Trypanosomatid Pathogens

Subjects: Microbiology

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Unicellular eukaryotes of the Trypanosomatidae family include human and animal pathogens that belong to the *Trypanosoma* and *Leishmania* genera. Diagnosis of the diseases they caused requires the sampling of body fluids (blood, lymph, peritoneal fluid, cerebrospinal fluid, etc.) or organ biopsies (bone marrow, spleen, etc.), which are mostly obtained through invasive methods. Body fluids or appendages can be alternatives to these invasive biopsies but their appropriateness remains poorly studied. To further address this question, we perform a systematic review on clues evidencing the presence of parasites, genetic material, antibodies, and antigens in body secretions, appendages, or the organs or proximal tissues that produce these materials.

Trypanosoma Trypanosomiasis Leishmaniasis Chagas disease Canine Leishmaniasis Human African Trypanosomiasis Sleeping sickness Animal trypanosomiasis Dourine Nagana Canine visceral leishmaniasis Feline leishmaniasis Diagnosis Noninvasive biopsy Urine Feces

1. Introduction

Unicellular eukaryotes of the Trypanosomatidae family include human and animal pathogens that belong to the *Trypanosoma* and *Leishmania* genera (including *Endotrypanum*) (Figure 1). *Leishmania* and possibly *Trypanosoma* are probably descended from the parasites of blood-sucking insects that survived accidental transmission to a vertebrate host during feeding [1].

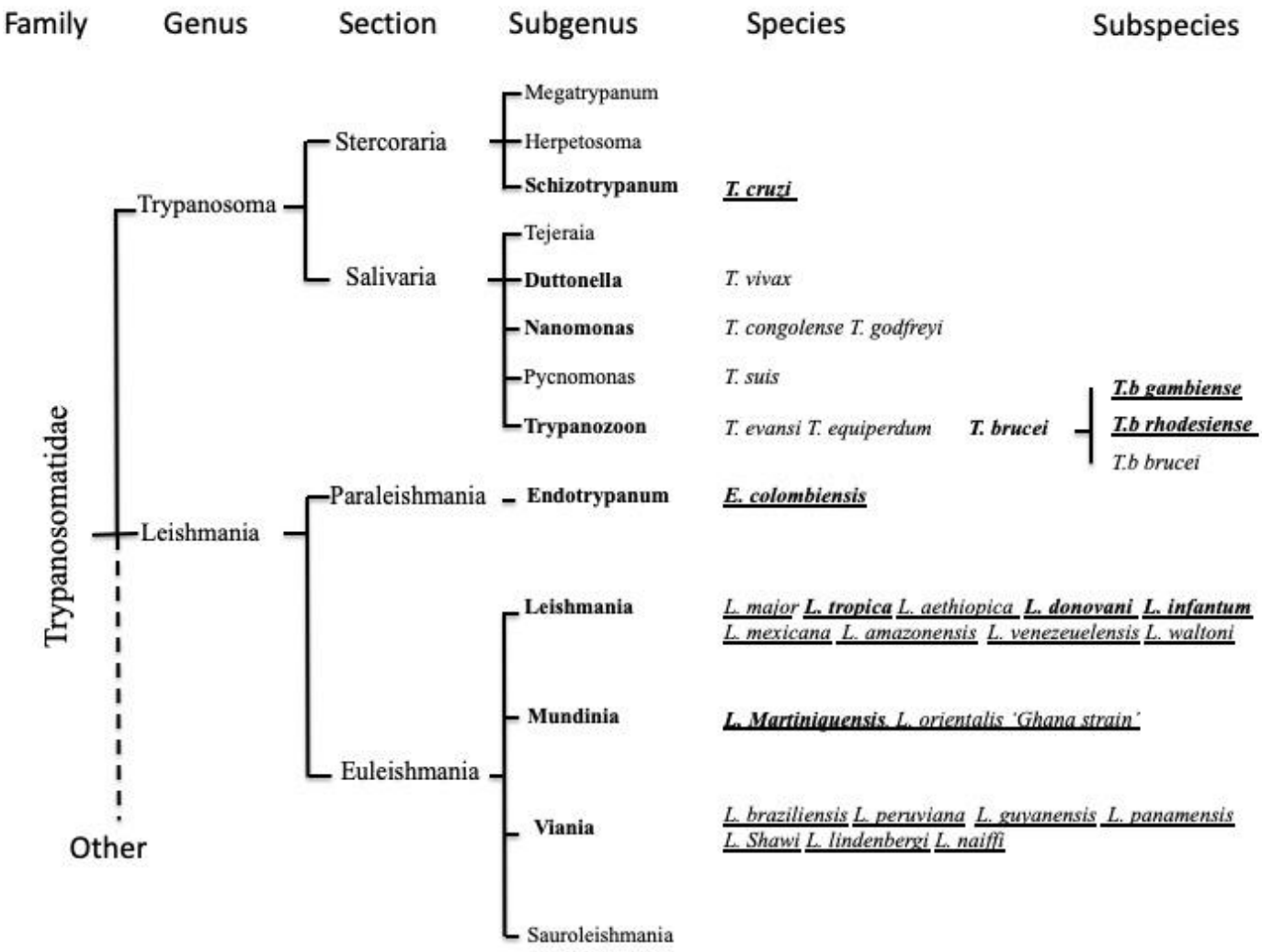

Figure 1. Classification of human and animal pathogenic trypanosomatids. Human pathogenic species are underlined, and pathogens causing systemic infection are in bold.

They possess a complex life cycle that includes arthropod vectors belonging to the Hemiptera and Diptera orders (Figure 2). Two *Trypanosoma* subspecies of *T. brucei* (*i.e., Trypanosoma brucei gambiense, T. brucei rhodesiense*) and *T. cruzi,* along with 21 species of *Leishmania,* are pathogenic for humans. They cause human African trypanosomiasis (HAT or sleeping sickness), Chagas disease (CD), and cutaneous (CL), muco-cutaneous (MCL) or visceral (VL) leishmaniases [2][3][4][5] (http://leishmania.ird.fr/). Occasional infections in humans with *T. evansi*, *T lewisi*, *T. brucei brucei* or *T. congolense* have been described, but little is known about the public health importance of these diseases [6].

