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Review

Novel Methods for Diagnosis Of Blood-Borne Protozoa

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ABSTRACT

The majority of parasitic infections are generally reported in tropical and subtropical climate regions but affect developed countries due to the increase in migration and international travel. They may cause growth and development retardation in children, and labor and power loss in adults. Thus, rapid and reliable diagnosis is the key step for early treatment. In recent years, new and rapid diagnostic methods, such as serologic testing, rapid antigen tests, new nucleic acid amplification tests, and proteomic methods, for diagnosis of blood parasites have been developed. In this review, methods still used for diagnosis of blood parasites are mentioned briefly, and newly developed or developing methods are discussed.

Keywords: Blood-borne protozoa, diagnosis, methods

INTRODUCTION

Two groups of parasites cause diseases in humans: the protozoan (single-celled parasites) and metazoan (multi-celled parasites). Sporozoans of *Toxoplasma, Plasmodium*, and *Babesia* and the flagellates of *Leishmania* and *Trypanosoma* are blood-borne protozoan infections (1, 2). Although the majority of parasitic infections are described in tropical and subtropical climate regions, developed countries are affected due to migration and international travel (1). Due to blood transfusions and organ transplantation, even those who did not travel to endemic regions may become infected with blood-borne protozoan infections (3). Rapid diagnosis of parasitic infections is important to determine appropriate treatment and prevent death. New techniques and tests used for diagnosis should be simple and rapid and prevent user bias during result interpretation. They also should have higher specificity and sensitivity (1). In our article, the basic methods used for laboratory diagnosis of blood-borne infections caused by protozoan pathogens are evaluated (Table 1), and new studies in the field of diagnosis are investigated.

CLINICAL AND RESEARCH CONSEQUENCES

Malaria

The *Plasmodium* genus infects a wide range of birds, mammals, reptiles, and amphibians via blood-feeding dipteran insects as vectors. Although there are at least 200 named *Plasmodium* species, only five infect humans, *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium vivax*, *Plasmodium knowlesi*, and *Plasmodium ovale* (4).

Classical Microscopy

The classical method for the diagnosis of malaria is the microscopic examination of thin and thick blood smears stained with Giemsa dye. Accurate interpretation varies according to the availability of trained and experienced laboratory technicians, quality of reagents, and light microscopes. The thick film contains two drops of blood that have been lysed on the slide by addition into a hypotonic solution. This releases intracellular parasites and helps examination of up to 30 layers of blood. The thick blood film is more sensitive (20 times) than the thin film, with a reported detection threshold of 10 to 50 parasites/μL of blood, or approximately 0.0002% to 0.001% parasitemia. Because of the high sensitivity, the thick film is ideal for screening and parasite detection. Under field conditions, the estimated sensitivity may be lower (100 to 500 parasites/μl of blood) (4). Microscopic examination of thick and thin blood films has additional benefits for the diagnosis of malaria. Microbiologists can show the presence of species and parasitemia by preparing smears in a short time (<1 h). The asexual forms can be distinguished, and mixed species causing infection can be shown by using this method. Microscopic examination can also provide information regarding the morphology and quantity of blood cells (5).

Other Microscopy Methods

Other less common microscopic methods are used for the identification of malaria parasites in the whole blood, including staining methods for nucleic acid and hemozoin. Acridine orange (AO), a DNA-binding fluorescent dye, excites at 490 nm and produces a yellow or apple-green fluorescence. This method requires a fluorescence microscope or light microscope with an

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Table 1. Diagnosis of blood-borne protozoan infections (1)

Pathogen	Microscopy	Serology	Molecular	Proteomic
Plasmodium spp.	Detection from blood smear	RDTs	LAMP, LAMP card test, real-time PCR	LDMS
Babesia microti	Examination of blood smears to detect parasite in the patients' RBCs	IIF, ELISA, immunoblot	PCR, real-time PCR	
Leishmania spp.	Detection of parasites in aspirates from spleen, bone marrow, or lymph nodes	ELISA, ICT strip test	PCR, NASBA, oligochromatography	
Trypanosoma brucei	Detection of parasite (trypomastigote) in the blood or CSF	CATT, micro-CATT, and LATEX (*T. b. gambiense*)	PCR, LAMP, real-time PCR	SELDI-TOF
Trypanosoma cruzi	Detection of parasite (trypomastigote) in blood smears	IIF, IHA, ELISA, immunoblot, radioimmunoprecipitaton, mix-ELISA, TESA-ELISA	PCR	SELDI, MALDI MS-MS
Toxoplasma gondii	Detection of parasites from blood, CSF, or stained tissue	Sabin-Feldman dye test, IIF, hemagglutination, capture ELISA, ISAGA, avidity ELISA, γinterferon ELISA	PCR	

