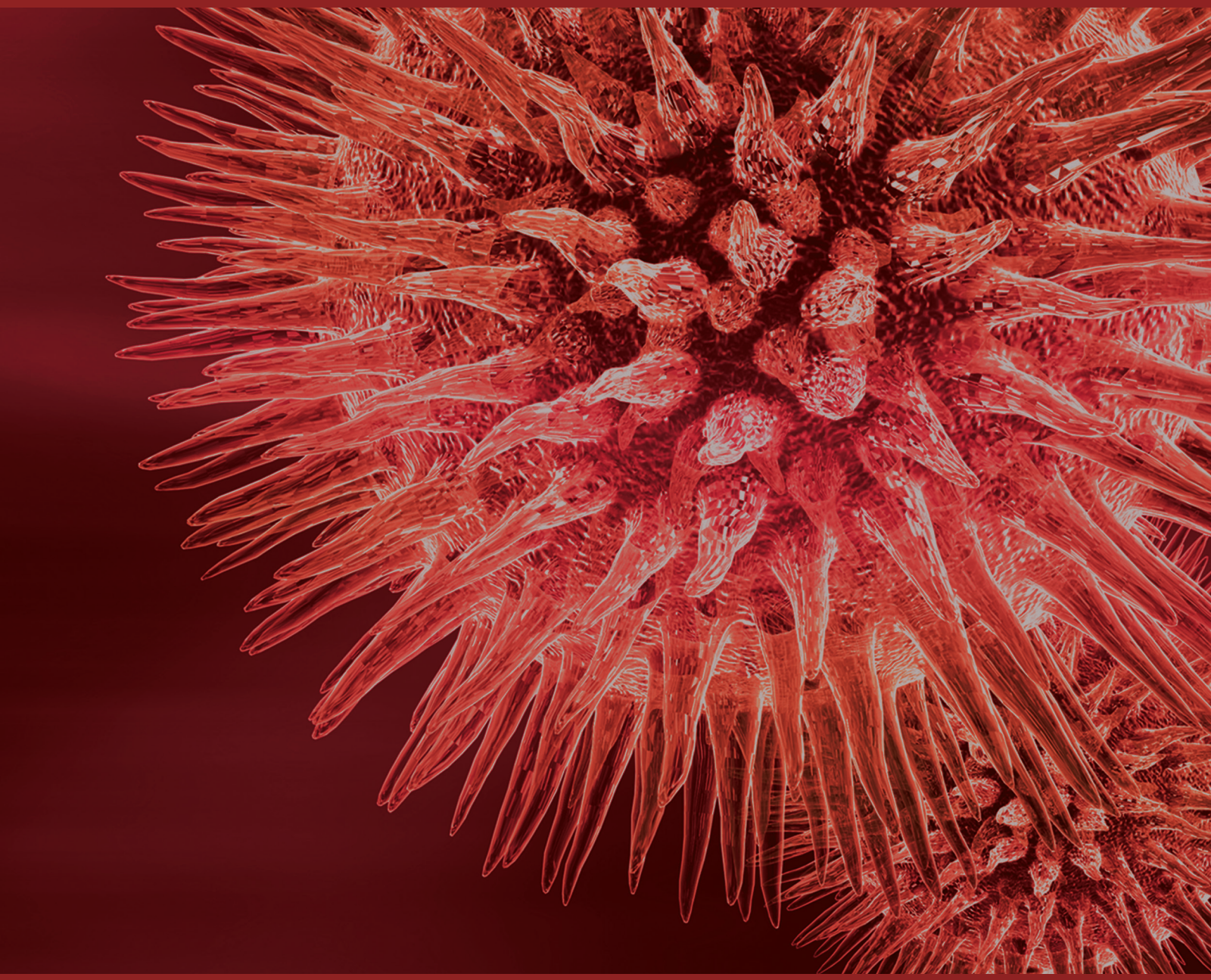
BioMed Research International

Challenges for Diagnosis of Malaria and Neglected Tropical Diseases in Elimination Settings

Guest Editors: Stephan Karl, Malcolm K. Jones, Lucía Gutiérrez, Brioni Moore, Eline Kattenberg, and Marcus Lacerda



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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 270756, 2 pages
http://dx.doi.org/10.1155/2015/270756

Editorial

Challenges for Diagnosis of Malaria and Neglected Tropical Diseases in Elimination Settings

Stephan Karl,[1,2] Malcolm K. Jones,[3] Lucía Gutiérrez,[4] Brioni Moore,[5] Eline Kattenberg,[6] and Marcus Lacerda[7]

[1]*Population Health and Immunity, Walter and Eliza Hall Institute of Medical Research, Parkville, VIC 3052, Australia*
[2]*Department of Medical Biology, The University of Melbourne, Melbourne, VIC 3052, Australia*
[3]*School of Veterinary Science, The University of Queensland, Brisbane, St. Lucia, QLD 4072, Australia*
[4]*Departamento de Biomateriales y Materiales Bioinspirados, Instituto de Ciencia de Materiales de Madrid, 28049 Madrid, Spain*
[5]*School of Medicine and Pharmacology, The University of Western Australia, Perth, WA 6009, Australia*
[6]*Vector-Borne Infectious Diseases Research Unit, Papua New Guinea Institute of Medical Research, Madang 511, Madang Province, Papua New Guinea*
[7]*Fundação de Medicina Tropical Dr. Heitor Vieira Dourado and Instituto de Pesquisa Leônidas e Maria Deane, Fiocruz, 69077-000 Manaus, AM, Brazil*

Correspondence should be addressed to Stephan Karl; karl@wehi.edu.au

Received 1 October 2015; Accepted 1 October 2015

Neglected tropical diseases (NTDs) represent a significant health burden for many countries in the developing world. Currently, there are 17 NTDs prioritized by the World Health Organization (WHO). These NTDs are endemic in 149 countries and affect an estimated 1.4 billion people. NTDs therefore also constitute a very significant burden on the already strained healthcare systems and the economies of many developing countries [1]. To complicate matters, there is not much interest in the development of new diagnostic tools for these diseases. Malaria, for instance, even not formally considered a neglected disease, poses many challenges in terms of the diagnosis of submicroscopic parasitemia [2], which seems to sustain the transmission of the disease in low endemic areas.

Many countries are facing scenarios where transmission of an NTD has been reduced to low levels. In addition, many NTDs cause asymptomatic infections, making the identification of residual transmission foci of these diseases a challenging task. Thus, in these very low transmission settings, with often undetectably low individual pathogen burdens, diagnostic challenges are considerable and the need for developing better techniques and strategies for diagnosis and epidemiological surveillance is strikingly evident.

In this special issue, authors contributed several reviews and original research papers describing current diagnostic challenges for African sleeping sickness, human cystic echinococcosis, dengue and chikungunya fevers, and schistosomiasis.

The development of reliable, highly sensitive, and species specific molecular techniques such as loop-mediated isothermal amplification (LAMP), which are potentially high throughput and more easily adaptable to remote field conditions, may allow for more accurate diagnosis and epidemiological surveillance of a number of NTDs such as *Trypanosoma brucei gambiense* (J. M. Kagira et al.). Particularly noninvasive molecular techniques, for example, approaches using saliva or urine samples, as discussed in several papers in this issue, would constitute significant progress over current techniques relying on microscopy using blood samples or lymph node aspirates (*T. b. gambiense*), which are often not

sensitive enough to provide definitive diagnosis. J. Bonnet et al. discuss these issues and review current developments for field-deployable human African trypanosomiasis diagnosis.

The need for the development of reliable serological markers for the detection of infection with *Echinococcus granulosus*, a highly neglected zoonotic tropical helminth infection, is discussed in the contribution of C. Sánchez-Ovejero et al. Reliable serology tools not only could enable better confirmation of ultrasound-based diagnosis of this disease but also could be used in cross-sectional surveys in order to obtain more accurate estimates of the burden of human cystic echinococcosis and other NTDs such as schistosomiasis [2].

The clinical differentiation of dengue and chikungunya virus infections is a particular challenge reviewed in the contribution of S. K. Mardekian and A. L. Roberts. Both viruses result in similar clinical symptoms, especially early in the infection. However outcomes and management strategies for dengue and chikungunya are very different. With the increasing global numbers of dengue and chikungunya infections, and with outbreaks occurring more frequently in southern Europe, northern Australia, and Florida, better understanding of the distinguishing features of these infections is critical [3].

The diagnosis of schistosomiasis is challenging as the number of eggs in faecal smears is frequently below the sensitivity threshold of the Kato-Katz faecal smear technique for direct egg detection. Several new approaches have been proposed recently to improve schistosomiasis diagnosis, via magnetic concentration techniques or field applicable flotation devices, as well as molecular and antigen-detection methods. In their contribution to this special issue, M. C. C. Espírito-Santo and colleagues review current laboratory diagnostic techniques for schistosomiasis diagnosis [4, 5].

The final adoption and deployment of diagnostics in a range of settings requires many factors to come together. The diagnostic must be technically feasible within the setting; the diagnostic must be affordable for sustained use in that setting and of course must be able to meet the demands of the many clinical and research questions asked about it. No diagnostic tool is perfect and there is ongoing demand for improvements to the current tools. The collected papers highlight a snapshot of the current state of play for diagnostics of just a few neglected tropical diseases.