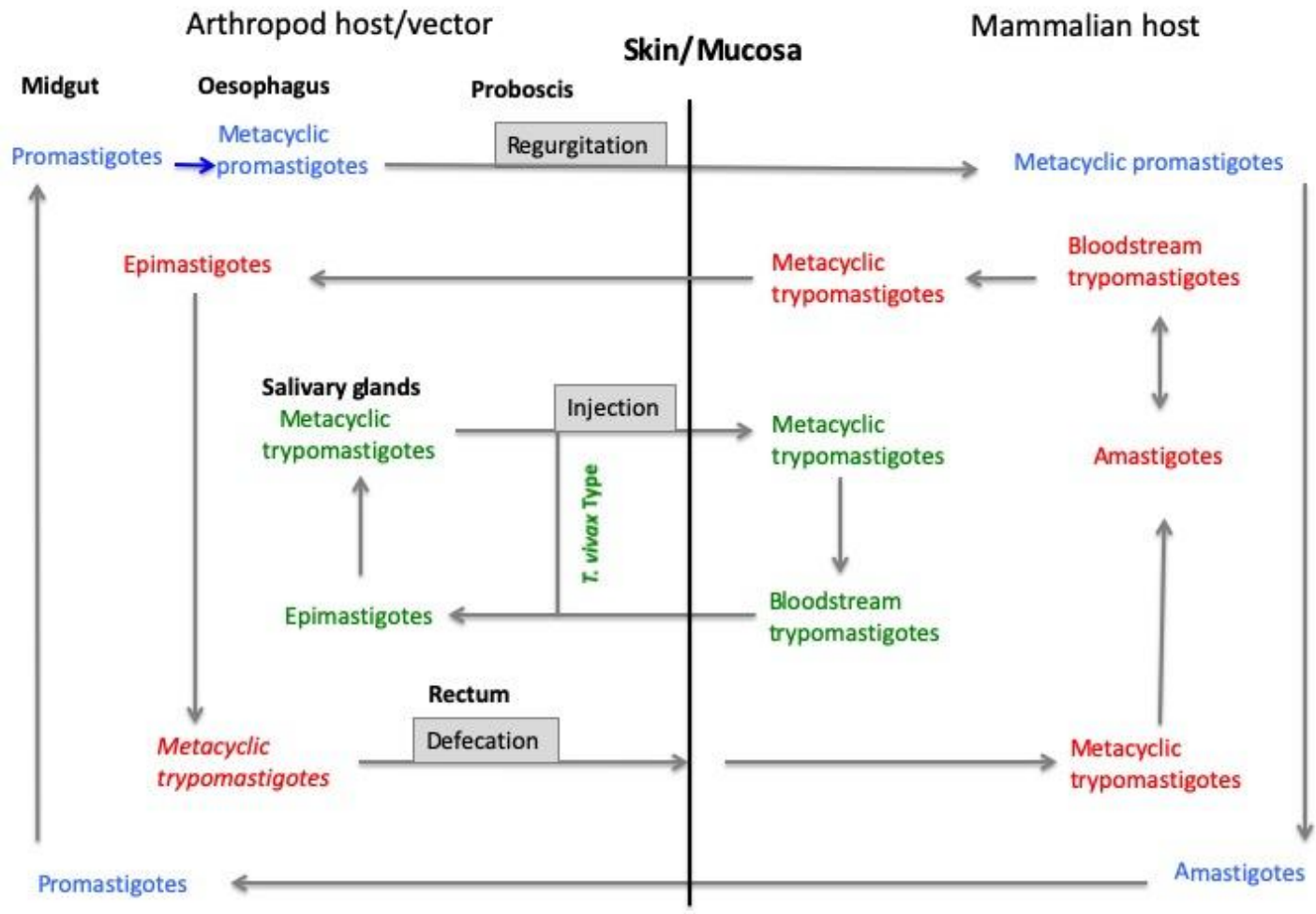

Figure 2: Developmental life cycle of Leishmania (blue), *T. cruzi* (red), and *Trypanosoma sp* (green)

In addition to their impact on human health, these diseases also affect domestic, feral or wild animals. Canine visceral leishmaniases (CVL) are mainly caused by L. infantum infection and occasionally by L. donovani or L. major. *Trypanosoma congolense*, *T. evansi*, *T. b. brucei*, *T. vivax*, *T. simiae*, *T. suis* and more rarely *T. godfreyi* affect livestock, causing animal trypanosomiasis, and *T. equiperdum* affects equids [7][8]. Altogether, worldwide, more than 30 million people are infected with these pathogens, and approximately 100,000 persons die every year from *Trypanosoma brucei* spp., *T. cruzi* or *Leishmania* spp. infections [9]. An estimated of 48 million cattle are at risk of contracting Animal Trypanosomiasis in Africa. African Animal Trypanosomiasis (AAT) causes about 3 million deaths in cattle every year (http://www.fao.org/paat/the-programme/the-disease/en/). A map of the worldwide distribution of African and American human trypanosomiasis, animal trypanosomiasis and leishamniasis is given in the Figure 3.

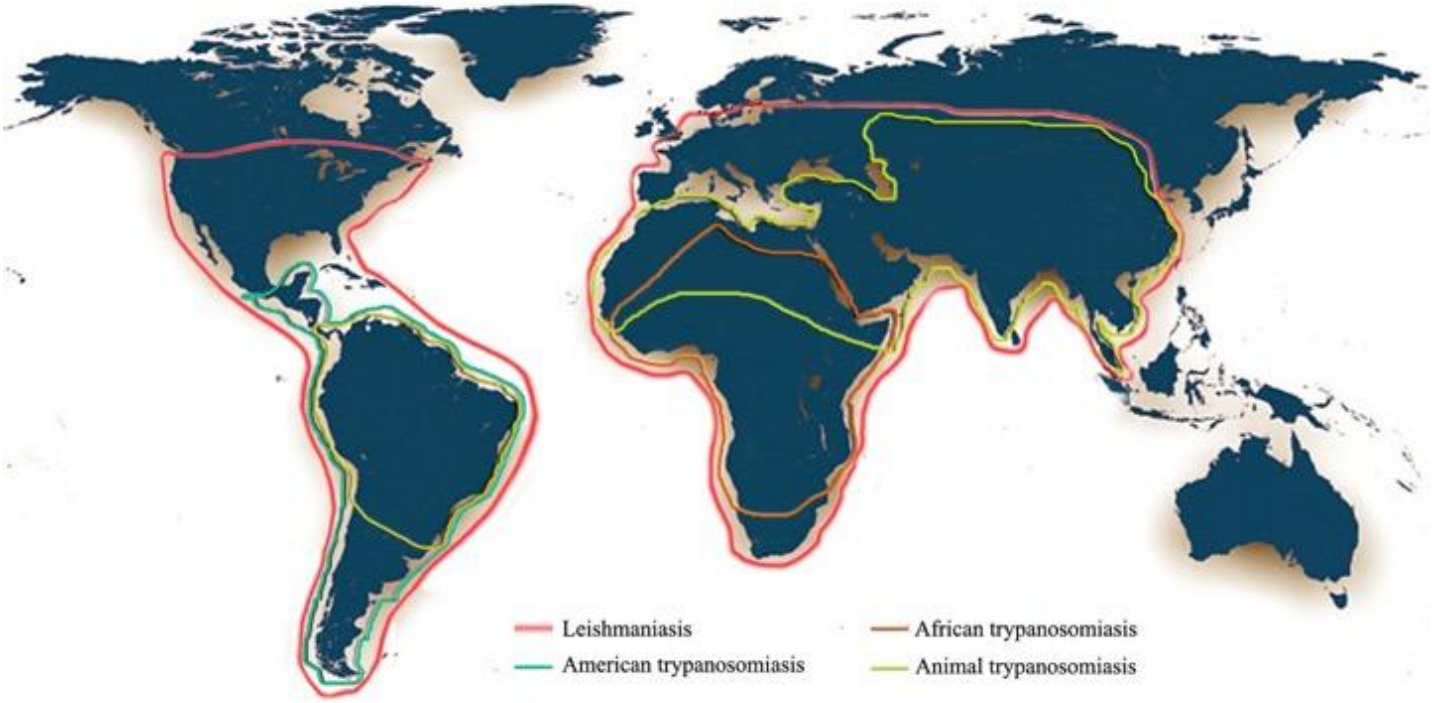

Figure 3. Worldwide distribution of African trypanosomiasis (Human African Trypanosomiasis), American Trypanosomiasis (Chagas Disease), Animal Trypanosomiasis, and Leishmaniasis. The map was drawn using adobe photoshop version 5.