ELISA: enzyme-linked immunosorbent assay; ICT: immunochromatography; PCR: polymerase chain reaction; NASBA: nucleic acid sequence-based amplification; CSF: cerebral spinal fluid; IIF: indirect immunofluorescence; ISAGA: immunosorbent agglutination assay; CATT: card agglutination test for trypanosomiasis; LAMP: loop-mediated isothermal amplification; LATEX: rapid latex agglutination test; SELDI-TOF: surface-enhanced laser desorption ionization time-of-flight mass spectrometry; MALDI-TOF: matrix-assisted laser desorption ionization time-of-flight mass spectrometry; IHA: indirect hemagglutination; TESA: trypomastigote excreted-secreted antigens; MS-MS: tandem mass spectrometry; RDT: rapid diagnostic test; LDMS: laser desorption mass spectrometry; RBC: red blood cell

adaptor. Some studies found the AO method has similar sensitivity and specificity to traditional Giemsa-stained thick films. It can reliably detect <100 parasites/μL or 0.002% parasitemia and allows for more rapid screening than with traditional Giemsa method. Malaria hemozoin pigment may be detected by dark-field microscopy or in histological tissue sections. Another quantitative buffy coat method requires a fluorescent microscope and has high sensitivity for *P. falciparum* (4).

Serology and Rapid Diagnostic Tests

P. falciparum histidine-rich protein 2 (PfHRP2), which exhibits polymorphism, is widely used as a diagnostic marker. Verma et al. (6) developed monoclonal antibodies (mAbs; b10c1 and Aa3c10) against the 105th amino acid at the C terminal of histidine-rich protein 2 antigens of *P. falciparum*. The sensitivity and specificity of the monoclonal antibodies were 95% and 96%, respectively. These data strongly suggest that the anti-C-terminal PfHRP2 mAbs b10c1 and Aa3c10 have merits for improving the existing malarial diagnostics. Rapid diagnostic tests for malaria include serology-based tests used especially in field studies (7). Rapid diagnostic tests (RDT) started to gain importance for detecting *Plasmodium* species. These tests are used to investigate malarial antigens and generally in forms of dipsticks or immunochromatographic cards (5). These tests have some target antigens. For example, histidine-rich protein 2 is produced only by *P. falciparum*. Plasmodial lactate dehydrogenase may be used to investigate *P. falciparum* or the genus. Aldolase enzyme may detect *Plasmodium* genus. Various RDTs can singly detect *P. falciparum*, *P. vivax*, or pan-malaria species (5). To be placed on the World Health Organization procurement paper, the *P. falciparum* and *P. vivax* test panels must obtain detection scores at least 75% and ≥75% at 200 parasites/μL, respectively; additionally, false positivity should be <5% (8). RDTs are fast, practical, and only need a minimum number of samples, but they are not at par with microscopy due to some disadvantages, such as they do not determine parasitemia, cannot differentiate sexual and asexual stages, and cannot make a specific diagnosis for *P. ovale*, *P. knowlesi*, or *P. malariae* (5). In addition, infections due to *P. falciparum* in South America be efficiently detected because of the lack of common histidine-rich proteins in this species (1). Table 2 shows the advantages and disadvantages of malaria RDTs.

Main Points:

- Rapid tests are expensive, are not the gold standard, and carry the risk of giving false-negative results.
- Molecular tests have improved over time and give promising results.
- There is still a need for new and extensive studies with variable species.

Molecular Methods

Polymerase chain reaction (PCR) is commonly used for nucleic acid amplification and detection of *Plasmodium* parasite DNA, with the 18S small subunit rRNA gene. Most PCR tests are developed in research laboratories. Some commercial PCR tests are available, but none of these were confirmed by the Food

Table 2. Advantages and disadvantages of malaria rapid diagnostic test (1, 4, 5)

Advantages	Disadvantages
Rapid and easy to use	Do not measure parasitemia
Subjective result	Do not distinguish sexual–asexual stages
Supports microscopy	No species–specific diagnosis for P. ovale, P. malariae, and P. knowlesi
Requires minimal patient samples	More expensive than blood smear
Appropriate for field use	Inefficient detection for P. falciparum in South America

and Drug Administration (FDA) (4). Poon et al. (9) developed a loop-mediated isothermal amplification (LAMP) test identifying the *P. falciparum* 18S rRNA gene *in vivo*. Compared with PCR, its sensitivity and specificity were 95% and 99%, respectively. In recent times, the LAMP has been further simplified as a card test. Yamamura et al. (10) used LAMP in combination with DNA filter paper and melting curve analysis to diagnose *P. falciparum*, and they reported that the sensitivity and specificity were 97.8% and 85.7%, respectively, compared with the microscopic method. Lee et al. (11) used a multiplex PCR method able to identify *P. knowlesi*.