Stephan Karl
Malcolm K. Jones
Lucía Gutiérrez
Brioni Moore
Eline Kattenberg
Marcus Lacerda

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 834371, 8 pages
http://dx.doi.org/10.1155/2015/834371

Review Article

Diagnostic Options and Challenges for Dengue and Chikungunya Viruses

Stacey K. Mardekian and Amity L. Roberts

Sidney Kimmel Medical College at Thomas Jefferson University, 117 South 11th Street, PAV 207, Philadelphia, PA 19107, USA

Correspondence should be addressed to Amity L. Roberts; amity.roberts@jefferson.edu

Received 10 March 2015; Revised 23 June 2015; Accepted 25 June 2015

Academic Editor: Marcus Lacerda

Dengue virus (DENV) and Chikungunya virus (CHIKV) are arboviruses that share the same *Aedes* mosquito vectors and thus overlap in their endemic areas. These two viruses also cause similar clinical presentations, especially in the initial stages of infection, with neither virus possessing any specific distinguishing clinical features. Because the outcomes and management strategies for these two viruses are vastly different, early and accurate diagnosis is imperative. Diagnosis is also important for surveillance, outbreak control, and research related to vaccine and drug development. Available diagnostic tests are aimed at detection of the virus, its antigenic components, or the host immune antibody response. In this review, we describe the recent progress and continued challenges related to the diagnosis of DENV and CHIKV infections.

1. Introduction

Dengue virus (DENV) and Chikungunya virus (CHIKV) are single-stranded, positive-sense RNA viruses. DENV belongs to the family Flaviviridae and genus *Flavivirus* of which there are 5 known serotypes (DENV1–5). CHIKV belongs to the family Togaviridae and genus *Alphavirus* of which there are 3 known strains (Asian-West African; East-Central; South African) [1]. The genome of each virus is approximately 11 kb in length [1, 2]. The DENV genome encodes three structural (C, prM, and E) and seven nonstructural (NS1, NS2B, NS3, NS4A, NS4B, and NS5) proteins [3]. The CHIKV genome encodes three structural (C, E1, and E2) and four nonstructural (nsP1–4) proteins [1].

Both viruses are arthropod-borne viruses (arboviruses) sharing a common vector: mosquitos of the *Aedes* genus, specifically *A. aegypti* and *A. albopictus* [4]. Both viruses circulate in similar geographic regions. In nonendemic regions, travel-associated infections are an important consideration for patients with a recent travel history who present with fever. Concurrent infection with both viruses, transmitted from either two different mosquitos or one dually infected mosquito, is possible [5, 6]. For DENV, transmission has also been reported to occur via infected blood products, organ donation, and prenatal and/or perinatal vertical transmission [7].

While DENV and CHIKV present similarly as an acute febrile illness, these two viruses have vastly different management strategies and outcomes. The majority of CHIKV infections are self-limiting with chronic joint disease being the most common long-term outcome, and fatality is exceedingly rare. Nonsteroidal anti-inflammatory drugs (NSAIDs) are the mainstay treatment for CHIKV, but NSAIDs should be avoided until DENV is confidently ruled out, as NSAIDs are contraindicated in DENV infection [8]. DENV is likewise commonly a self-limiting illness, yet this diagnosis necessitates stricter monitoring due to the potential for more significant morbidity and mortality. Infection with one serotype of DENV confers lifelong immunity to that particular serotype but only short-term immunity to the other serotypes; subsequent infections with a different serotype increase the risk of severe complications [7].

2. Epidemiology

The majority of DENV and CHIKV infections affect people residing in endemic areas, which include most of the tropical

and subtropical regions in the world. Many of these areas serve as popular tourist destinations and, consequently, dengue-related infections have recently surpassed malaria and gastrointestinal infections as the most common cause of fever among travelers [23]. The major endemic regions include Southeast Asia, the Western Pacific, the Eastern Mediterranean, Africa, and the Americas [9]. Specific countries with cocirculation and coinfections of DENV and CHIKV include India, Sri Lanka, Gabon, Cameroon, Madagascar, Indonesia, Singapore, and Thailand [24]. In the United States, autochthonous outbreaks of DENV have been reported in Hawaii and along the Texas-Mexico border, and outbreaks of both DENV and CHIKV have recently occurred in southwest Florida [6, 25].

3. Clinical Presentation

These two viruses share a similar geographic distribution; unfortunately, their clinical manifestations also show substantial overlap. The typical incubation periods for DENV and CHIKV are 4–7 days and 3–7 days, respectively [4]. Patients infected with either virus typically present with acute onset of fever, myalgia, and headache, and some patients experience a maculopapular rash and/or gastrointestinal symptoms [4, 6].

A classification scheme for DENV, put forth by the World Health Organization (WHO) in 2009, includes criteria for probable dengue and severe dengue [9]. Most DENV infections are either asymptomatic or mild and self-limited, but there are "warning signs" that may suggest which patients may progress to severe disease and require stricter medical management [9]. Severe dengue may manifest as significant plasma leakage, hemorrhagic complications, and/or severe organ impairment, so early recognition of DENV infection is imperative [9]. Compromising the sensitivity of the WHO classification scheme is the fact that patient age influences the type and severity of symptoms; Low et al. found that fewer older adults reported symptoms of myalgia and arthralgia, as well as mucosal bleeding, which is one of the primary "warning signs" [26].

The clinical course for CHIKV is likewise typically mild and self-limited. The hallmark presentation of CHIKV is a bilateral migratory arthralgia, often intense, affecting mainly the small joints of the extremities [1, 4]. However, most children with CHIKV report only mild arthralgia [8]. The major long-term complication is persistence of joint pain and stiffness, which may last years after resolution of the initial infection [1]. Rarely, CHIKV infection is associated with neurologic, ophthalmologic, and hemorrhagic disease [4, 5].

While neither infection possesses a defining clinical feature, there are suggested trends in the symptomatology and complete blood count (CBC) results that may help differentiate between the two infectious processes. It is suggested that, at initial presentation, significantly more DENV patients have thrombocytopenia (platelets $< 100 \times 10^9$/L) and associated minor bleeding complications such as petechiae and nose bleeds, while patients with CHIKV are more likely to have arthralgia. Leukopenia is common to both infections at initial presentation but tends to be more pronounced in DENV patients; CHIKV patients tend to have higher white blood cell (WBC) counts (>3.6 or 5.0×10^9/L according to two separate authors) than DENV patients [4, 6, 8]. During the course of illness, DENV patients are more likely to have abdominal pain and the CBC will demonstrate leukopenia, neutropenia, and thrombocytopenia that is more frequent and more pronounced than in CHIKV patients. In contrast, CHIKV patients may show a shorter duration of fever, conjunctivitis, acute arthritis, and more prominent arthralgia affecting multiple joints [6]. While these trends in clinical findings may be helpful, they are neither specific nor consistent enough to be considered diagnostic.

Unfortunately, there is no single clinical or laboratory marker available for distinguishing DENV or CHIKV infection from each other or from other acute febrile illnesses. Therefore, both of these viruses must be initially included in the differential diagnosis for a patient with suspicious clinical symptoms who is living in or returning from travel to an endemic area. Clinical features can serve, at best, as a guide for favoring one virus over the other, as patients may present atypically, either by lacking the "classic" signs or symptoms as mentioned above, or by presenting in an uncharacteristic manner. Laboratory diagnostic tests are thus essential for accurate identification of the causative virus.

4. Methods for Diagnosis

A wide variety of laboratory diagnostic methods are available to aid in the diagnosis of DENV and CHIKV infections. The premise of these tests is detection of the virus, viral components (antigens or nucleic acid), or the host immunologic response to the virus [10]. Therefore, selection and interpretation of testing depends on the kinetics of viremia and antibody response, which differ between primary and secondary infections. Other factors influencing test choice include the purpose of testing and availability of resources. Each type of test offers unique advantages and disadvantages, and a combination of tests may be employed in order to increase diagnostic confidence. For a summary of available tests for DENV and CHIKV infection, see Tables 1 and 2, respectively.

4.1. Overview of Currently Available Tests. The acute febrile phase of infection corresponds to the period of viremia, which lasts typically from 5 days after onset of fever for both DENV and CHIKV. During this time, diagnosis rests on isolation of the virus, viral RNA, or viral antigen from the specimen. Isolation of DENV or CHIKV can be performed via mosquito inoculation or cell culture; CHIKV isolation can also be accomplished by intracerebral inoculation of mice [16]. Virus may be recovered from serum, plasma, whole blood, or tissues collected at autopsy. Mosquito inoculation is the most sensitive isolation method but is impractical for routine diagnosis due to the highly specialized requirements and high maintenance costs [3]. Cell culture is in wider use, with preference given to the mosquito cell line C6/36 (cloned

Table 1: Diagnostic tests for DENV infection.

Premise of test	Method	Sample types	Sensitivity (%)	Specificity (%)	Advantages	Disadvantages	References
Detection of virus	Virus isolation	Serum, plasma, whole blood, and fresh or FFPE tissues	71.5–84.2 (mosquito inoculation); 40.5 (cell line-based)	100	Greatest specificity Allows for further characterization of isolate	Technical, laborious Variable sensitivity Narrow window of detection (viremic period)	[3, 9, 10]
Detection of viral antigen	NS1 detection via ELISA	Serum, urine, and CSF	54.2–93.4 (serum); 73.9–76.9 (urine) 50 (CSF, if neurological symptoms)	71–80 (serum); 100 (CSF, if neurological symptoms)	Early diagnosis Rapid tests available	Does not differentiate between serotypes Lower sensitivity in secondary infections	[11–13]
Detection of viral nucleic acid	RT-PCR	Serum, plasma, whole blood, fresh or FFPE tissues, urine, and saliva	48.4–98.2	100	Rapid turnaround time	Expensive reagents and specialized equipment	[3, 9, 14]
	Real-time RT-PCR		58.9–100	100	Multiplex available (can identify all serotypes from single sample; less potential for contamination)		
	Isothermal amplification methods (NASBA, LAMP)		98.5	100	Does not require specialized equipment (i.e., thermocyclers)		
Detection of host antibody response	MAC-ELISA	Serum	61.5–99	79.9–97.8	Detection of IgM is considered diagnostic	Cross-reactivity among serotypes (not serotype-specific) and with other flaviviruses (false-positives)	[3, 9, 14, 15]
	IgG ELISA				Can distinguish primary from secondary infection using paired sera	Later diagnosis (need postconvalescent sample)	
	IgM/IgG ratio				Distinguishes between primary from secondary infection	Later diagnosis	
	IgA	Serum and saliva	93 (serum); 70–92 (saliva)	88 (serum); 97 (saliva)	Option for testing saliva (easier sample to obtain) Better sensitivity and specificity in secondary infection	Lower sensitivity in primary infection	

Table 2: Diagnostic tests for CHIKV infection.