2. Diagnosis and detection of Trypanosomatid's infections

The diagnosis relies on the detection of parasites (parasitological methods), Nucleic acid (Molecular methods), antibody or antigens (Immunological methods) (Figure 4).

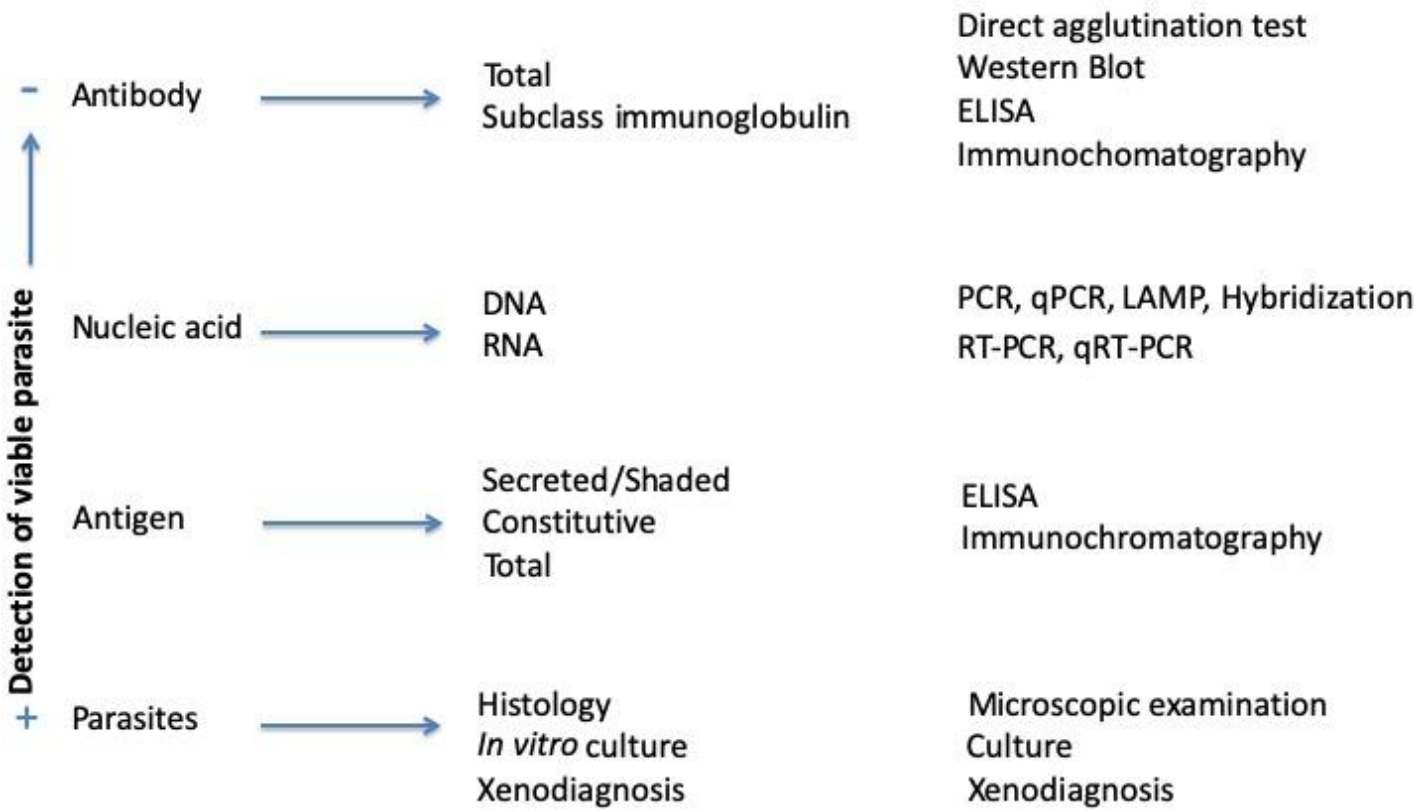

Figure 4. Overview of the methodologies in use to detect infections caused by trypanosomatid parasites.PCR: polymerase chain reaction. qPCR: quantitative polymerase chain reaction. RT-PCR: Reverse transcript polymerase chain reaction. qRT-PCR quantitative reveverse transcript polymerase chain reaction. LAMP: loop-mediated isothermal amplification. ELISA: enzyme-linked immunosorbent assay.

Microscopic examination of biopsy represents the simplest methodological approach to diagnose infection and detect pathogens. The hard identification at the species/subspecies level and its low sensitivity are limitations. Like microscopic examination, the *in vitro* parasites cultivation presents the advantage of being relatively simple to perform but has low sensitivity and requires sophisticated laboratory equipments. Xenodiagnosis is more complex than the other parasitological approaches but do not require biological sampling. Molecular methods involved polymerase chain reaction (PCR) or isothermal amplification of the genetic material. PCR is relatively simple to perform and to visualize. Refinements in PCR technologies included the development of nested PCR and of multiplexed PCR methodologies that have increased sensitivity and discriminative capacity of the test. Other refinement in the detection of the amplified product includes PCR-ELISA (Enzyme-Linked Immunosorbent Assay). PCR-RFLP (Restriction fragment Lenght Polymorphism) allows detecting variation between DNA fragments patterns, generated by restriction enzyme digestion caused by alternative nucleotides at the restriction sites that

can be used for *Leishmania* and trypanosome species discrimination. PCR-HRM (High Resolution Melting) detects dsDNA alternatives by ascertaining changes in the fluorescence intensity, of a DNA-intercalating dye, during the dissociation process of double-stranded DNA (dsDNA) to single-stranded DNA (ssDNA). It was applied with success to *Leishmania* and *T. cruzi* detection, and species and DTU delineation [10][11]. Oligochromatography-PCR (OC-PCR) provides a simple and rapid format for detection of PCR or nucleic acid sequence-based amplification (NASBA) products, visualized on a dipstick by hybridization with a gold-conjugated probe. This detection format takes only 5–10 min and requires no equipment other than a water bath and a pipette [12][13]. Loop-mediated isothermal amplification (LAMP) uses the strand displacement activity of a DNA polymerase to amplify the dsDNA target with four primers designed to recognize six distinct regions. Amplification is completed in a single step at an isothermal temperature [14]. LAMP can be more sensitive than conventional PCR for the detection of *Leishmania* and *Trypanosoma* species [15][16]. The Dermal diagnostic tests or Leishmanin skin test (LST)/Montenegro test is based on the delayed type hypersensitivity (DTH) reactions raised following intradermal injection of killed *Leishmania* promastigotes into the skin forearm. It does not require biological sampling. Indirect immunofluorescence (IFAT) relies on the use parasite layered on a fluorescent glass slide that is used to test the presence of anti-parasites antibodies in the patient serum. This methodology was assayed for the serodiagnosis of Chagas disease, sleeping sickness, leishmaniasis and animal trypanosomiasis [17][18][19][20]. IFAT methodology is often used for surveillance program. Western blot allows to visualize antigens targeted during antibody response. It presents the advantage of being more sensitive and specific than ELISA (see below). The direct agglutination test (DAT), further modified for detection of the agglutination activity on a card (CATT), allows the visualization of the precipitin activity. It uses whole micro-organisms as a mean of looking for serum antibodies. CATT is a commonly serological test for HAT and is still in use for AAT serodiagnosis [21][22]. The agglutination methodology can also be performed with antibody coated latex beads to trap antigen. KAtex, a commercialized latex agglutination test, is developed for the diagnosis of visceral leishmaniasis and use a specific *Leishmania* antibody coated on latex particles [23]. Enzyme linked immunoabsorbent assays (ELISA) can be performed to detect and quantify antibodies or antigens in samples. Alternatively, sandwich ELISA can be used to detect circulating parasite's antigens, that informs on the ongoing infectious process. Immunochromatography (ICT) or lateral flow test is based on a series of capillary beds that has the capacity to transport fluid spontaneously. The analyte is deposited on the dipstick and then spontaneously migrates to the first element that acts as a sponge to hold an excess of sample fluid. Once soaked, the fluid migrates to the second element in which antibody or antigen is present in conjunction with colored particles. The analyte migrates to the third component of the test, on which antibodies are immobilized to stop the flow. The methodologies used for typing detect trypanosomatidae parasites and diagnosing infections are summarized in the Table 1.