Other Diagnostic Laboratory Methods

Rapid diagnosis of malaria is important for infection control. Investigation of the hemozoin pigment of *Plasmodium* via laser desorption mass spectrometry (LDMS) has been evaluated as a sensitive (<10 parasites/μL) method to detect *P. falciparum* species cultured in human blood. In mice, the hemozoin pigment has been detected via LDMS in 0.3 μL of blood within 2 days of infection independently of the inoculating dose of 10^6, 10^4, or 10^2 parasite-infected erythrocytes. Investigators suggested that LDMS for hemozoin may become a faster screening test compared with light microscopy for low-level parasitemia <0.1% (12, 1). Additionally, LDMS was shown as a faster and more sensitive alternative test than microscopy in pregnant women (13, 1). Recently, a device was designed for noninvasive rapid detection of *P. falciparum* in patients with malaria. The principle of the test was based on detecting vapor bubbles around the hemozoin via transdermal optical excitation and acoustic detection. This instrument was suggested as a cheap and practical diagnostic tool that can be useful for clinicians and researchers. However, the test still needs to be developed and evaluated by using multiple cases (14).

Babesiosis

The *Babesia* genus infects wild and domestic animals worldwide via primarily ixodid tick vectors. More than 100 named *Babesia* species exist, but only several are known to regularly infect humans. *Babesia microti* causes the majority of infections in humans in the United States. *Babesia duncani* infections have also been reported here. Most cases of this infection are due to the bite of an infected ixodid tick (4).

Microscopy

Similar to malaria diagnosis, the traditional diagnostic method for babesiosis is classical microscopy using thick and thin blood films the confirmed by Giemsa dye. *Babesia* spp. may present a diagnostic challenge on blood films because of the many morphologic similarities shared with *Plasmodium* spp. (specifically *P. falciparum*) (4). Generally, *Babesia* parasites demonstrate greater pleomorphism in size and shape compared with *P. falciparum* parasites. Ovoid, elliptical, pear, racket, and spindle shapes may commonly be seen. Differentiating various human *Babesia* spp. by their morphological appearance is not possible. Molecular methods are required for species identification of the species (4).

Isolation Procedures

A biotest was performed via inoculation of the patient's blood into the peritoneum of laboratory rodents to confirm the disease. However, using such labor-intensive methods in routine testing is difficult, and it requires a long waiting time to receive the result (2-4 weeks) (15, 16).

Serologic Methods

Commercial serological tests are used, e.g. immunofluorescence assay for immunoglobulin M (IgM) or immunoglobulin G (IgG) antibody versus *Babesia microti* detection to diagnose babesiosis (15, 16). Antibodies against *B. microti* antigens typically appear 2 weeks after the onset of illness. They could be detectable for several years after infection. The recommended diagnostic method is the indirect immune fluorescent assay (IFA), which detects serum antibodies against *B. microti* with a relatively high sensitivity (88%-96%) and specificity (90%-100%) (4). Although enzyme-linked immunosorbent assay (ELISA) and immunoblotting methods are available, these tests are not yet standardized, and they require confirmation with IFA (1).

Molecular Methods

Currently, the PCR is the reference method for the diagnosis of babesiosis (16). PCR is recommended if the pathogen species cannot be identified based on the blood smear or if the diagnosis is unclear, and medical history and clinical symptoms indicate babesiosis infection (15, 16). The sensitivity of PCR for 18S rRNA gene was between 5 and 10 pathogens/μL of blood, corresponding to 0.0001% parasitemia (16). Rozej-Bielicka et al. (17) developed a multiplex PCR method to identify pathogen species, including *Babesia divergens*, *B. microti*, *Babesia venatorum*, and *Babesia canis* in a variety of biologic samples. The researchers reported that the method was practical and cheap for use in screening and for diagnostic purposes. Currently, no FDA-approved *Babesia* PCR assays are available (4).

Leishmaniasis

Leishmania spp. are protozoal members of the family Trypanosomatidae. *Leishmania* spp. cause leishmaniasis, which is a zoonosis of obligate intracellular parasites transmitted to humans by bites from infected female sand flies. Depending on the species, *Leishmania* spp. infection can manifest with different forms, such as cutaneous, diffuse cutaneous, mucocutaneous or visceral disease (18). Various microscopic and cultural methods were developed to detect this parasite (18-20).