Premise	Diagnostic method	Sample types	Sensitivity (%)	Specificity (%)	Advantages	Disadvantages	References
Detection of virus	Virus isolation (*in vivo* or *in vitro*)	Serum, plasma, whole blood, and fresh or FFPE tissues	Variable	100	Highly specific	Technical, laborious Requires biosafety level 3 containment May take 1-2 weeks	[1]
Detection of viral antigen	ELISA or immunochromatographic assay (ICA)	Serum and CSF	85 (serum) 80 (CSF)	89 (serum) 87 (CSF)	Early diagnosis	Commercial assays not widely available Requires biosafety level 3 containment	[16, 17]
Detection of viral nucleic acid	RT-PCR	Serum and dried blood spots	100	Up to 100	Highly sensitive and specific Rapid turnaround time Multiplex available	Expensive reagents and specialized equipment	[13, 16, 18–20]
	Real-time RT-PCR		100	Up to 100	Multiplex available	Expensive reagents and specialized equipment	
	Isothermal amplification methods (RT-LAMP)		100	95.25	Does not require specialized equipment (i.e., thermocyclers)		
Detection of host antibody response	ELISA	Serum CSF	IgM: 17 (serum); 48 (CSF) IgG: 45 (serum); 63 (CSF)	IgM: 95 (serum) IgG: 53 (serum)	Widely available Relatively cheaper and easier to perform Rapid bedside tests are available	Possible cross-reactivity with other alphaviruses Elevated IgM does not distinguish recent past infection from acute infection	[4, 16, 17, 20–22]
	IFA	Serum	85–97	90–98	Sensitive and specific Commercially available	Lack the ability to quantify antibodies, are subjective, and require special equipment and training	
	PRNT	Serum			Very specific for alphaviruses; gold standard for confirmation of serologic test results	Requires the use of live virus (requires Biosafety level 3 containment)	

from *A. albopictus*) or AP61 (cloned from *A. pseudoscutellaris*) [9, 16]. Other less sensitive options include mammalian cell cultures such as Vero, LLC-MK2, and BHK-21 [3]. The resultant virus isolate may be further characterized during subsequent *in vitro* studies, such as genome sequencing, virus neutralization, and infection studies [3]. Virus isolation is highly specific and has a theoretical detection limit of a single viable virus, although, in practice, the sensitivity is only approximately 40.5% in cell line-based virus isolation. It also requires highly trained operators, a dependence on sample integrity and a short viremia period, thus providing a narrow window of opportunity from illness onset. Virus isolation followed by an immunofluorescence assay for confirmation requires days to weeks [9, 16]. Therefore, despite its advantages, this approach is not widely used in routine diagnostic laboratories and may serve more use in surveillance purposes. A more recent development in viral isolation is described by Patramool et al., who used anionic polymer-coated beads to isolate DENV and CHIKV [27]. This may prove a useful strategy to monitor the status of circulating mosquitos in regions at risk for outbreaks with these arboviruses. Compared to traditional isolation techniques, this method provides reduced cost, good sensitivity, and rapidity, which is conducive to simultaneous analysis of a large number of samples [27].

Compared to virus isolation, viral nucleic acid detection techniques performed on acute-phase specimens offer better sensitivity with a much more rapid turnaround time. Viral nucleic acid can be detected for a few additional days beyond the period of viremia. Detection of viral nucleic acid can be accomplished by reverse transcriptase polymerase chain reaction (RT-PCR), real time RT-PCR, or isothermal amplification methods. All of these methods involve three basic steps: viral RNA extraction, amplification, and detection and characterization of the amplified product [9]. There is a wide variety of specimen types that can be tested with RT-PCR, including blood, serum, plasma, and fresh or formalin-fixed paraffin-embedded tissues. For DENV, urine and saliva have been found to be suitable specimen types as well [3]. Testing urine samples by real-time RT-PCR provides a larger window of detection that extends well past the viremia period; DENV RNA may be detected in urine up to day 16, compared to day 8 for blood specimens [28]. The ability to test urine and saliva is advantageous in patients for whom blood samples are difficult to obtain, such as in newborns and patients with hemorrhagic syndromes [14].

RT-PCR using primers designed for structural and nonstructural domains has been found to be useful in the rapid diagnosis of CHIKV. The combination of RT-PCR/nested PCR has proved efficient for specific detection and genotyping of CHIKV. Loop-mediated isothermal amplification (LAMP) assays can be rapidly carried out at a single temperature in a water bath, with visually detectable results, and comparable sensitivities to conventional PCR [17].

Detection of viral antigens is another diagnostic methodology available for DENV infection. Nonstructural protein 1 (NS1) antigen is a highly conserved glycoprotein produced during the virus replication process, and a soluble form of NS1 accumulates in high concentrations in the serum of patients with both primary and secondary DENV infections [29, 30]. Several commercial assays, consisting of both rapid tests and enzyme-linked immunosorbent assay (ELISA) kits, are available for the detection of the NS1 antigen. Serum is the most common sample type. DENV NS1 can also be detected in urine samples during the acute phase of DENV infection, which provides an opportunity for the development of a rapid noninvasive test [11]. Lastly, NS1 antigen may be detected in the cerebrospinal fluid (CSF) of patients with neurological symptoms [12]. A downfall is that these tests do not differentiate between dengue serotypes, as NS1 is highly conserved by all serotypes. Additionally, these tests are most successful during the acute phase of illness and lose sensitivity once the period of viremia ends. The sensitivity of NS1 has also been found to be lower in DENV secondary infections, which is thought to be due to assay interference by anti-NS1 antibodies which are present more frequently in secondary infections [10, 29]. An antigen-based commercial detection assay is not widely available for CHIKV, and the ones described thus far in the literature have unclearly established performance characteristics [21, 22].

After the period of viremia, the methods described thus far become much less sensitive for diagnosis. At this point, the best diagnostic strategy entails detection of antibodies indicative of host immune response to the virus. However, the caveat is that individuals in endemic areas often have immunologic levels to these viruses. Serologic methods include ELISA, indirect immunofluorescence assays (IFA), hemagglutination inhibition (HI), and microneutralization (MNt) [1]. ELISA and IFA are rapid and sensitive techniques for detecting virus-specific antibodies and can distinguish between IgG and IgM. For techniques that cannot make this distinction (HI and MNt), it is required to compare paired serum samples (acute and convalescent phases) to establish recent infection.

For DENV, serologic methods are most commonly employed, in particular IgM capture ELISA [4]. IgM antibodies are detectable in 50% by days 3–5 after onset, 80% by day 5, and 99% by day 10 after initial symptoms. They may persist for months; hence DENV IgM antibodies are a reliable marker of recent but not necessarily acute infection [29]. IgG antibody response develops a few days after the onset of IgM antibodies, and IgG may persist for many years [29]. Serologic confirmation of infection requires demonstration of a fourfold rise in antibody titer between acute and convalescent phase sera, or by demonstration of IgM antibodies specific for the virus [16]. Patterns of antibody response differ between primary and secondary infections, with primary dengue invoking stronger and more specific IgM response than in secondary, which have stronger and more rapid IgG response. Prior vaccination against other *Flavivirus* (Japanese encephalitis virus; Yellow-fever virus) or prior infection with nondengue flaviviruses (including West Nile) can potentially influence antibody responses measured in some assays [4]. The recent introduction of rapid diagnostic kits that offer combined detection of NS1 and IgM/IgG antibodies was an effort to create a point-of-care test with better performance characteristics [13]. Evaluation

of some of these combined tests has revealed diagnostic sensitivity of 89–93% and specificity of 75–100% [3, 13].

A combination of molecular and IgM antibody detection assays is recommended for diagnosis of CHIKV infection. Some advocate adopting an algorithmic approach, wherein the IgM capture ELISA is used as an initial screening tool followed by the use of rapid molecular assays in CHIKV IgM negative samples, to facilitate rapid diagnosis during outbreaks [18].

4.2. Simultaneous Testing for DENV and CHIKV. Because infection with DENV and CHIKV should be on the differential diagnosis together at the initial patient presentation, tests that screen for these viruses simultaneously are preferred to test for them separately. CHIKV and DENV are not readily differentiated serologically due to cross-reactivity of their serocomplexes, so there is a reliance on molecular detection methods for this purpose [19]. A one-step duplex conventional RT-PCR assay for distinguishing DENV and CHIKV has been reported [20]. Saha et al. developed a highly sensitive and specific, rapid one-tube duplex RT-PCR assay which provides a result within 110 minutes [19]. Two authors have described a one-step multiplex real-time RT-PCR assay that can simultaneously detect and quantitate RNA for all DENV serotypes and CHIKV. Cecilia et al. report a sensitivity of 100% for DENV and 95.8% for CHIKV, while the specificity was 100% for both viruses when compared to conventional RT-PCR [24]. Pongsiri et al. report an assay sensitivity of 97.65% and specificity of 92.59% when compared to conventional RT-PCR [31]. Real-time reverse transcription-loop-mediated isothermal amplification (RT-LAMP) is a sensitive alternative to real-time PCR for use in field applications [18]. A RT-LAMP method has been described in which a reverse transcription and amplification was designed in one step with two tubes under the same reaction conditions for the rapid identification and quantitative detection of RNA for CHIKV and DENV, respectively [32]. This assay has a sensitivity of 100% and specificity of 95.25%. The LAMP reaction can be ended within one hour under isothermal conditions and does not require sophisticated instruments, making this method adaptive to field diagnosis. Additionally, the use of a turbidimeter allows for quantitative detection of viral load [32]. For RT-PCR assays described above, the one-step process reduces the chance of contamination and there is lack of cross-reactivity between related *Flavivirus* groups and DENV [19].