Methodologies	Quantification	Culture	CD detection	AT detection	HAT detection	*Leishmania* detection

					& identification	& identification	& identification	& identification
		PCR/qPCR /Multiplex	yes	no	yes/**DTU**	yes/**sp**	yes/**sp**	yes/**sp**
		PCR-OC	no	no	NA/**NA**	yes/**sp**	yes/**sp**	yes/**sp**
		PCR-ELISA	yes	no	NA/**NA**	yes/**sp**	yes/**sp**	yes/**sp**
	PCR	PCR-HRM	no	no	yes/**DTU**	NA/**NA**	NA/**NA**	yes/**sp**
DNA/RNA-based Methods		PCR-RFLP	no	no	yes/**DTU**	yes/**sp**	yes/**sp**	yes/**sp**
		PCR-sequencing	no	no	yes/**DTU**	yes/**sp**	yes/**sp**	yes/**sp**
		PFGE	no	yes	yes/**NA**	yes/**sp**	yes/**sp**	no/**sp**
	Other	NASBA	no	no	NA/**NA**	NA/**NA**	yes/**sp**	yes/**sp**
		LAMP	poss	no	yes/**no**	yes/**sp**	yes/**sp**	yes/**sp**
Non DNA-based Methods	Parasitology	Microscopic examination	yes	no	yes/**no**	yes/**no**	yes/**no**	yes/**no**
		In vitro parasite culture	no	yes	yes/**no**	yes/**no**	yes/**no**	yes/**no**
		Isolation in experimental animals	no	no	yes/**no**	NA/**NA**	yes/**no**	yes/**no**
		Xenodiagnosis	no	no	yes/**no**	NA/**NA**	yes/**no**	yes/**no**

	Dermal diagnostic tests	no	no	NA/**NA**	NA/**NA**	NA/**NA**	yes/**no**
Immunology/Serology	ELISA Ab	no	no	yes/**no**	yes/**no**	yes/**no**	yes/**no**
	ELISA Ag	no	no	yes/**no**	yes/**no**	NA/NA	yes/**no**
	IFAT	no	no	yes/**no**	yes/no	yes/no	yes/**gen**
	ICT Ag	no	no	NA/NA	NA/NA	NA/NA	yes/g**en**
	ICT Ab	no	no	NA/NA	NA/NA	yes/no	yes/**no**
	DAT/CATT	no	no	yes/yes	yes/**yes**	yes/**yes**	yes/**yes**
	Western blot	no	no	yes/no	NA/NA	NA/NA	yes/**sp**
Protein-based methods	MLEE	no	yes	no/DTU	no/sp	no/sp	no/**sp**
	MALDI-TOF	no	yes	no/**DTU**	NA/**NA**	no/**sp**	no/**sp**

Table 1. Methodologies to diagnose Chagas disease (CD), animal trypanosomiases (AT), human African trypanosomiasis (HAT), and leishmaniosis and/or to detect their respective causative agents.

gen: genera. **sp**: species. **DTU**: discrete typing unit. **NA**: not available. **DAT**: direct agglutination test. **CATT**: card agglutination test for trypanosomiasis. **MLEE**: multilocus enzymatic electrophoresis. **MALDI-TOF**: matrix-assisted laser desorption ionization-time of flight. **ICT**: immunochromatographic test. **ELISA**: enzyme-linked immunosorbent assay. **PCR-OC**: polymerase chain reaction with oligochromatography. **LAMP**: loop-mediated isothermal

amplification. **Ab**: Antibody. **Ag**: Antigen. **PFGE**: pulse field gel electrophoresis. **NASBA**: nucleic acid sequence-based amplification. **PCR**: polymerase chain reaction. **HRM**: high melting resolution. **RFLP**: restriction fragment length polymorphism.

3. Systematic review of Non-invasive Sampling Strategies for the Diagnosis and Detection of Trypanosomatid Pathogens and Infections

The selection of the appropriate biopsy for diagnoses relates to the physiopathology of the diseases, reflecting the disseminative capacity (tissue or organ tropism) of these pathogens within its host. Therefore, the diagnosis of these diseases requires the sampling of body fluids (blood, lymph, peritoneal fluid, cerebrospinal fluid, etc.) or organ biopsies (bone marrow, spleen, etc.), which are mostly obtained through invasive methods. Alternative biological samples, such as body secretions (milk, saliva, urine, semen, nasal secretion, lacrimal fluid, earwax, sweat, feces, etc.) or appendages (nail, hair, bristles, etc.) that are constantly produced, might be an interesting alternative to invasive biopsies. No- invasive biological sampling that do not require trained professional and are easy and safe to collect would render the diagnosis more convenient. We address the interest of such biological material, via a systematic review of the published literature and meta-analysis on data extracted from a defined pool of published paper. A schematic overview is given in the Figure 4.