Microscopy

Classical microscopy is the gold standard method for diagnosis of visceral leishmaniasis (VL). The specificity of the method is high; however, sensitivity is variable (20). Amastigotes may be recognized by size, shape, staining properties, and the presence of a kinetoplast. After Giemsa staining, the cytoplasm appears bluish, and the kinetoplast and nucleus appears red-purple. Because amastigotes cannot be stained with mucicarmine, periodic acid–Schiff, or silver stain, they can be differentiated from intracellular fungi by using these dyes (18).

Culture

For culture, the samples must be collected aseptically. The tissues should be minced before culture. Schneider's Drosophila medium with 30% fetal bovine serum and Novy, MacNeal, and Nicolle's medium (NNN) can be used as culture media. Culture media, incubated at 25°C, could be checked twice a week for the first 2 weeks and once a week thereafter for up to 1 month before the culture is declared negative. Promastigote stages of the pathogen can be detected microscopically in wet mounts (18). In 2004, a microcapillary diagnostic test based on culture method was developed for cutaneous leishmaniasis (CL). Higher sensitivities and shorter periods for promastigote emergence were reported for this method (19).

Animal Inoculation

For diagnosis of leishmaniasis, animals, such as hamsters can be inoculated with the patient material. For cutaneous and mucocutaneous leishmaniasis, animals should be inoculated intranasally, and for VL, they should be inoculated intraperitoneally. Positive identification may take 2-3 months (18).

Serology

The most hopeful antigen for serologic diagnosis of VL are antigens related to kinesin. An immunochromatographic strip test developed using rK39 antigen can be used for mass screening in endemic regions (21). Magalhães et al. (22) tested three antigen mixtures (poly-histidine-tagged polypeptides) and found it was useful for canine or human VL, among 13 identified through different screenings. This investigation provided similar results with high sensitivity for both canine (88%) and human (84%). In a recent study, the enolase enzyme of *Leishmania braziliensis* was cloned, and rEnolase recombinant protein was tested for serodiagnosis of canine and human VL. Thus, the ELISA test with rEnolase indicated diagnostic sensitivity and specificity of 100% and 98.57% for canine VL, and 100% and 97.87%, respectively, for VL in humans. The search for antibodies against rEnolase was reported to improve the serodiagnosis of VL (23). Coelho et al. (24) evaluated the diagnostic properties of cytochrome c oxidase and IgE-dependent histamine-releasing factor proteins in canine VL and human tegumentary leishmaniasis. ELISA tests using these recombinant proteins showed 100% sensitivity and specificity for serodiagnosis of both infection forms. These proteins showed better diagnostic performance than the *Leishmania* antigen extraction or recombinant A2 protein (24).

Molecular Methods

Because of its high sensitivity and reliability, PCR is the most important molecular diagnostic method for *Leishmania* infections. Some PCR protocols use different targets, such as ribosomal RNA, DNA of kinetoplast, mini-exon RNA or internal transcribed spacer (ITS) (1). In addition, assessment of antimicrobial drug treatment and determination of clinical outcomes can be performed using nucleic acid sequence-based amplification (NASBA) by amplifying the RNA sequences. In combination with oligochromatography, NASBA may be used to monitor the progression from active disease to cure (25). Niazi et al. (26) developed a nano-diagnostic method using a NASBA method and gold nanorods for colorimetric measurement targeting 18S rRNA of *Leishmania* and reported that the sensitivity and specificity were 100% and 80%, respectively. In a study in Iran, ITS-rDNA, Hsp70, and Cyt b genes were used to accurately identify *Leishmania* spp. and investigated in clinical samples from three important regions where CL is common. By using the combination of the three genes, 231 *Leishmania* parasites were identified correctly among 360 clinical samples, and this method was more sensitive than routine laboratory methods that can only detect 203 *Leishmania* parasites (27). Sagi et al. (28) developed a practical swabbing test, combined with highly sensitive multiplex PCR for detection of *Leishmania* infections. They found that this combination was very practical and more sensitive than classical microscopy (28). Multilocus sequence typing and multilocus enzyme electrophoresis are used to identify *Leishmania* species and strains, but this depends on having culturable isolates, and in some cases, these methods were not discriminative enough (18).

Protein Analysis Methods

In recent years, proteomic methods started to gain importance in the diagnosis of parasitosis. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) spectrometric method was used to detect *Leishmania* pathogens isolated from cultures (29). They reported that they created a free web-based application for *Leishmania* species and set up a data library containing fingerprints of the pathogens' spectra. Researchers also identified differentially expressed proteins in the inflammatory region of CL, revealing increased caspase-9 expression. Immunological analyses validated the involvement of caspase-3, caspase-9, and granzyme B in tissue damage in CL cases (30). Duerte et al. (31) investigated some antigenic proteins to validate CL immunoscreening results and the coding regions of some antigens, such as enolase, tryparedoxin peroxidase, eukaryotic initiation factor 5a, and beta-tubulin. After being cloned in a vector, the serodiagnostic performances of the proteins were evaluated for CL. These proteins had sensitivity and specificity levels ranging from 82.5% to 100%. The study suggested the use of these antigenic proteins for diagnosis of CL (31).