4.3. Sending Out Samples. Within the United States, CHIKV testing is performed at the Centers for Disease Control and Prevention (CDC), a limited number of select state health departments, and one commercial laboratory. The CDC's Arbovirus Diagnostic Laboratory at the Division of Vector-Borne Diseases (DVBD) is located in Fort Collins, CO. Test results are normally available 4 to 14 days after specimen receipt, but reporting times may be longer during summer months when arbovirus activity increases. Initial serological testing is performed using IgM capture ELISA and IgG ELISA. If the initial results are positive, further confirmatory testing is performed which may delay the reporting of final results. All results are sent to the appropriate state health department.

The CDC Dengue Branch, located in San Juan, Puerto Rico, provides DENV testing free of charge to submitting physicians and state and private laboratories. A "Dengue Case Investigation Form" must accompany the specimen. One potentially problematic issue with sending samples to this laboratory is that an international shipping license is required. Another challenge, especially for underdeveloped countries, is specimen preservation during shipment. The CDC recommendation is that the serum specimen is frozen immediately after separation and sent on dry ice, or alternatively kept refrigerated and sent in cold packs.

4.4. Future Test Developments. Other diagnostic methodologies may be available for future use in the laboratory diagnosis of DENV and CHIKV infection. One technique becoming an increasingly popular serological option in arbovirology is microsphere-based immunoassay (MIA). This technology is based on detection by flow cytometry of antigen or antibody attached to microspheres or beads. This is a much more rapid test than MAC-ELISA and also has the potential for performance in multiplex [33]. Similarly, microarray technology, which focuses on detection of nucleic acid fragments corresponding to different pathogens, is useful to screen a sample for the many pathogens on a wide differential diagnosis for infectious symptoms in a given region [10]. Finally, mass spectrometry could be applied to this field of diagnosis, proving especially useful in determining viral serotypes and genotypes during an outbreak [9].

5. Conclusion

Confirmation of DENV or CHIKV infection requires laboratory diagnosis. Molecular assays are more sensitive for diagnosis in the early stages of illness (2–5 days after onset) when antibodies are not detected. However, in the later stages of illness, the sensitivity of molecular methods decreases due to the onset of a brisk immune response and corresponding reduction in viral load. At this stage, the IgM ELISA is a more sensitive diagnostic test.

An ideal diagnostic test meets certain key criteria: affordability by those at risk of infection, specificity, sensitivity, ease of use, rapid results, little reliance on equipment, and delivery to those in need [29]. The ideal test should also be part of a multiplexed assay for other pathogens causing acute undifferentiated fever, such as malaria [17]. Progress for DENV and CHIKV diagnostic testing has been made. Generally, tests with high sensitivity and high specificity require more complex technologies and technical expertise, while rapid tests may sacrifice sensitivity and specificity for the advantages of speed and ease of performance. It is difficult to find a balance between accessibility of a diagnostic method and the confidence in the test results. Antigen detection assays seem most promising for rapid and early diagnosis in rural areas. In this regard, development of DENV diagnostic tests is ahead of those for CHIKV, but clearly both of these arboviruses are important causes of disease in their shared endemic regions and in travelers to these areas.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of the paper.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 583262, 10 pages
http://dx.doi.org/10.1155/2015/583262

Review Article

Overview of the Diagnostic Methods Used in the Field for Human African Trypanosomiasis: What Could Change in the Next Years?

Julien Bonnet,[1,2] Clotilde Boudot,[1,2] and Bertrand Courtioux[1,2]

[1]*INSERM, U1094, Tropical Neuroepidemiology, Limoges, France*
[2]*UMR_S 1094, Tropical Neuroepidemiology, Institute of Neuroepidemiology and Tropical Neurology, Université de Limoges, CNRS FR 3503 GEIST, 87000 Limoges, France*

Correspondence should be addressed to Bertrand Courtioux; bertrand.courtioux@unilim.fr

Received 12 March 2015; Revised 19 June 2015; Accepted 2 July 2015

Academic Editor: Stephan Karl

Sleeping sickness is a parasitic infection caused by two species of trypanosomes (*Trypanosoma brucei gambiense* and *rhodesiense*), transmitted by the tsetse fly. The disease eventually affects the central nervous system, resulting in severe neurological symptoms. Without treatment, death is inevitable. During the first stage of the disease, infected patients are mildly symptomatic and early detection of infection allows safer treatment (administered on an outpatient basis) which can avoid death; routine screening of the exposed population is necessary, especially in areas of high endemicity. The current therapeutic treatment of this disease, especially in stage 2, can cause complications and requires a clinical surveillance for several days. A good stage diagnosis of the disease is the cornerstone for delivering the adequate treatment. The task faced by the medical personnel is further complicated by the lack of support from local health infrastructure, which is at best weak, but often nonexistent. Therefore it is crucial to look for new more efficient technics for the diagnosis of stage which are also best suited to use in the field, in areas not possessing high-level health facilities. This review, after an overview of the disease, summarizes the current diagnosis procedures and presents the advances in the field.

1. General Presentation of the Disease

Human African Trypanosomiasis (HAT), or sleeping sickness, is a vector-borne parasitic disease endemic in sub-Saharan Africa. This disease is caused by an extracellular parasite called *Trypanosoma* (genus) *brucei* (species). Three subspecies exist, which possess identical morphological characteristics (presence of a flagellum, a kinetoplast, and a nucleus) but differ in their ability to infect various hosts. *Trypanosoma brucei brucei* (*T.b.b.*) is a domestic animal parasite, which transmits Nagana disease, which is not pathogenic to humans [1]. The destruction of *T. b. brucei* is caused by two trypanolytic factors (TLF) complex content in human serum. Both TLF complexes include apolipoprotein L-1 (APOL1) and haptoglobulin-related protein (Hpr). Hpr has been thought for a long time to be the active trypanolytic component of TLF. But now there are more and more confirmative evidences showing that APOL1 is the trypanolytic factor of normal human serum [2]. This parasite has proven particularly useful for research purposes. Regarding the 2 human pathogens [3], *T. b. gambiense* is an anthroponotic parasite found in 24 countries of central and western Africa and causes a chronic syndrome. *T. b. rhodesiense* is zoonotic and is endemic in 13 countries of eastern and southern Africa and causes an acute syndrome [4]. However, increasingly, the spread of *T. b. rhodesiense* has been found, especially in Uganda, where the 2 diseases forms overlap. *T. b. gambiense* is present in the north while *T. b. rhodesiense* is present in the south, but this distribution remains artificial due to population migrations and climatic changes [5].

Recently, its prevalence has dropped, largely because of the implementation of controls and intervention programs. It belongs to the group of Neglected Tropical Diseases. Neglected Tropical Diseases are diseases that develop mainly among the poorest populations. Currently HAT is one of 17 priority Neglected Tropical Diseases recognized by WHO

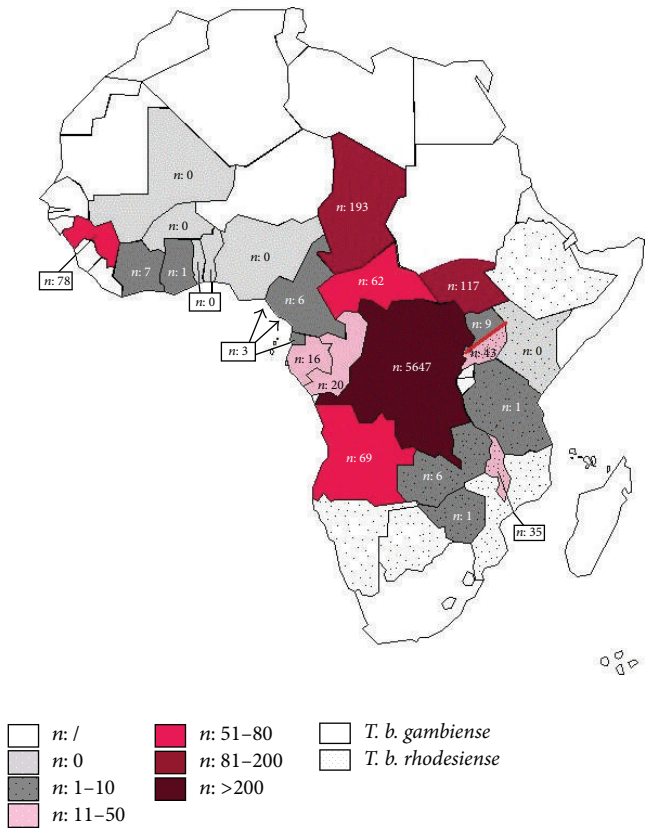

Figure 1: Number of new cases of HAT reported in 2013 to the WHO [1].

(World Health Organization) as Malaria, HIV, and others [6]. HAT is considered to be a huge threat to public health. Three severe epidemics have ravaged African populations. The first occurred at the end of the 19th century, the second during the 1920s, and the most recent began at the end of the 1970s and tends to be controlled today [1]. This disease outbreak is essentially linked to diverse social, economic, and political issues. Indeed, 36 sub-Saharan African countries are affected [4, 7], especially poor and remote rural regions (Figure 1). Furthermore, current estimations show that 70 million people live at risk of contracting HAT infection. Among these, 57 million people are at risk of developing *gambiense* HAT and 12.3 million people are at risk of contracting *rhodesiense* HAT [4]. This disease is considered by WHO to be one of the Neglected Tropical Diseases, for which it is necessary to establish population screening and disease control measures [4, 6].

This disease is transmitted by the bite of the tsetse fly during its blood meal. The *Glossina* vector belongs to the Diptera order. *Glossina* is viviparous and both the male and female are capable of spreading disease [1]. Many subgenus flies are involved in the transmission of parasites: *G. palpalis palpalis* and *G. p. gambiensis* transmit *T. b. gambiense* and *G. morsitans* transmits *T. b. rhodesiense* [8]. These flies need particular conditions to survive (temperature 16°C–38°C, 50%–80% relative humidity) [6]. However, the *Glossina* is classed as a "bad vector," because it loses parasites at every blood meal, and because the female produces only 10 larvae during its lifetime [9].

