida; PCR (2): Leishmania and Trypanosoma spp.; PCRs (3): Trypanosoma spp.; PCRs (4): Leishmania spp.; PCR (5): Leishmania donovani complex (L. donovani/ L. infantum); PCR (6): L. infantum.

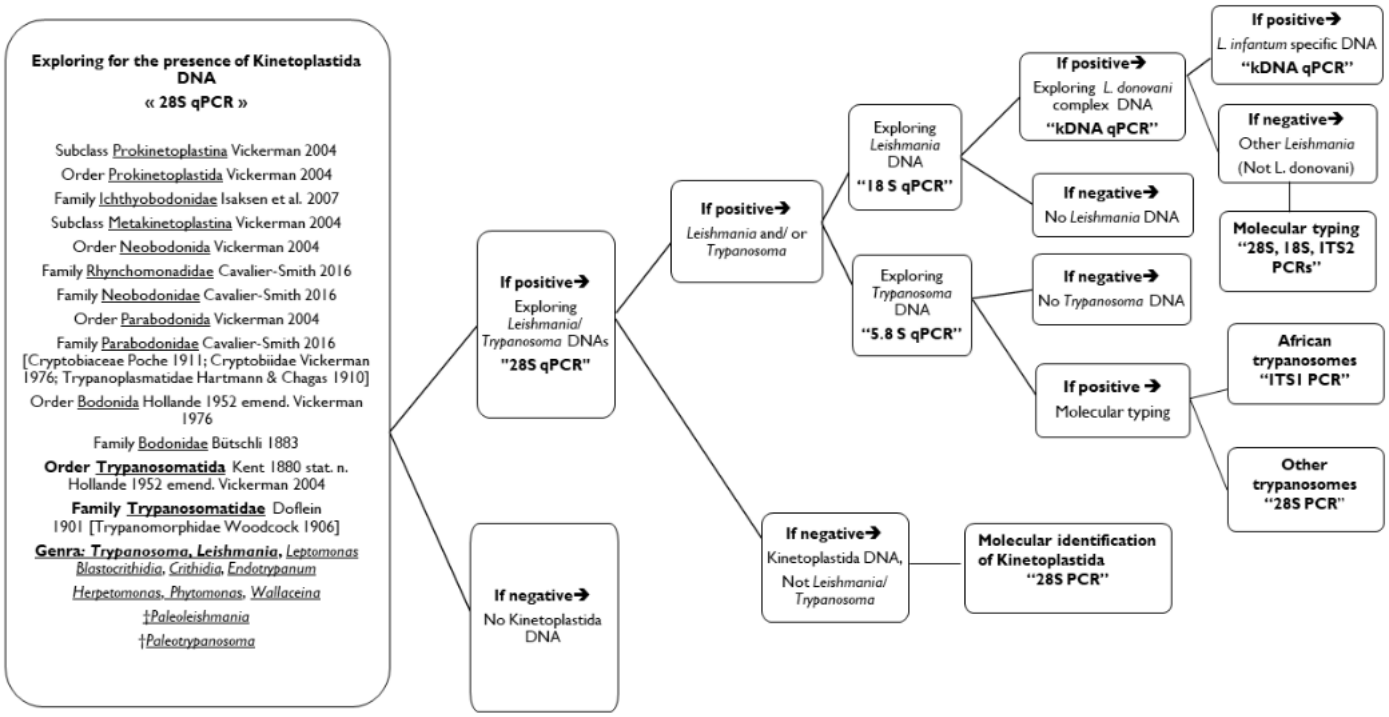

Figure 2

Proposal of molecular approach for the complete diagnosis of Kinetoplastida in human and animal samples.

Supplementary Files

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- Graphicalabstract.tif