African Trypanosomiasis

African trypanosomiasis is generally seen in the tsetse fly belt of Mid-Africa. The Gambian form of sleeping sickness, noted for its chronicity and accounting for 99% of the sleeping sickness cases, is caused by *Trypanosoma brucei gambiense*. The Rhodesian (East African) form, noted for its acute morbidity and mortality within months of infection, is caused by *Trypanosoma brucei rhodesiense* (18).

Microscopy
In addition to staining thin and thick blood smears, determining the buffy coat is recommended to detect the parasites. The parasites can be detected on thick blood smears when numbers are >2000/mL by determining the hematocrit concentration in a capillary tube or by quantifying buffy coats when numbers are >100/mL, and by anion-exchange chromatography when numbers are >4/mL (32). In suspected and confirmed cases of trypanosomiasis, lumbar puncture is mandatory to rule out central nervous system involvement. Cerebrospinal fluid (CSF) examination must be conducted by using centrifuged sediments (33).

Culture and Animal Inoculation
Rats and guinea pigs have also been used to detect trypanosomiasis. *T. brucei rhodesiense* is more adaptable to cultivation (Tobie's medium) and animal infection than *T. brucei gambiense;* however, cultivation methods are not practical for most clinical laboratories (18).

Serologic Methods
Serologic techniques, including IFA, ELISA, indirect hemagglutination assay, card agglutination trypanosomiasis test (CATT), and LATEX/*T. b. gambiense* has been used for epidemiologic screening. However, these methods have not been approved by the FDA. Serologic tests are normally used for screening. For a definitive diagnosis, microscopic observation of trypomastigotes is needed (18). A CATT (CATT/*T. brucei gambiense*) developed in 1978 for West African trypanosomiasis diagnosis is a cheap, rapid, and high-sensitivity test; however, it may give high false positives for infections accompanied by malaria (1). Similarly, micro-CATT and LATEX/*T. b. gambiense* tests are used especially in endemic regions; however, they need to be confirmed by microscopy (1). The CATT and LATEX/*T. b. gambiense* have good negative predictive values. Markedly elevated serum and CSF IgM concentrations have diagnostic value (18).

Molecular Methods
For diagnosis of African trypanosomiasis infections, the *T. b. rhodesiense* serum resistance-associated (*SRA*) gene is used with PCR and LAMP techniques in CSF samples from patients (34, 35). Becker et al. (34) completed real-time PCR with primers synthesized to target the 177-bp repeated satellite DNA of the parasite. They reported that the method was rapid and sensitive for use in routine laboratories. Diagnosis of African trypanosomiasis infections was investigated with the *SRA* gene used with PCR and LAMP techniques in CSF samples from patients (34, 35).

Protein Analysis Methods
Recently, researchers reported the discovery of serum proteomic signature for diagnosis of human African trypanosomiasis by using surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) mass spectrometry and data-mining algorithms. The new method, coupled with biochemical characterization of the proteins that contribute to the signature, provides stronger and novel tools to create improved diagnostic tests (36).

American Trypanosomiasis
Trypanosoma cruzi causes Chagas disease, also called American trypanosomiasis, a zoonotic infection. Triatomine insect infected with protozoa from other contacts with animals transmits the trypanosomes when the triatomine deposits its feces on the skin of the host and then bites (18).

Microscopy:
Trypomastigotes can be detected by using wet mounts of blood, examining blood smears, or the concentrated buffy coat. Giemsa stain is used for both amastigote and trypomastigote stages. *Leishmania donovani* and *T. cruzi* infections can be differentiated by other methods, such as PCR, immunoassay, culture, serologic tests, and animal inoculation (18).

Culture and Animal Inoculation
In laboratories, aspirates, blood, and tissues can be cultured. Generally, the NNN medium is chosen. Cultures, incubated at 25°C, should be examined for epimastigote forms at least twice weekly during the first 2 weeks and once weekly thereafter for up to 1 month before being considered negative. In advanced laboratories, rats or mice may be inoculated, and their blood can be investigated for trypomastigotes (18).

Xenodiagnosis
In this method, trypanosome-free reduvid bugs are allowed to feed on individuals suspected with Chagas disease. The feces, hemolymph, hindgut, and salivary glands can be examined microscopically for flagellated forms for 3 months, or PCR methods can be used to detect infected bugs and provide rapid diagnosis (37). Xenodiagnosis is positive in <50% of seropositive patients (18).