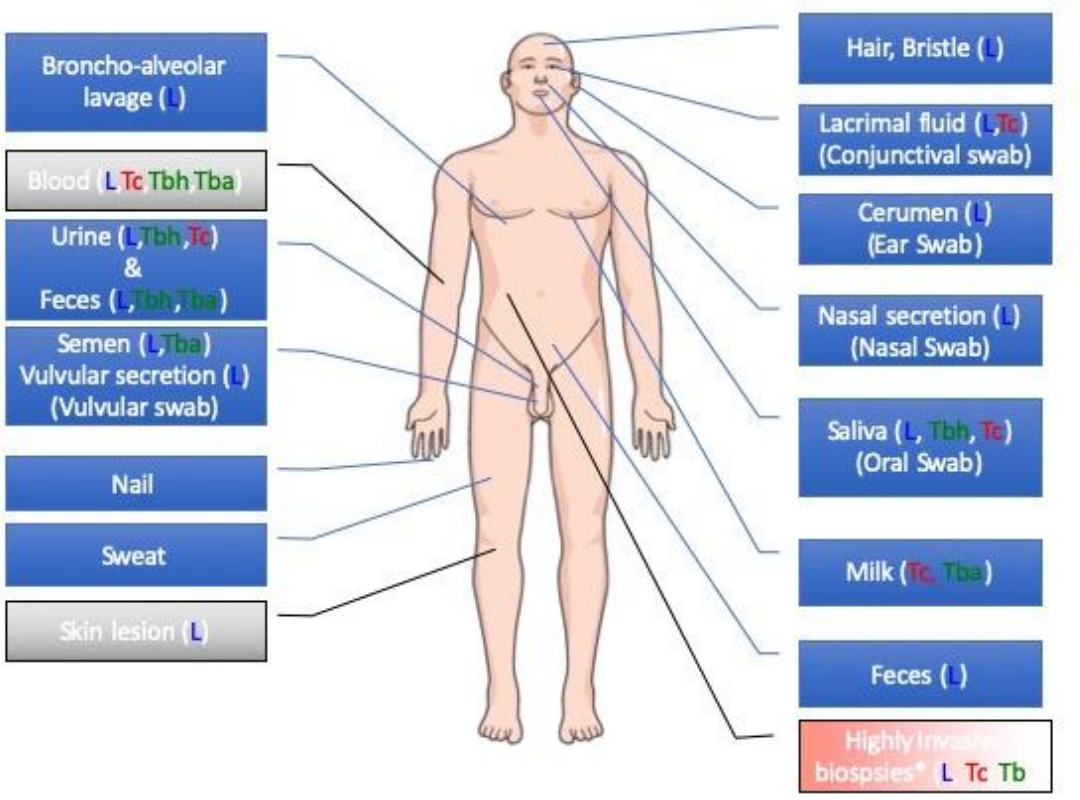

Figure 4: Schematic representation of the compiled evidence on the presence of *Trypanosoma* and *Leishmania* parasites in body secretions or appendages. Tba: *Trypanosoma* species responsible for animal trypanosomiasis; Tbh: *Trypanosoma* species responsible for human African trypanosomiasis; Tc: *Trypanosoma*

cruzi (American trypanosomiasis, Chagas disease); L: *Leishmania* spp. Blue boxes represent material obtained through noninvasive methods, while gray and red boxes represent materials obtained through invasive or highly invasive methods.

3.1. Urine

Urine is an easy-to-collect secretion that is produced daily. Therefore, a large amount of information has been gathered on the presence of trypanosomatid parasites within this liquid.

Human and Animal Leishmaniases. The survival capacity of *Leishmania* in urine is known since 1966 [24]. *In vitro,* urine can promote the growth of the *Leishmania* promastigote and can be used as a low-cost culture adjuvant alternative to serum [25]. The first evidence of the presence of *Leishmania* in the urine of patients infected by *donovani* came in the 1930s through the detection of Leishman-Donovan bodies in the urine of infected patients [26]. The presence of viable *Leishmania* parasites in the urine of infected individuals is documented [27][28][29]. The crossing of the glomerular barrier by *Leishmania* is thought to be a consequence of VL renal lesions and renal failure [30][31]. Tubulointerstitial involvement and glomerulonephritis are the main causative agents of the proteinuria disorder, which is common in most patients with a clinical episode of leishmaniasis [32][33][34]. In infected individuals, urine represents a fluid from which parasite DNA is easily extracted for detection and species identification [35], and has been probed in the urine of patients [28][36][37][38] and in animal reservoirs [39][40]. These searches were performed in VL caused by *L. infantum* [28][36][37][38]; in CL and VL-HIV+ patients infected by *L. martiniquensis* [41]; in CL due to *L. major* or *L. tropica* [38]; in South American cutaneous and mucocutaneous leishmaniasis caused by *L. braziliensis*, *L. guyanensis* or *L. peruviana* and in canine visceral leishmaniasis [37][39][40]. The presence, in urine, of precipitin activities directed against several microorganisms has been known since 1948 [42]. The nature of these activities was formerly attributed to antibodies in 1965 [43]. In 1983, the presence of anti-*Leishmania* antibodies in urine was demonstrated [44][45]. Since then, the anti-*Leishmania* antibody response in patient urine to diagnose VL has been further investigated. ELISA, which uses recombinant antigens or whole antigen preparations as well as the direct agglutination test (DAT), were used to test for disease diagnosis using patient urine [45][46][47][48][49][50][51][52][53]. Immunochromatographic tests to detect rk39 antibodies are currently commercialized and have been thoroughly tested in urine [51][54][55][56][57]. Antibodies present in urine directed against rKP42, a kinesin-related protein and a homolog of rK39, also showed remarkable sensitivity and specificity for VL [58]. This specificity and sensitivity were comparable to those obtained with ELISA performed using acetone-treated *L. donovani* promastigote antigens or DAT. The detection of the antibody response against *Leishmania* infection, due to *L. major*, *L. tropica* or *L. infantum*, was also investigated using Western blot [38][59]. IgA or IgG are detected in the urine of dogs suffering from, leishmaniasis [60], where antibodies directed against *L. infantum* are present [61][62][63]. Because of the persistence of antibodies after cure, these tests cannot be used to diagnose VL in people with a past history of VL. A search for *Leishmania* proteins in urine has therefore been undertaken [44,59,64-66], as well as for changes in the urinary proteome of infected individuals [67]. In *L. infantum*-infected patients, iron superoxide dismutase, *L. infantum* tryparedoxin, and *L. infantum* nuclear transport factor 2 (Li-ntf2) were identified by mass spectrometry analysis [65,66]. When used in a multiplex ELISA test, these biomarkers show a sensitivity superior to 80% for VL diagnosis caused by *L. infantum* but fail to accurately diagnose VL due to *L. donovani* [65,66]. In *L.*

donovani-infected patients, two biomarkers showing a sensitivity of approximately 82% were characterized [68]. A low-molecular-mass heat-stable leishmanial carbohydrate antigen has allowed the development of a latex agglutination test (KAtex) to be commercialized [69]. Its efficiency was thoroughly tested in various VL endemic areas [23,54,70-76]. The KAtex test in urine might be useful for the detection of VL within the clinical case definition: fever for more than two weeks, splenomegaly and no previous history of VL [77].

Human African Trypanosomiasis & Animal Trypanosomiases. No information on the presence of DNA, antibodies or antigens in the urine of human individuals affected by sleeping sickness was collected during the systematic review. The sole evidence on the presence of genetic material in urine comes from an experimental infection of vervet monkeys by *brucei.* In this model*, Trypanosoma*a DNA could be amplified from urine, with LAMP as early as 17 days postinfection [78]. Biochemical changes associated with trypanosome infection are published on animal models infected by various *Trypanosoma* species. Rabbits infected by parasites of the *T. brucei* subgroup showed a progressive increase in proteins released in the urine [79,80]. The presence of fibrinogen and fibrin degradation products in the urine of rabbits infected by *T. brucei* is suggestive of a glomerular permeability change[79]. Mice or *Microtus montanus* infected by *T. b. gambiense* showed an increase in the excretion of aromatic amino acid catabolites [81-84]. In mice infected with *T. evansi,* the concentration in phenylpyruvic acid, 4-hydroxyphenylpyruvic acid, and indole-3-pyruvic acid correlates with parasitemia and returns to normal following suramin treatment [85]. These metabolites are also detected in dogs and donkeys experimentally infected [85]. The high rate of aromatic amino acid catabolism by African trypanosomes was associated with the large decrease in free serum levels of aromatic amino acids and with alterations in host tyrosine and phenylalanine metabolism. These events correlate with the pathology of sleeping sickness and the depletion reported in certain amino acids (tryptophan), which would lead to the depletion of essential metabolites such as serotonin and the toxicity of end products such as phenylpyruvate reviewed in [86]. Changes in the urinary proteome of patients suffering from sleeping sickness were observed, notably, in proteins related to several infectious processes. These changes can be the rationale for developing noninvasive tools aimed at tracking the disease stage [87]. In animal models, *T. brucei* parasites are observed in the kidney glomeruli of infected rats, and *T. lewisi* in the kidney capillaries [88,89]. *T. musculi,* a parasite specific to mice, resides in the blood and lacks intracellular stages. After immune clearance of the flagellates from the general circulation, mice became resistant to reinfection. However, long after parasites are no longer detected in the peripheral blood, they still persist in the vasa recta of the kidneys in a peculiar biological stage [90], releasing molecular determinants in the urine as potential diagnostic biomarkers.