During the blood meal, the infected tsetse fly injects its saliva to prevent the coagulation of the host blood, and the metacyclic trypomastigote trypanosomes are injected subdermally into the host [4]. The trypanosomes proliferate at the site of inoculation and then transform into bloodstream trypomastigotes form during the first disease stage. That form can then multiply by binary fission, in different body fluids (blood, lymph), and can move to the cerebrospinal fluid (CSF), signaling the beginning of the second disease stage. If a new, noninfected tsetse fly bites the infected host, it can ingest parasites, in their bloodstream trypomastigote form, which can move to the fly midgut, where some will differentiate into procyclic trypomastigotes. Afterwards, the parasites migrate from the midgut to the salivary gland and transform into epimastigotes. In the salivary gland, the epimastigotes further transform into metacyclic trypomastigotes and await a new fly blood meal (Figure 2).

HAT clinically evolves in two stages and the symptoms for the *T. b. gambiense* and *T. b. rhodesiense* forms are often the same, but their frequency, severity, and kinetic appearance differ. Indeed, *T. b. rhodesiense* can cause patient death within 6 months, whereas *T. b. gambiense* patients can survive for more than 10 years [10, 11].

The first stage is called the hemolymphatic or bloodstream stage and is characterized by an intermittent fever,

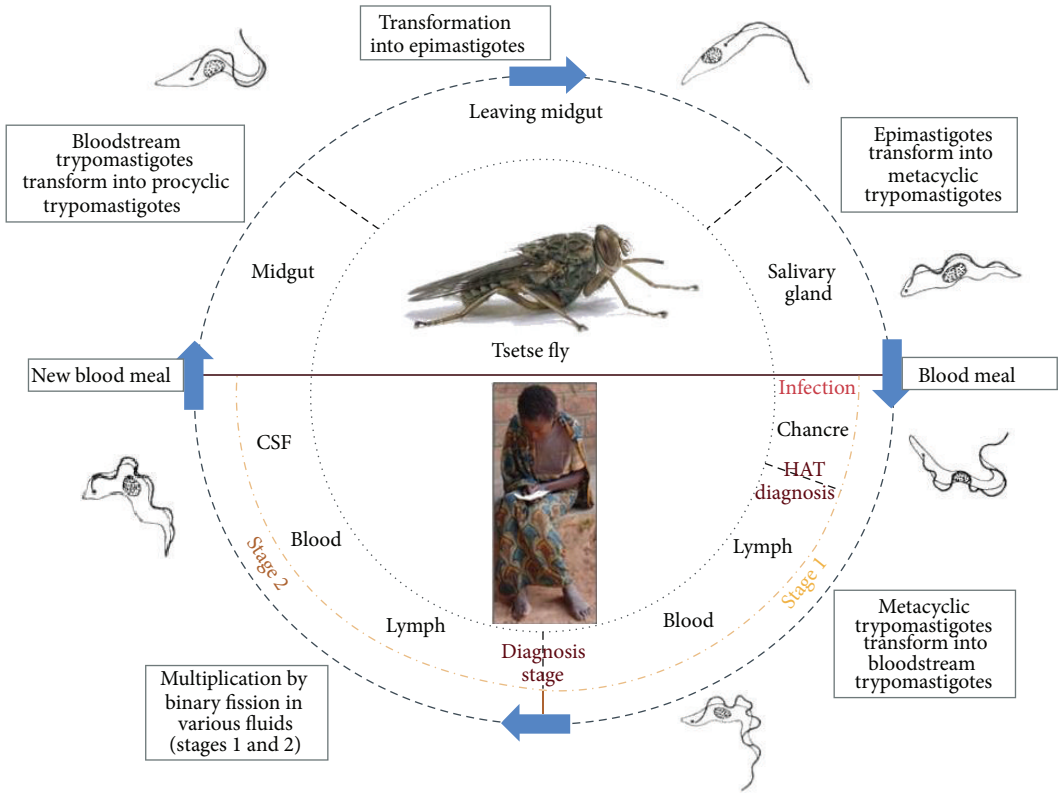

Figure 2: Life cycle of HAT.

headaches, pruritus, lymphadenopathy, asthenia, anemia, and hepatosplenomegaly [1, 4]. Once the parasites cross the blood-brain barrier (BBB), the meningoencephalic stage begins and the major symptoms are neuropsychiatric and include sleep disturbances, abnormal movement, limb paralysis, hemiparesis, irritability, aggressive behavior, and psychotic reactions [1, 4, 10]. This second stage is fatal if untreated.

Moreover, the impact on quality of life is potentially devastating, as affected subjects are unable to work for several years, which engenders poverty and social exclusion. Treatment development and therapeutic management are therefore very important. Treatments are separated into two groups.

The first group of treatments is composed of Pentamidine (Pentacarinat) and Suramin (Moranyl), and these treatments are mainly used during early disease stages. Pentamidine is the drug of choice for treatment of the *T. b. gambiense* form, while Suramin is used for *T. b. rhodesiense* treatment. However, Suramin cannot be used against *T. b. gambiense* in Western and Central Africa, because there is a risk of adverse reaction if combined with the medication used to treat *Onchocerca spp.* [1]. Pentamidine is administered intramuscularly and Suramin through intravenous injection. Pentamidine is generally well tolerated, despite side effects including hypoglycemia, nausea and vomiting, and injection site pain. Suramin can cause severe reactions, such as allergic reaction, hypersensitivity, nephrotoxicity, hematuria, or peripheral neuropathy [12].

Second stage treatments include Melarsoprol (Arsobal), Eflornithine (DFMO or α-Difluoromethylornithine), and the more recently developed Eflornithine/Nifurtimox combination therapy (NECT) [13]. Melarsoprol and Eflornithine are administered by intravenous injection; Nifurtimox is given orally. Melarsoprol is the only medication which can be used to treat both HAT forms [1, 12], although one of the known side effects is an increased risk of a potentially fatal encephalopathic syndrome. NECT has now become the standard first-line treatment for CNS stage *T. b. gambiense* HAT. Concerning CNS stage *T. b. rhodesiense* HAT it is intravenous Melarsoprol which is the first-line treatment [14]. However, Eflornithine causes similar adverse drug reactions as antineoplastic agents [15]. Nifurtimox can only be used in association with Eflornithine, against *T. b. gambiense*, and increases the efficacy of Eflornithine. All of these treatments require clinical surveillance during the therapeutic care. This is a major drawback for people with no access to health structures. In Table 1 are grouped the different dosages of the drugs currently used to fight against HAT.

Aside from the not insignificant adverse effects of these medications, a degree of drug resistance has evolved in the 15 to 50 years that these treatments have been employed, including Pentamidine, Melarsoprol, and Eflornithine [13, 16]. Therefore, the development of new, effective, and safe therapies is essential to advance the fight against HAT.

Recently, two new candidate drugs have been proposed. Fexinidazole, the 2-substituted 5-nitroimidazole, belongs to

Table 1: Drugs dosage used against THA.

	Pentamidine	Suramin	Melarsoprol	Eflornithine	Eflornithine/Nifurtimox
Dosage	4 mg/kg/day during 7 days	100–200 mg the first day and maximum 1 g/injection for 7 days	2.2 mg/kg/day for 10 days (for *T.b.g*) 3 × 3.6 mg/kg/day for 7 days (for *T.b.r*)	100 mg/kg/6 h during 14 days	200 mg/kg/12 h for 7 days (Eflornithine) + 5 mg/kg/3x day for 10 days (Nifurtimox)

the nitroimidazole class of drugs [17, 18]. This pharmacological class includes many active compounds, several of which target trypanosomes. Fexinidazole was discovered in the 1980s by the Drugs for Neglected Diseases initiative (DNDi) and was developed jointly with Sanofi. In studies, Fexinidazole exhibited trypanocidal properties and demonstrated the potential to become a safe, short-course oral treatment for both HAT stages. Furthermore, this therapy, currently undergoing phase 2/3 clinical trials in treatment centers in the Democratic Republic of Congo (DRC) and Central African Republic (CAR), may avoid the necessity of disease stage screening and treatments requiring several days of hospitalization [17, 19].

The other potential treatment candidate, Benzoxaborole or SCYX-7158, is a by-product of the family of oxaboroles, developed by Anacor Pharmaceuticals. This drug proved to be highly effective in preclinical studies and is in phase 1 clinical trials today, as a single dose oral treatment for both HAT stages [18, 20]. This drug would be the ideal candidate to use for disease elimination, if current trials prove successful.

To date, these ideal treatment options are not available in the field and treatment remains "stage dependent" with serious side effects and potential complications during the second stage of the disease. Improvement in staging diagnosis and early screening methods are current challenges which would avoid delayed patient treatment.

2. Management of the Disease in the Field: Diagnosis

Diagnosis should be made as early as possible, in order to avoid disease progression to the neurological stage, which may necessitate complex and potentially unsafe treatments. Exhaustive screenings require major investment in personnel and material resources. In Africa such resources are often limited, especially in remote areas where the disease is most common. As a result, many infected people may die before diagnosis or treatment.

2.1. The Diagnosis of HAT Is Based on Active Screening (Figure 3). Antibody and parasite detection are needed for adequate patient examination and successful diagnosis in the field [1]. In this review, we only present the most currently used technics in the field and propose how to put them into practice for field diagnosis of HAT in the context of a prospective campaign with a proposition of possible decision tree (Figure 3).