Serologic Methods
Blood and saliva are used for the diagnosis of Chagas disease. Complement fixation (Guerreiro–Machado test), chemiluminescence, IFA, indirect hemagglutination, and ELISA are used for serological testing. Most of these tests use an epimastigote antigen, and cross-reactions have been reported for patients infected with *Trypanosoma rangeli*, *Leishmania* spp., *Toxoplasma gondii*, and hepatitis (18). The secretory antigens of *T. cruzi* can potentially be used in the serologic diagnosis of Chagas disease. Umezawa et al. (38) developed recombinant antigens, including B13, 1F8, and H49 antigens to create a *T. cruzi* mix-ELISA kit. The sensitivity and specificity of the kit were 99.7% and 98.6%, respectively. Sánchez-Camargo et al. (39) evaluated 11 different RDTs for detecting *T. cruzi* antibodies in serum banks. These tests relied on different testing principles, such as particle agglutination, immunochromatography, immunofiltration, or immunoblot. They found that 8 of 11 tests were useful to detect infections (39).

Molecular Methods
PCR test was used to detect as few as one trypomastigote in 20 mL of blood and was found useful in treatment follow-up. However, it is not routinely available in the field. Real-time PCR using multiple gene targets has been advocated to improve the detection of positive patients. More target genes are needed due to polymorphism (18).

Protein Analysis Methods
New data suggest that both MS platform-dependent and platform-independent biomarker-based tests may be beneficial for

Table 3. Guide to general interpretation of *Toxoplasma* serology results obtained with IgG and IgM commercial assays (2)

IgG result	IgM result	Report/interpretation for humans (except infants)
Negative	Negative	No serological evidence of infection with *Toxoplasma*
Negative	Equivocal	Possible early acute infection or false-positive IgM reaction. Obtain a new specimen. If the results remain the same, the patient is probably not infected with *Toxoplasma*.
Negative	Positive	Possible acute infection or false-positive IgM result. Obtain a new specimen for IgG and IgM testing. If results for the second specimen remain the same, the IgM reaction is probably a false positive.
Equivocal	Negative	Indeterminate. Obtain a new specimen for testing or retest this specimen for IgG in a different assay.
Equivocal	Equivocal	Indeterminate. Obtain a new specimen for both IgG and IgM testing.
Equivocal	Positive	Possible acute infection with *Toxoplasma*. Obtain a new specimen for IgG and IgM testing. If results for the second specimen remain the same or if the IgG test becomes positive, both specimens should be sent to a reference laboratory with experience in the diagnosis of toxoplasmosis for further testing.
Positive	Negative	Infected with Toxoplasma for >1 year.
Positive	Equivocal	Infected with Toxoplasma for probably >1 year or false-positive IgM reaction. Obtain a new specimen for IgM testing. If results with the second specimen remain the same, both specimens should be sent to a reference laboratory with experience in the diagnosis of toxoplasmosis for further testing.
Positive	Positive	Possible recent (within the last 12 months) infection or false-positive IgM reaction. Send the specimen to a reference laboratory with experience in the diagnosis of toxoplasmosis for further testing.

subjects with latent Chagas disease. Rather than replacing antibody-based and PCR testing, mass spectrometry assays will help build more complementary information about the diagnosis in the future (1).

Toxoplasmosis

Toxoplasma gondii protozoan parasite causes toxoplasmosis, and it is one of the most common parasitic infections in humans. This disease is most typically asymptomatic. However, in select clinical situations, it can cause severe disabilities. Thus, accurate and timely diagnosis is important (2).

Microscopy

Diagnosis by microscopic examination of patient samples is rare. Secretions, exudates, sterile fluids, and tissues are potential samples for direct observations; however, they are generally not chosen. Giemsa staining in CSF (40) or heparinized fluid samples fixated in methanol may identify *T. gondii* tachyzoites, whereas the same sample may identify *T. gondii* cysts in tissue samples (2).