Chagas Disease. Evidence on the capacity of *cruzi* to survive in urine came along with those on *Leishmania* [24], and *T. cruzi* amastigotes have been occasionally detected in the kidney [91]. Parasite DNA was detected in the urine of experimentally infected pigs (*Sus scrofa*) or mice [92-94]. The crossing of *T. cruzi* to urine in experimentally infected mice is apparently independent of renal injuries [94]. The presence of DNA in urine is associated with the presence of parasite DNA in blood and heart and with a high level of parasite DNA in blood, but not with the presence of parasites in kidney or kidney injury [94]. The detection of antigens within the urine of patients suffering from acute or chronic CD [95-97] has opened up some new innovative approaches for diagnosis. The presence of these urinary antigens is generally associated with active or recent infections. A number of *T. cruzi* urinary antigens can be identified and classified according to their molecular weight, such as the 80 kDa iron binding protein or the

150-160 kDa antigen. These antigens were detected by the use of antibodies raised against an immunodominant epitope of *T. cruzi*. In addition, parasite tubulin was also detected in urine as well as a set of immunoreactive antigens [98-100]. To develop a diagnostic test based on a capture ELISA system, a panel of polyclonal antibodies was produced against membrane antigens or trypomastigote excreted/secreted antigens. The test performed on urine from patients positive for ELISA capture against sera demonstrated a 100% positivity [101]. Antigens are present in urine at low concentrations and are susceptible to degradation after collection. These characteristics limit the sensitivity and the reliability of all urinary-based antigen detection. The use of nanoporous hydrogel particles produced with poly(N-isopropylacrylamide) (poly(NIPAm)) and N,N9-methylenebisacrylamide (BAAm) coupled to chemical baits via amidation reaction has the potential to concentrate and preserve the antigens [102] for its application using urine [103]. The test, called Chunap (Chagas urine nanoparticle test), has been further developed and evaluated for congenital transmission of *T. cruzi*. In this condition, it showed more than 90% sensitivity and more than 95% specificity [104]. It also demonstrated good sensitivity in HIV-*T. cruzi* coinfected cases [105].

3.2. Feces

Human and Animal Leishmaniases. Most of the references that document the findings of *Leishmania* in human feces were published during the 1920s and l930s [106,107]. The detection of *Leishmania* amastigotes and its DNA in the feces of a dog infected by *infantum* is documented [108]. More recently, a screening of wild gorilla fecal samples revealed the presence of promastigotes and amastigotes of *L. major* within these samples [109]. Nevertheless, this finding has been a matter of debate [110,111]. More recently, a large diversity of trypanosomatid parasites in the feces of great apes, but no *Leishmania* DNA, was evidenced [112]. Since the 1920s, at the time Donovan bodies were detected in human feces, no additional information on the detection of parasites or the DNA of *Leishmania* in human feces has been published. The only other clues on the presence of *Leishmania* DNA in the human gut come from studies performed on pre-Columbian mummies using next-generation sequencing. These analyses highlight the presence of DNA related to *Leishmania* and *T. cruzi*, without being able to firmly identify *Leishmania* at the species level [113,114].

Human African Trypanosomiasis & Animal Trypanosomiases. The ITS1 region of *b. brucei, T. b. gambiense*, *T. b. rhodesiense* and *T. b. evansi* was successfully amplified from DNA isolated from fecal samples of experimentally infected mice [115] and *T. b. rhodesiense* and/or *T. b. gambiense* DNA was detected in the feces of wild gorillas [112,115].

Chagas Disease. Megacolon is a pathological affliction that occurs in chagasic patients [116]. Evidence of the presence of *cruzi* DNA in the gut of pre-Columbian mummies is documented, depicting that the disease has a long evolutionary history with humans in South America [113,114,117,118]. The tissue tropism of various *T. cruzi* isolates was investigated in a mouse model of infection. In these experiments, parasite DNA was detected in the small intestine and rectum of the animals [119,120]. In infected mice, the gut is the primary site of parasite persistence in the BALB/c model of chronic Chagas disease and is associated with a perturbation in the gut microbiome [121,122]. In opossums (*Didelphis marsupialis*), one of the multiple wild reservoirs of *T. cruzi*, the

developmental cycle that usually occurs in the intestine of the triatomine vector can take place in the anal odoriferous glands [123]. In human feces, to our knowledge, no information is currently published.

3.3. Saliva/Oral Swab/Sputum

Oral swab, saliva, and sputum are the easiest and least-invasive sampling methodologies for the detection of infectious pathogens. Although bronchoalveolar lavage is not considered a noninvasive method to collect biological samples, it does not cause damage to tissues.

Human and animal leishmaniases. The presence of viable *Leishmania* parasites in the saliva of infected patients was demonstrated in 1934 by Forkner [124]. More recently, *braziliensis* was recovered from the saliva of a person suffering from cutaneous leishmaniasis [125]. A large number of studies describe the successful detection and identification of *Leishmania* DNA in saliva or oral swabs, with PCR or other methodologies of DNA amplification (LAMP). The DNA was amplified in *L. martiniquensis*-HIV positive and negative patients [41,126-129] but also in kala-azar patients infected by *L. donovani* [73,130] and in dogs suffering from CVL [131,132]. In 1994, a report discussed the presence of agglutinating anti-*Leishmania* activity in the saliva of kala-azar patients [133]. The capacity of anti- *Leishmania* antibodies present in the saliva to be used to diagnose CVL and VL was investigated more recently. For CVL, the detection of IgG2 and IgA antibodies targeting specific recombinant K39 protein (rK39) in saliva demonstrated the usefulness of this test to diagnose CVL and to differentiate between seropositive and seronegative dogs [134]. In humans, a preliminary experiment involving the detection of rK39 antibodies demonstrated 99.2% sensitivity and 100% specificity for *Leishmania* diagnosis using patient sputum [56]. Interestingly, KAtex shows a higher sensitivity to diagnose Mediterranean visceral leishmaniasis with oral fluid than with urine, even if this test was originally conceived to be used with urine [36].

Human African Trypanosomiasis & Animal Trypanosomiases. Trypanosome-specific IgG can be detected in the saliva of *b. gambiense*-infected HAT patients using ELISA. Nevertheless, the antibody concentration is at least 250-fold lower in saliva than in serum [135]. The ELISA performed on the saliva of a cohort of 208 individuals, including 78 parasitologically confirmed patients, demonstrated a robust sensitivity and specificity (>90%) comparable with CATT performed on sera [136]. Since then, no additional experiments have been performed.

Chagas Disease. The first evidence on the presence of *cruzi* in the saliva of experimentally infected dogs dates from 1966 [137,138]. More recently, an ELISA that detected and quantified the IgG response to *T. cruzi* was developed using saliva from infected patients. The methodology was tested with success on saliva from patients with chronic infection, which is characterized by the absence of blood circulating parasites [139,140]. The oral swab was also tested to detect fragments of *Trypanosoma* DNA (*Trypanosoma dionisii*, *T. rangeli* and *T. cruzi*) to evaluate the potential reservoirs for *T. cruzi* in gallery forest bats [141].