2.1.1. Antibody Detection

CATT (Card-Agglutination Trypanosomiasis Test). CATT is a serological test, useful for initial population screening to identify suspected cases. The test was developed in the late 1970s. It can be carried out on blood, capillary blood obtained from a finger prick, or blood from impregnated filter papers [21]. Antigen used for the test CATT is complete bloodstream forms of *T. b. gambiense* variable antigen type LiTat 1.3. This test can be performed on plasma or serum dilutions for which it is more specific than the CATT on blood and is therefore used to reduce the number of false-positive reactions, often before parasitological examinations. The sensitivity of CATT on blood is about 91%, with a range of 78–99.8%, and negative predictive values as high as 99–100% have been reported in mass population screening [22, 23]. False-negative CATT results may be obtained for patients infected with strains of trypanosomes that do not express the LiTat 1.3 gene, resulting in lower sensitivity of CATT in some endemic areas [24, 25]. Despite a specificity of about 97%, the positive predictive value of the CATT remains limited when the test is used for mass screening in populations in which the overall prevalence of *gambiense* HAT is low [23, 26–28]. False-positive results are found for patients with other parasitic diseases, such as malaria and filariasis, or a transient infection with *T. b. brucei*. Parasite CATT titration is done by some control programs after all parasitological examinations. This titer also depends on the country [6]. Because of its simplicity, reliability, and low cost, it is used in all control programs for serological screening of populations at risk for *T. b. gambiense* infection.

2.1.2. Parasite Detection

(i) Lymph Node Examination. The lymph node palpation is realized only for patient with a positive CATT. The fluid is examined rapidly after puncture. The sensitivity of lymph node palpation and aspiration varies from about 40% to 80% depending on parasite strain, stage of disease, and the prevalence of other diseases which may cause lymphadenopathy [6].

(ii) mAECT (Mini Anion Exchange Centrifugation Technique or mAECT). Parasitological investigation with minicolumns by anion exchange can be carried out on venous blood. Patient blood cells are negatively charged, while trypanosomes remain neutral, so that they can be separated by anion-exchange chromatography at pH 8 [29, 30]. For mAECT, 400 μL of blood is applied onto a column containing

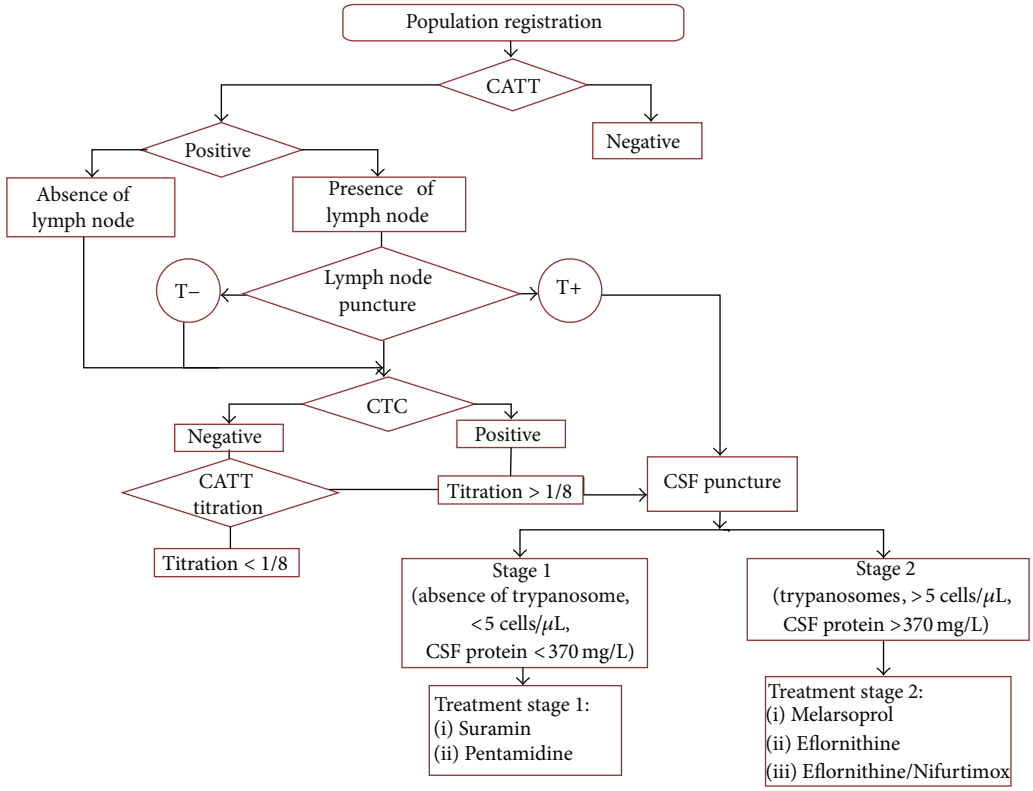

Figure 3: Decision tree of HAT stage diagnosis.

diethylaminoethyl cellulose. The blood cells stay on the gel, and the eluant containing the trypanosomes is collected in a tube. Trypanosomes are concentrated at the bottom of the tube by low-speed centrifugation (1000 g for 15 min), and the tip of the tube is examined in a special holder under a microscope (10 × 10 or ideally 10 × 16 magnification) for the presence of trypanosomes. The large blood volume used in the mAECT allows detection of fewer than 30 trypanosomes/mL, resulting in a high diagnostic sensitivity of 77% (68.8–92.1%) for mAECT [6]. This technic is time consuming and needs materials (buffer, column, etc.) and good technicians. Its use in the field is discussed by some authors and programs.

(iii) CTC (Capillary Tube Centrifugation). The CTC technic is done on capillary tubes containing anticoagulant which are filled to three quarters (about 50 μL) with finger-prick blood. The dry end is sealed with plasticine or by flame, avoiding heating of the blood and killing the trypanosomes. Trypanosomes are concentrated in the same layer as the white blood cells (WBCs), between the plasma and the erythrocytes, by high-speed centrifugation (12 000 g for 5 min) in a hematocrit centrifuge. The capillary tubes are mounted in a special holder or between a microscope slide and a coverslip, and the empty space between the glass surfaces is filled with water to reduce diffraction. The capillary tubes are examined at low magnification (10 × 10) for mobile parasites at the junction of the WBC layer and the plasma layer. If available, use of 16x ocular lenses facilitates recognition of trypanosomes. The detection limit of the CTC is estimated to be about 500 trypanosomes/mL. To increase its sensitivity, examination of at least four capillary tubes per person is recommended. The sensitivity is about 56% (39–80%) [6].

Disease stage identification, by examination of the cerebrospinal fluid (CSF), obtained by lumbar puncture, helps to establish the degree of progression of the disease and subsequently to determine the most appropriate treatment in each case (Figure 3).

2.2. Stage Diagnosis. Differentiation between the two stages can only be done by examination of the CSF after lumbar puncture. The detection of trypanosomes in CSF by microscopy alone has limited sensitivity and has a poor reproducibility rate. The number of parasites circulating in CSF can be very low, generating false negative results. An increased white blood cell (WBC) count in CSF is an indicator of meningitis and can help to increase the sensitivity of parasite detection. The WHO diagnostic criteria, which require the presence of trypanosomes in the CSF or a WBC count of more than 5 cells per μL, or both [1], are the most widely used guidelines for diagnosing late stages of the disease. Some clinicians use a higher white blood cell count cutoff point such as 20 cells per μL, especially for diagnosing CNS *T. b. gambiense* HAT. A consensus about the optimum WBC count of 10 cells per μL has been suggested for staging HAT [13].

There are reports of some patients with CSF white blood cell count of 20 cells per μL or less being treated successfully

with an early-stage drug like pentamidine, which highlights the possibility of an intermediate stage of infection [31–33]. This intermediate stage is characterized by parasites which have crossed the BBB but have not yet spread to the brain parenchyma.

Another potential parameter which may assist with late-stage diagnosis is the measurement of CSF IgM concentrations, which, due to synthesis within the spinal cord, are increased early in disease development in cases where there is CNS involvement. However all of these approaches have an intrinsic drawback; there is no gold standard of CNS HAT diagnosis with which to compare any new methods [14]. Furthermore, WBC counting is not specific to sleeping sickness, and alternative diagnoses or coexisting diseases, such as malaria, syphilis, HIV infection, tuberculosis, and toxoplasmosis, need to be investigated and excluded [34–38]. Most of the articles which report on the staging of sleeping sickness disease agree that WBC counting must be supported and confirmed by newer, more advanced diagnostic procedures.

3. New Research Pathways to Improve the Diagnosis of HAT

3.1. Screening of the Population. Existing diagnostic procedures are complex and cumbersome to implement because they require specialized mobile teams, trained to carry out rapid testing using invasive protocols. Research on this disease seeks to develop simplified tests which enable the integration of activities related to HAT diagnosis within the public health infrastructure. Thus, the target of HAT phase-out by 2020, as stipulated in the WHO roadmap and the London Declaration on Neglected Tropical Diseases, will have to be achieved through the development of rapid tests which are easy to produce on a large scale [6]. Several promising tests are under development.

Lateral flow immunochromatographic devices can detect low concentrations of antibodies targeting antigens in biological fluids [39, 40]. This technology can be used to develop rapid diagnostic tests (RDTs) that detect anti-trypanosome antibodies in human finger-prick blood samples. These RDT-based lateral flow devices are simple to use and easy to read and have stability characteristics that allow wide distribution and availability in remote endemic areas. The first RDTs for HAT diagnosis are currently being tested in the field. The tests were developed by Standard Diagnostics (SD BIOLINE HAT) and Coris Bioconcept (Sero-K-SeT) [41, 42]. They are based on a device using native surface glycoproteins (VSG) LiTat 1.3 and LiTat 1.5 to test for anti-trypanosome antibodies [41, 42]. Both tests show good ranges of sensitivity and specificity when compared to CATT [42]; however, improvements are still needed especially to facilitate test production and cost. Thus, recombinant antigens are currently being produced in line with these objectives.

The second prototype device, which uses the potential ISG65 diagnosis [43], is based on a combination of recombinant and native ISG65 VSG MiTat 1.4 [44]. ISG 65 is one of two well-characterized type 1 invariant surface glycoproteins, which have moderately abundant transmembrane domains, expressed in *T. brucei* [45].