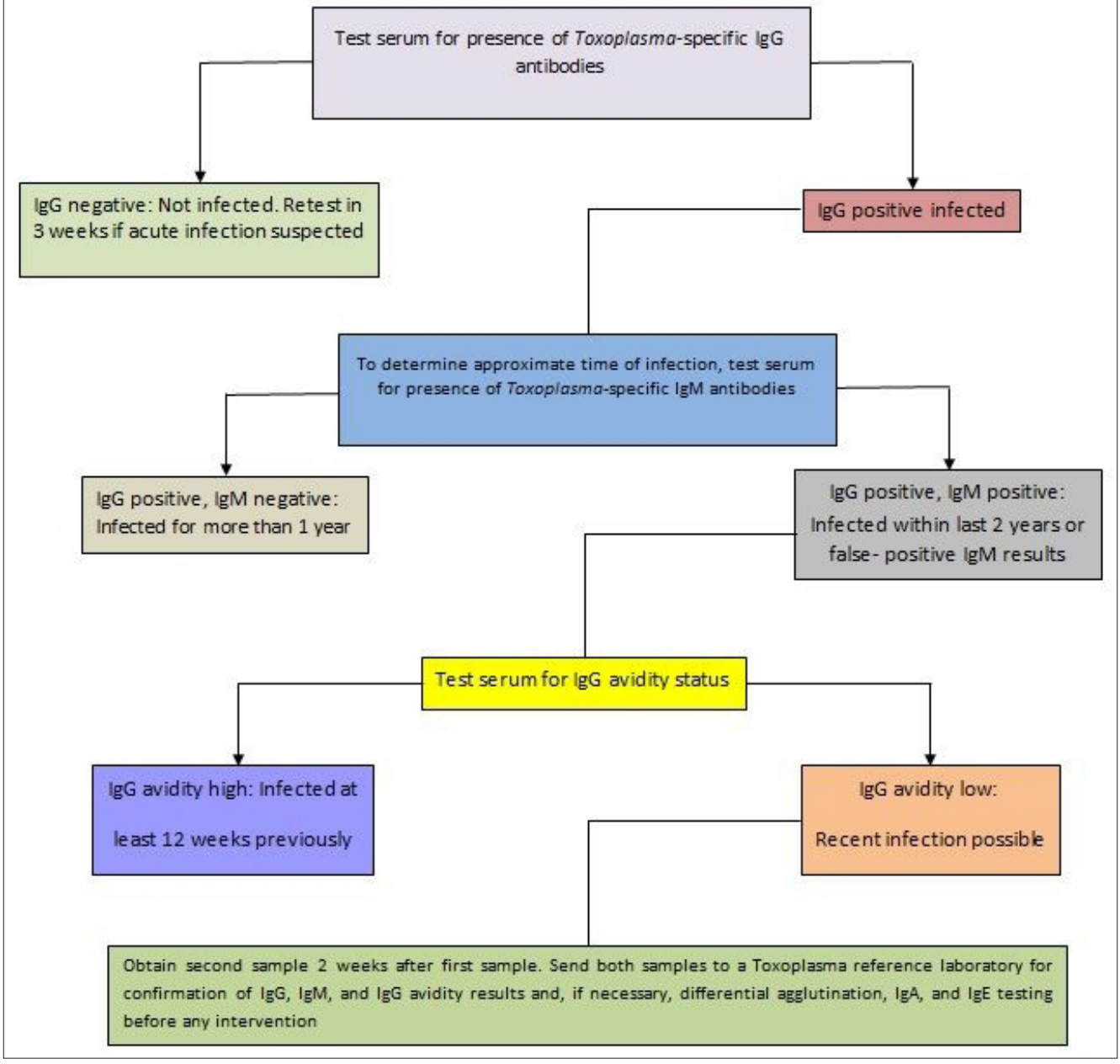
Isolation Procedures

Toxoplasma gondii isolation can be performed by inoculating patient sample into either mice or cell culture. However, the success of this method is limited. *T. gondii* grows in tissue culture cells. Cytopathic effects may be shown on direct examination after 24 to 96 h. Isolation in the cell culture technique allows faster diagnosis than the inoculation of microorganism in mice (2).