3.4. Conjunctival Swab/Lacrimal Fluid/Occular

A swab is a small piece of soft material used for taking a small amount of substance from a body. The conjunctival or corneal swab, a routine practice to perform biological sampling to diagnose eye infection, has been applied to

detect trypanosomatid pathogens.

Human and animal leishmaniases. In humans, ocular lesions are usually associated with systemic signs [142-144]. Ocular pathologies are documented in patients suffering from cutaneous [145-150], diffuse cutaneous [151] or post-kala-azar leishmaniasis [142] and in VL [152,153]. In dogs, keratoconjunctivitis and kerato-uveitis are described as the most usual symptoms, occurring in 16–80% of affected dogs [154,155]; keratoconjunctivitis is also observed in feline leishmaniasis [156]. *Leishmania* has been isolated from the aqueous humor of a patient suffering from leishmaniasis [143]. In naturally infected dogs, anti-*Leishmania* IgG was detected in the aqueous humor, although at a level not related to the serum level of IgG [157,158]. In dogs, histopathological investigations depicted the presence of plasmatic cells and macrophages bearing amastigote forms of *Leishmania*, in the ciliary body, sclerocorneal limbus, iris, and lacrimal duct but also in smooth and striated muscles [158-161]. *Leishmania* were observed in squamous carcinoma cells from conjunctival swab samples in an HIV+ patient [162]. *Leishmania* DNA can be detected and quantified by qPCR in the lacrimal glands of symptomatic dogs [163]. All these clues have prompted testing the efficiency of the conjunctival swab for CVL diagnosis [164-167] and tracking asymptomatic dog infections [168] but also for diagnosing feline leishmaniasis [167,169,170]. In addition, the detection of *Leishmania* DNA in conjunctival swabs has also been applied to track *infantum* wild reservoirs [171,172].

Human African Trypanosomiasis & Animal Trypanosomiases. In humans, eye pathologies associated with trypanosome infections remain unusual [173], and an investigation for the presence of parasites, DNA or antibodies within conjunctival swabs has not been performed. In dogs infected by *b. brucei*, the eyes are one of the most severely affected organs, and infection by *T. evansi* can provoke blindness [174,175]. Experimental infections of cats with *T. brucei* [176] and of cats and goats with *T. evansi* highlight their disseminative capacity in the eye, with their presence being detected in the aqueous humor [177,178].

Chagas Disease. In 1935, Romana first described the “unilateral schyzotrypanosomic conjunctivitis” associated with acute *cruzi* infection later known as Romana’s sign [179]. The invasion of the human host by *T. cruzi* occurs in various ways but mainly via skin lesions or the conjunctival way [180,181]. *T. cruzi* parasites deposited on the conjunctiva, via the manipulation of contaminated bug feces, are drained with tears into the nasolacrimal duct and nasal cavity. Then, *T. cruzi* infects the most proximal tissues lined with cuboidal and columnar epithelial cells [181,182]. Surprisingly, reports on eye pathology in CD patients are very scarce. Recently, the first case of *Trypanosoma cruzi*–associated retinitis was diagnosed [183]. The presence of *T. cruzi* amastigotes in the conjunctiva, corneal stroma, the adjacent ocular muscle and the interstitial macrophages of *Thrichomys apereoides* (Rodentia, Echimyidae) experimentally infected with *T. cruzi* is documented [184].

3.5. Genital Organs: Semen/Vulvular Secretion

Some trypanosomatid infections impact male and female reproductive organs, causing infertility [185]. *Leishmania* infection provokes a decrease in sperm quality, genital lesions, testicular amyloidosis, chronic prostatitis and epididymal inflammation [185]. Chagas disease is associated with male hormonal changes and a loss in sperm

quality due to parasitic load. In females, it is associated with the invasion of the placenta and hormonal changes linked to overproduction of inflammatory cytokines in the oviduct and uterus. In sleeping sickness, an inpairment in the spermatogenic cycle due to damage in the pituitary gland as well as damage to the reproductive organs is reported. In females, impairment in the estrus cycle due to pituitary gland damage is noticed [185].

Human and animal leishmaniases. Leishmaniasis does not belong to the broad list of potential sexually transmitted infections (STIs). Nevertheless, some evidence suggests that venereal transmission of leishmaniasis does occur in dogs and humans [186-189]. In humans, lesions in the male genitalia associated with the presence of parasites are well documented [190-192], with the presence of parasites [193,194]. In dogs, genital lesions associated with visceral leishmaniasis and the shedding of *Leishmania* in the semen of naturally or experimentally infected dogs is described and can lead to infertility [195,196]. In the prepuce and glans of male symptomatic dogs, heavy parasite burden has been detected and is associated with inflammation, testicular degeneration, atrophy, an absence of spermatogenesis, and necrosis [197]. In these dogs, immunohistochromatography showed that 75% of symptomatic dogs and 35% of asymptomatic dogs were positive for *Leishmania* in the testis. These percentages rose to 95% and 60% for symptomatic and asymptomatic leishmaniasis, respectively, in the epididymal duct. The detection of *Leishmania* parasites in semen has been evidenced through parasite culture [29], microscopic observation or immunohistology [195,197], and polymerase chain reaction [195,196,198]. A CVL experimental infection of 8 female dogs pinpoints that vulvar swab is at least as sensitive as oral swab for the detection and quantification of *Leishmania* kDNA, and this methodology is proposed to confirm *Leishmania* infection in seropositive dogs [199]. The presence of *L. infantum* amastigotes in the genital tract of naturally infected bitches is documented [200].

Human African Trypanosomiasis & Animal Trypanosomiases. In humans suffering from sleeping sickness, sterility or infertility, menstrual disorder, a loss of libido, impotence, and amenorrhea are reported [201]. Testicular damages and clinical manifestations are described [202], and sexual transmission is very occasionally observed [203]. *Trypanosoma equiperdum,* responsible for dourine, is a sexually transmitted disease of Equidae [204-206]. A loss of fertility is observed in infected animals and is associated with the detection of parasites in semen [207,208]. For *Trypanosoma vivax,* in addition to tsetse flies, transmission routes include transplacental and sexual routes, and parasites were detected in the semen of infected animals [203,209]. In naturally acquired or experimentally induced animal trypanosomiasis caused by *brucei* or *T. congolense*, a decrease in semen production associated with an alteration in spermatogenesis is recorded [210-214]. Histological lesions characterized by testicular degeneration, epididymitis and epididymal epithelial hyperplasia were detected in the same animals and suggested the participation of the parasite in the pathogenic mechanism of reproductive damage, frequently reported in infected animals [209,211,215]. In experimentally-infected mice, bioluminescent imaging confirmed the localization of viable trypanosomes in infected mice [216] with an accumulation in the epididymal adipose tissue and in the epididymis [217].