3.2. Advances in CSF Stage Diagnosis. The diagnosis of stage HAT is a key component in the therapeutic care of patients due to the high toxicity of some drugs including Melarsoprol that lead to arsenical encephalopathy in 5% of cases. So there is an urgent need to develop a quick, reliable, easy to perform, and cheap diagnostic test that can be used for HAT staging. The research and development of methods for disease staging have been revitalized, especially through an initiative launched by FIND and WHO in the 2000s and several alternative staging biomarkers and tools are under investigation.

3.2.1. Antibodies. Many published studies have investigated disease stage diagnosis, at the molecular level. In blood, and particularly plasma samples, some studies have observed decreased levels of cytokines such as IFN-γ or IL-10 and NO after treatment. These markers may be compared to control subject plasma [46]. Staging studies have primarily focused on CSF as the ideal body fluid for examination, due to its proximity to the CNS. Some research has focused on CSF antibodies. An alteration in the protein concentration of CSF, such as an increase in albumin or immunoglobulin, could indicate a BBB dysfunction or increased intrathecal synthesis of proteins [47]. We have known since the 1980s that the increased concentration of immunoglobulin in the CSF and the absence of a switch between IgM and IgG are characteristic of the immune response in the brain. More recently, some publications have demonstrated that the increased intrathecal IgM fraction is a sign of the presence of a brain inflammatory process, not necessarily connected to damage of the BBB in Stage 2 HAT patients [48]. Intrathecal IgM levels are considered by many to be superior to WBC counting as a parameter for HAT staging, especially for *T. b. gambiense* cases.

3.2.2. Cytokines and Chemokines. Another field of research being explored for the development of new diagnostic procedures for HAT staging is the modulation of immune-effectors such as cytokines and chemokines. The neuroinflammation seen in late stage HAT presents some characteristics such as the early activation of macrophages and astrocytes, the upregulation of inflammatory cytokines, and the presence of Mott cells (plasma cells containing IgM). Activated astrocytes and macrophages are two important sources of pro- and anti-inflammatory cytokines and chemokines in the brain. The level of these cytokines and chemokines has been measured for the investigation of their diagnostic potential both in *T. b. gambiense* and *T. b. rhodesiense*. Cytokines and chemokines are also associated with the recruitment of leukocytes to the site of inflammation and their passage through the BBB, but also with the increase of WBC observed in CSF during the second stage of HAT. The most interesting cytokines and chemokines used for staging sleeping sickness are IL-10, IL-6, IL-1β, CCL-3, CXCL-8, SLPI, Lipocalin 2,

ICAM-1, VCAM, MMP-9, MMP-2, CXCL-10, and CXCL-13 [48, 49], which permit the activation and amplification of the immune response and allow leukocytes which are sequestered in the perivascular space to transmigrate across the basement membrane and the glial limitans to reach the brain parenchyma [50]. A recent study initially evaluated the most promising molecules such as CXL-10, CXCL-13, ICAM-1, VCAM-1, IgM, MMP-9, and B2MG and confirmed their capacity to act as accurate staging markers [50]. Furthermore, Neopterin as a new marker for staging of HAT was introduced and validated [51, 52]. Neopterin is an indicator of activation of the cellular immune response and has good potential not only as a staging marker but also for treatment outcome. The possibility of establishing a quick blood test for additional lateral disease testing, which is appropriate for field application, is advancing and is currently the primary focus of research and development [53]. This study was conducted only on *T. b. gambiense* patients. Some studies describe different outcomes for *T. b. rhodesiense* patients, largely due to the different neuropathogenesis of the two diseases [54]. In addition to being good staging markers, the level of these molecules seems to correlate with the severity of the neurological symptoms and therefore may assist with screening for the advanced second HAT stage [14]. The downside of these markers is the lack of specificity. Indeed, 80% of the CSF proteome is composed of blood derived proteins [55], and only the remaining 20% are produced in the brain, and so they are rarely considered to be specific to the neuroimmune response [47]. Moreover, these molecules are not specific markers of sleeping sickness, and other diseases such as malaria, which is also largely present in the countries affected by HAT, may also be responsible for the increased levels of these cytokines and chemokines. The vast majority of studies regarding this topic advocate the combination of multiple markers to increase staging accuracy [56, 57].

3.2.3. Proteomics. Another approach currently under investigation is the evaluation of the changes in protein expression between pathological and healthy conditions. Only a few studies have established first and second stage HAT disease CSF protein profiles. Previous studies have shown a large increase in the amount of immunoglobulins for stage 2 patients [46, 48], but they also show 73 proteins which are differentially expressed between the two stages. Two of these proteins, osteopontin and beta-2-microglobulin, were confirmed to be accurate markers of first and second stage patients [58]. It is important to research and study new protein biomarkers, particularly for discriminating stage 2 and stage 1 of the disease, and this is possible thanks to progress in matters of protein and peptide analysis with the evolution of mass spectrometry, for example, [59].

3.2.4. Polysomnography. In recent years, research has been conducted on the most typical clinical manifestation of HAT: the alteration of the normal sleep-wake cycle [1]. Polysomnography has been used for these studies. Polysomnography is a medical examination which involves the recording of several physiological variables, such as respiratory and heart rate, and carrying out other tests including an electroencephalogram, an electromyogram, and an electrooculogram, during patient sleep, in order to investigate sleep disorders. Studies show a high number of Sleep Onset Rapid Eye Movement Periods (SOREMP) in stage 2 patients during their sleep, not only restricted to nighttime, but also during daytime sleep too. Treatment with Melarsoprol seems to reduce the appearance of SOREMPs. In spite of the successful outcomes of these studies and the noninvasive nature of this diagnostic tool, polysomnography is largely neglected due to the difficulty in establishing the necessary environment for such examinations, which require high-tech and bulky material, trained personnel, and extended examination periods [32]. It is therefore difficult to use as a diagnostic tool in the field. Moreover, this diagnostic tool is not specific because the observed increase in SOREMPs may be attributed to other sleep disorders. In addition, SOREMPs may be detected early in the disease and so are not specific markers of stage 2 HAT.

3.2.5. DNA Amplification. Carrying out PCR to amplify specific parasite DNA sequences obtained from blood, CSF, urine, or saliva samples has been proposed for staging of the disease. The loop-mediated isothermal amplification (LAMP) technic for staging HAT disease is promising and shows high specificity and sensitivity. Furthermore, for this technic, the target DNA is amplified at a constant temperature, so this test can be used in the field with minimal equipment or in the low level laboratories available in HAT endemic countries. The test can be performed on fresh blood samples, or even on blood samples which are dried on microscopy slides or on ordinary filter papers. Moreover, no gel electrophoresis is required, as positive results can be visually identified (fluorescence, white precipitate, or color change) and the analysis of several samples may be carried out simultaneously. Sets of specific primers were designed and validated and the reproducibility was verified using samples obtained from HAT patients [60–62]. This test is currently used mainly in DRC and Angola to see if there is a good candidate disease staging and is employed up to 24 months after treatment is completed, to see if it can be used to confirm disease remission (http://www.finddiagnostics.org/programs/hat-ond/hat/molecular_diagnosis.html). A similar technic for RNA amplification has recently been introduced. The Trypanozoon-specific real-time nucleic acid sequence-based amplification (NASBA) assay allows the detection of parasite 18S ribosomal RNA [63].

Recent study using CSF PCR of *T. b. gambiense* patients for stage determination indicated a good staging accuracy of PCR especially for stage 2 patients before treatment. But the presence of parasite DNA or RNA in CSF of stage 2 HAT patients should be interpreted with care as the low specificity of molecular test [63].

However, for the posttreatment follow-up, molecular biology seems to be for several authors not a good marker. The specificity and sensitivity of a diagnostic PCR mainly depends on the DNA sequence targeted by the primers. Therefore it is important to continue research for optimizing amplification,

by designing new primers [64]. This technic is not quite optimal in a the field and is still debatable within scientific community.

4. Conclusion

After more than 50 years of neglect, the international community has recognized the need to eliminate sleeping sickness in Africa. The signing of a partnership agreement in 2001 between the WHO and Aventis announced the advent of a new era in the fight to eliminate the *Trypanosoma* parasite from the African continent. Private partners, Non-government Organizations (NGOs), institutional partners, and the Belgian and French governments are fully engaged, working hand in hand with relevant organizations (WHO, FAO, etc.), with the objective to find new diagnosis tests. The successful elimination of the disease in Africa needs a better management of patients. The research for new stage biomarkers for sleeping sickness is a key for the eradication of the disease since actually no dependent stage treatment is accessible by all the people concerned by HAT.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 867846, 7 pages
http://dx.doi.org/10.1155/2015/867846

Research Article

Loop Mediated Isothermal Amplification for Detection of *Trypanosoma brucei gambiense* in Urine and Saliva Samples in Nonhuman Primate Model

Maina Ngotho,[1] John Maina Kagira,[2] Beatrice Muthoni Gachie,[3] Simon Muturi Karanja,[4] Maxwell Wambua Waema,[3] Dawn Nyawira Maranga,[1] and Naomi Wangari Maina[3]

[1]*Animal Science Department, Institute of Primate Research (IPR), P.O. Box 24481, Karen, Nairobi 00502, Kenya*
[2]*Animal Health and Production Department, College of Agriculture and Natural Resources, Jomo Kenyatta University of Agriculture and Technology (JKUAT), P.O. Box 62000, Nairobi 00200, Kenya*
[3]*Biochemistry Department, College of Health Sciences, Jomo Kenyatta University of Agriculture and Technology (JKUAT), P.O. Box 62000, Nairobi 00200, Kenya*
[4]*Public Health Department, College of Health Sciences, Jomo Kenyatta University of Agriculture and Technology (JKUAT), P.O. Box 62000, Nairobi 00200, Kenya*

Correspondence should be addressed to Naomi Wangari Maina; nmaina@jkuat.ac.ke

Received 9 February 2015; Revised 22 April 2015; Accepted 3 May 2015

Academic Editor: Malcolm Jones

Human African trypanosomiasis (HAT) is a vector-borne parasitic zoonotic disease. The disease caused by *Trypanosoma brucei gambiense* is the most prevalent in Africa. Early diagnosis is hampered by lack of sensitive diagnostic techniques. This study explored the potential of loop mediated isothermal amplification (LAMP) and polymerase chain reaction (PCR) in the detection of *T. b. gambiense* infection in a vervet monkey HAT model. Six vervet monkeys were experimentally infected with *T. b. gambiense* IL3253 and monitored for 180 days after infection. Parasitaemia was scored daily. Blood, cerebrospinal fluid (CSF), saliva, and urine samples were collected weekly. PCR and LAMP were performed on serum, CSF, saliva, and urine samples. The detection by LAMP was significantly higher than that of parasitological methods and PCR in all the samples. The performance of LAMP varied between the samples and was better in serum followed by saliva and then urine samples. In the saliva samples, LAMP had 100% detection between 21 and 77 dpi, whereas in urine the detection it was slightly lower, but there was over 80% detection between 28 and 91 dpi. However, LAMP could not detect trypanosomes in either saliva or urine after 140 and 126 dpi, respectively. The findings of this study emphasize the importance of LAMP in diagnosis of HAT using saliva and urine samples.