Serologic Tests

Because of its high sensitivity and specificity, the Sabin–Feldman dye test has been accepted as gold standard method (1). This method was used by several laboratories, whereas most laboratories focus on novel methods, including immunofluorescent antibody assays, hemagglutination tests, immunosorbent agglutination assays, and capture ELISAs (1). Chronic and acute infections can be differentiated by using *Toxoplasma*-specific IgM antibodies. The most important use regarding IgM test results is that a negative reaction essentially excludes recent infection. Table 3 presents a guide for *Toxoplasma* IgG and IgM serology result interpretation (2). The *Toxoplasma* IgG avidity test is an important tool to discriminate between past and recently acquired infections. During acute infection, IgG antibodies bind antigen weakly or have low avidity; whereas, during chronic infection, antibodies bind antigen more strongly or with high avidity. Thus, the avidity test works on this principle. A high-avidity result indicates that the infection was acquired >3 to 5 months before. However, low-avidity result does not indicate a recently acquired infection because low-avidity antibodies may be detectable a year post-infection (2). Testing for *Toxoplasma*-specific IgA antibodies should be performed in addition to IgM assays for newborns suspected with a congenital infection. *Toxoplasma*-specific IgE antibodies may also contribute to the determination of acute infections, although reports of the utility of IgE antibody detection have been mixed (2). Figure 1 shows an algorithm for serological testing for immune status, and acute acquired infection is shown. A new method developed for the postnatal diagnosis of toxoplasmosis is based on measuring interferon-gamma levels in full blood cells stimulated by *Toxoplasma* antigens with ELISA (41). The interferon-gamma release test (IGRA) shows activation of lymphocytes after *T. gondii* antigen stimulation and distinguishes infected individuals from those who are not. This test is a practical and economical method to show cell-mediated immunity against the pathogen (42). Begeman et al. (43) used the *Toxoplasma* ICT IgG-IgM point-of-care test for diagnosis of congenital toxoplasmosis (CT) and showed that the new test was 100% sensitive and specific for identification of *Toxoplasma* infections. Zacche-Tonini et al. (44) evaluated conventional serology (Q-Preven™ and ELFAVIDAS™) and flow cytometric assays for early serodiagnosis of CT. In conclusion, they proposed a novel algorithm with high accuracy (97.1%),

Figure 1. Algorithm for serodiagnosis of toxoplasmosis in children older than 1 year



including screening with Q-Preven™IgM assay at the birth, followed by flow cytometric IgG avidity analysis and ELFAVIDAS™ IgM during the first month of life. These assays have a high-performance for early serological diagnosis of CT. Baschirotto et al. (45) designed a novel test using a liquid microarray method. They evaluated different antigens to detect IgGs against *T. gondii*, and rubella. The performance of 6 of 13 antigens was sufficient to be used in a multiplex PCR assay for diagnosis of the *T. gondii* infection. The test was reported to have 100% sensitivity and specificity for detection of *T. gondii* infection. The test seems to have a potential for prenatal infection screening of pregnant women after some modifications (45).

Nucleic Acid Detection Methods

Recently, different molecular methods, including PCR, real-time PCR, nested PCR, and LAMP, were designed for diagnosis of toxoplasmosis. PCR test was found useful to detect pathogens in amniotic fluid, placental, and cerebral tissues, and aqueous humor and vitreous fluid (46). PCR method with amplification of repeating B1 gene of *T. gondii*, 18S rRNA, P30, 529-bp repeat fragment, or AF146527 element was used for molecular diagnosis. Nested PCR was used to increase specificity of DNA amplification, and it was found to be useful to detect pathogens found in low amounts in the specimens (46, 47). Berredjem et al. (48) used PCR studies of peripheral blood and amniotic fluid samples

for early toxoplasmosis diagnosis in pregnant women. Samples with PCR amplification were divided into two, with nested PCR to increase *T. gondii* in the B1 region and PCR-ELISA using major surface antigen P30 gene primers. With regard to the PCR assay using peripheral blood and amniotic fluid, both B1 and P30 primer sets performed equally well and therefore appear adequate for *Toxoplasma* identification. However, the B1 gene proved to be valuable in PCR for *T. gondii* detection better than the P30 gene (48). Mousavi et al. (49) reported that the performance of the B1 gene was better than the RE gene for molecular diagnosis of toxoplasmosis. Real-time PCR seems to be a very sensitive molecular diagnostic test that can detect the DNA region even at low concentrations. In addition, these tests are fast, more sensitive, and reproducible when compared to classical PCR. They can also be used for monitoring the therapeutic response and prognosis of the infection (46). Parasite load can also be investigated by using this method. Varlet-Marie et al. (50) evaluated a novel test, the Iam TOXO Q-LAMP (DiaSorin, Italy) assay, using a reference real-time PCR method (laboratory developed). This LAMP method was found to be less sensitive than real-time PCR at very low parasite load. However, both methods yielded identical results qualitatively.

CONCLUSION

Although novel and rapid diagnostic instruments are being investigated and even used by advanced microbiology units, the results often need to be confirmed by microscopy, which is accepted as the gold standard (1). Commercially available rapid antigen and antibody detection kits are easy to use and suitable for mass screening. However, rapid tests are expensive, are not the gold standard, and may give false-negative results. Molecular tests, including PCR, real-time PCR, NASBA, oligochromatography, and LAMP have improved during the past decade. PCR methods for all six blood parasites and LAMP technique for *Plasmodium* spp. and *T. brucei* can be used to diagnose parasitic infections. Recently, MALDI MS, LDMS, and SELDI-TOF proteomic techniques that analyze the protein expression of the parasites have begun to give promising results and opened new horizons for the future of diagnosis of blood-borne parasites. However, new and larger studies with different species are still needed to standardize and optimize these novel techniques.

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