Chagas Disease. Sexual and transplacental transmission have epidemiological relevance [218,219] and the possibility of sexual transmission of *cruzi* has been discussed since the discovery of Chagas disease. In 1911, Vianna described testis lesions in experimentally *T. cruzi*-infected guinea pigs [219]. Human orchitis was described

in 1916 [220]. In human, evidence on the infection of the testis by *T. cruzi* during the acute phase of the disease dates from 1982 [221]. The first conclusive evidence of sexual transmission in a model has only been published in 2016 [222]. Previous observations of *T. cruzi* colonization in several tissues of the urogenital system in infected mice as well as the presence of parasites mixed with spermatozoa suggested that *T. cruzi* could potentially be transmitted via the sexual route [223,224]. Moreover, successful infections of healthy mice were obtained by intravaginal instillation of blood trypomastigote forms of *T. cruzi* and also by similar instillation in the penis *[225]*. A low probability of sexual transmission by crossbreeding of immunosuppressive females with acutely infected males was evidenced in a murine model [226]. *T. cruzi* sexual transmission in mice was successfully obtained (100% mating) through mating between chronically infected males and naive females or infected females and naive males; the transmission was corroborated by both serological and molecular techniques in all cases. Few data exist in humans, but the presence of *T. cruzi* was reported in seminiferous tubes and ovarian cells of children who succumbed to Chagas disease, or in menstrual blood of infected patients [227,228]. Also, *T. cruzi* amastigote forms were observed in epithelial cells of cervical tissues in a reactivation case of *T. cruzi* infection in a young immunocompromised patient [229].

3.6. Milk

The presence of parasites of the Trypanosomatidae family in milk has been probed in view of a maternal transmission risk and food contamination.

Human and animal leishmaniases. Attempts to test the capacity of *Leishmania* to survive and proliferate in milk were undertaken as early as the 1930s; some evidence on the adequacy of this medium to support *Leishmania* survival was published [230]. Histopathological investigation of female dogs suffering from CVL probed the presence of *Leishmania* amastigotes in the mammary glands [197]. Nevertheless, the presence of *Leishmania* in milk has not yet been reported in patients suffering from leishmaniasis.

Human African Trypanosomiasis & Animal Trypanosomiases. The investigation of trypanosomes in milk has a long history of research and has first focused on the risk of the transmission of pathogens, mainly the risk of transmission of *evansi*. Evidence on the presence of *T. evansi* in the milk of lactating cow comes from the work of Zwick and Fisher and described by Henry and Guilhon in 1944 [231]. During the 1910-1930 period, a set of experimental procedures was employed to detect the presence of various species of *Trypanosoma* species in the milk of experimentally or naturally infected animals [231]. Nathan-Larrier reported that mice and rats experimentally infected by *T. equiperdum* show trypanosomes in their milk [232].

Chagas Disease. Because *cruzi*, originally named *Schyzotrypanum cruzi*, possesses the capacity to cross the epithelium and to infect via the oral route [231,233,234], the presence of this pathogen in the milk has been searched. *T. cruzi* was found in the milk of experimentally infected mice [232,235,236], and several reports describe the presence of this pathogen in the milk from pregnant women [237-240], as reviewed by Norman and Lopez-Vélez [241]. In most cases, the presence of *T. cruzi* in the milk of pregnant women has been attributed to contamination by infected blood due to nipple bleeding [241]. Nevertheless, the capacity of *T. cruzi* to invade the

mammary gland of infected females has been undertaken and These histological investigations on mice demonstrate the presence of *T. cruzi* amastigotes in the mammary gland alveoli, excretory ducts, the connective tissue envelope of the ducts, inter- and intralobular connective tissue, histiocytes, adipose tissue, the sebaceous glands of the nipple, striated muscle fibers beneath the nipple, and inside the duct lumen [242]. Such proximity of *T. cruzi* parasites with colostrum or milk argues for the inactivation of *T. cruzi* by pasteurization or microwave treatment [243,244].

3.7. Nasal Secretion

Human and animal leishmaniases. The presence of *donovani* parasites has been detected in the nasal secretions of patients as early as 1936 and reconfirmed sixty years later [26,124]. Parasite DNA can efficiently be detected in this secretion [132]. Parasite DNA has also been detected in the clinically unaffected nasal mucosa of patients infected by *L. braziliensis* [245]. Among the clinical presentation of human leishmaniasis, mucocutaneous alterations are described. They are mainly present in South America and are caused by a restricted number of *Leishmania* species [4]. Nevertheless, this uncommon presentation is also reported to be caused by some Old World species [246], suggesting that nasal secretion deserves further investigation to be confirmed as a positive fluid for *Leishmania* detection.

Human African Trypanosomiasis & Animal Trypanosomiases. No information gathered during the study.

Chagas Disease. No information gathered during the study.

3.8. Ear Swab/Cerumen

Human and animal leishmaniases. *Leishmania* DNA has been detected and quantified in the cerumen of infected dogs [247]. A recent publication demonstrates that cerumen-qPCR expresses the highest sensitivity (87.5%) to detect genetic materials, followed by hair (lesions: 78.57%, healthy skin: 62.5%), and blood (68.75%) [248]. The ear skin of infected dogs bears a high parasite load compared to other corporal zones and tends to be more infective to sand flies than that of the abdomen [249]. The usefulness of ear swab was investigated in CVL-positive dogs, and a positivity of 43% was recorded [132]. In addition, ear lesions caused by *mexicana* (Chiclero's ulcer) are known, but the lesions at this site can be caused by other *Leishmania* species [250].

Human African Trypanosomiasis & Animal Trypanosomiases. No information gathered during the study.

Chagas Disease. No information gathered during the study.

3.9. Hair/Bristles

Appendages such as hair, bristles, and nails are not referenced as target tissues for trypanosomatid survival and proliferation. Therefore, only a few studies were performed using these materials for the investigation of trypanosomatid infection.

Human and animal leishmaniases. The first series of analyses was performed on dog hair by searching for markers of infection via the analysis of volatile organic compounds. This approach is based on the hypothesis that illnesses can modify odors exhaled by individuals [251] and that canine *Leishmania* infection involves the liberation of some volatile compounds specific to the infection [252,253]. Therefore, with this methodology, it is not the infectious agent that is detected, nor the immunologic response but the set of volatile compounds exhaled by the dogs. Although hair is not known as a target tissue for *Leishmania*, an investigation of *Leishmania* DNA was undertaken on CVL in a mouse experimental model of infection with *major* but also in the hair of wild mammals or Leporidae [252,254,255]. In mice, the DNA of *L. major* is detected near the inoculation site but also in hair collected in body areas far from the infection site [255]. The first evidence on the usefulness of PCR performed on hair to act as a biomarker of infectiousness of the host came from CanL cases [256]. The rationale for such an accumulation of parasite DNA into the hair of the infected animal is not entirely understood. The hypothesis of a « transdermal elimination » process has been raised. This process, observed as a secondary component of primary skin diseases, includes the elimination of endogenous substances but also exogenous infectious organisms, such as *Mycobacterium tuberculosis* or HIV [257]. It requires the direct incorporation of the parasite DNA among skin and hair keratinocytes at the site of inoculation. The intracellular infection of keratinocytes with *Leishmania* has not been detected following the infection of C57Black/6j mice [258], but the presence of *Leishmania* amastigotes has been observed in the hair follicles of patients with cutaneous leishmaniasis [257].

Human African Trypanosomiasis & Animal Trypanosomiases. No information gathered during the study.

Chagas Disease. No information gathered during the study.

4. Conclusions

This literature analysis reveals striking facts and gaps in the usefulness of body fluid secretions and/or appendages for the diagnosis of infection caused by Trypanosomatidae parasites. Nevertheless, diagnostic protocols that use new non-invasive biological samples should be of help to track disease in endemic areas with limited resources but they should also be of help to track diseases evolution and clinical and/or chemotherapy success.

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