1. Introduction

Human African trypanosomiasis (HAT) is a tropical disease that is endemic in several countries in sub-Saharan Africa. Control of sleeping sickness relies on passive case detection and it is considered to be the most cost-effective when compared to active case detection [1]. Sleeping sickness caused by *T. b. gambiense* is currently responsible for over 90% of all HAT cases [1]. Screening of the population at risk is done by antibody detection with the Card Agglutination Test for Trypanosomiasis (CATT) and confirmed by parasitological methods. Serological tests have varying sensitivities and cannot decisively differentiate between active and cured cases. Furthermore, cured patients can remain CATT seropositive for up to three years due to persisting circulating antibodies, thus prohibiting the use of antibody tests for assessment of treatment success [2]. The parasitological detection techniques also have limitations. The methods are time consuming, tedious, and prone to subjectivity. In addition, low detection rates may occur since *T. b. gambiense* infection is characterized by low parasitemia [3]. False negatives (CATT negative) but parasitemic cases have also been reported [4].

These limitations imply the need for more sensitive and specific diagnosis.

The amplification of DNA has emerged as one of the diagnostic techniques used in studies of infectious diseases [5]. Species specific genes have been used to characterize trypanosomes [6]. The discovery of the *T. b. gambiense*-specific glycoprotein (TgsGP) gene that is specific to the *T. b. gambiense* subspecies heralded its use as a probe for diagnosis. It is the only subspecies-specific gene for *T. b. gambiense* and encodes a 47 kDa VSG-like receptor protein [7]. Amplification of this gene using PCR has successfully been used in clinical samples [8]. However, challenges of the DNA extraction protocols may affect diagnosis of trypanosome infections [9] and requirements of expensive automated thermal cyclers make PCR impractical for adoption in the field [10].

Loop mediated isothermal amplification (LAMP) is performed under isothermal conditions and relies on autocycling strand displacement DNA synthesis [11]. It requires a simple heating device and is rapid and results are easily viewed by several detection formats. The autocycling reactions lead to accumulation of a large amount of the target DNA and by-products such as magnesium pyrophosphate allowing for rapid detection using varied formats. LAMP uses four to six specially designed primers recognizing six to eight regions of the target DNA sequence resulting in a high specificity. It has been used in detection of the *Trypanozoon* subgenus [12], *T. b. rhodesiense* [13], and recently Group 1 *T. b. gambiense* [14]. The test has high sensitivity and specificity and does not require specialized equipment, and this makes it a suitable diagnostic test in resource poor settings and would therefore be ideal diagnosis of neglected diseases such as HAT.

The importance of experimental animal models includes controlled conditions and planned sampling among other. The vervet monkey (*Chlorocebus aethiops*) has been developed as a model for early stage HAT caused by *T. b. gambiense* [15]. Using this animal model, the performance of LAMP based on the *TgsGP* gene was assessed in detection of *T. b gambiense* in serum, CSF, saliva, and urine.

2. Materials and Methods

2.1. Trypanosomes. *Trypanosoma b. gambiense* isolate IL3253 was used in this study. It was isolated from a human HAT patient from Sudan in 1982. The isolate was cryopreserved in liquid nitrogen and for infective purposes the parasites were subinoculated into immunosuppressed donor Swiss mice. At peak parasitemia, heart blood was obtained by cardiac puncture and parasites harvested and diluted to 10^5/mL using phosphate saline glucose.

2.2. Experimental Animals. Six adult vervet monkeys of both sexes weighing between 2.0 and 5.0 kg were used in this study. They were trapped from the wild in an area known to be nonendemic for human trypanosomiasis. The animals underwent a 90-day quarantine during which they were screened for zoonotic diseases and treated for ecto- and endoparasites. They were also trained for ease of adaptation and maintained on commercial pellets (Unga Feeds Ltd., Nairobi, Kenya) supplemented with fresh fruits and vegetables. Drinking water was provided *ad libitum*. The monkeys were housed in stainless steel cages at ambient room temperatures of 18–25°C, under biosafety level II animal holding conditions. At the end of the experiment period, the animals were euthanized by injection with Euthatal (20% sodium pentobarbitone, Rotexmedica®, Trittau, Germany) via the femoral vein.

2.3. Study Design. Six monkeys were infected intravenously with approximately 10^5 trypanosomes in 1 mL of phosphate saline glucose. The infected monkeys were monitored for a total period of 180 days after experimental infection. Parasitaemia was estimated daily using methods previously described using the rapid matching method [16] and haematocrit centrifuge technique [17].

2.4. Sample Collection. The monkeys were anaesthetized on weekly basis with ketamine hydrochloride (Rotexmedica, Trittau, Germany) at a dosage of 10 mg/kg body weight for sample collection. The samples were collected before and after infection on a weekly basis. Three mL of blood from the femoral artery and 1.5 mL of cerebrospinal fluid (CSF) via lumbar puncture were collected. Saliva samples were obtained by placing swabs under the animals tongue for ten minutes to allow for adequate wetting. Thereafter the swabs were placed in dry cryovial tubes. The urine was obtained via a collection apparatus placed on the bottom of the monkey cage and stored in 50 mL falcon tubes. This was done in the early morning prior to sedation. All samples were collected and stored at −20°C.

2.5. DNA Extraction. DNA was extracted from serum, urine, CSF, and saliva samples using genomic DNA isolation kits (Zymo Research, USA) as per manufacturer's instructions.

2.6. PCR. Amplification of *T. b. gambiense*-specific glycoprotein (*TgsGP*) gene was done using primer sequences as previously described [18]. The PCR reactions (nested) were performed as described [8] using 1 μL of extracted DNA in a 25 μL reaction mixture. The PCR amplification was performed by incubating the samples for 15 min at 95°C followed by 45 cycles of 1 min at 94°C, 1 min at 63°C, and 1 min at 72°C and a final extension at 72°C for 10 min. Thereafter, amplified products were analyzed by electrophoresis in 2% agarose gels. Gels were stained with ethidium bromide (0.5 μg/mL) (Sigma, USA) and viewed under UV illumination. The negative controls: purified DNA from *T. b. brucei* GUTAT1 and *T. b. rhodesiense* IPR001 and distilled water. The positive control was purified *T. b. gambiense* IL3253 DNA.

2.7. LAMP. The TgsGP primers as previously described were used [14]. The reaction mixture of 25 μL consisted of 40 pmol of the inner primers, 5 pmol of the outer primers, 20 pmol of the loop primers (Inqaba biotec, SA), 0.8M betaine (Sigma-Aldrich, St. Louis, MO, USA), 2.8 mM dNTPs mix, 1x Thermopol buffer (20 mM Tris-HCl pH 8.8, 10 mM KCl, 2 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100)

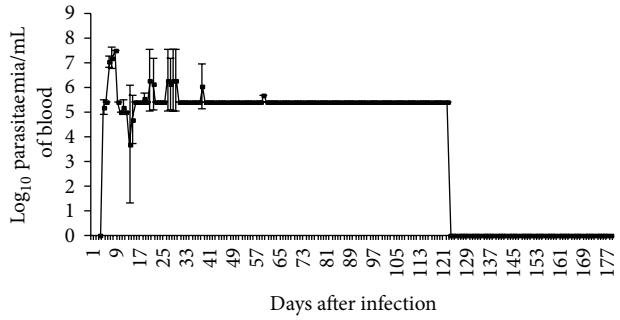

Figure 1: Mean daily parasitaemia of monkeys infected with *T. b. gambiense* IL3253. CSF: there was no CSF parasitosis observed during the experimental period.

(New England Biolabs, UK), and additional 4 mM $MgSO_4$, 8-unit *Bst* DNA polymerase (New England Biolabs, UK), double distilled water, and 2 μL of the template DNA. The positive and negative controls were similar to those used in the PCR reaction. The reactions were carried out in triplicate for 80 minutes in a Loopamp real-time turbidimeter LA320C (Eiken Chemical Co., Japan). Increase in turbidity indicates DNA amplification. After the reaction 1/20 dilution of SYBR green I dye (Sigma-Aldrich, St. Louis, MO, USA) was added to confirm the amplification.

2.8. Data Analysis. The percentage detection of the different tests and sample was determined and significant differences between the tests calculated using the chi-square (χ^2) test were determined. The differences were considered statistically significant when $p < 0.05$. The agreement between tests was quantified using Cohen's kappa statistic (k). Epicalc of EpiInfo 7 was used.

2.9. Ethics. All protocols and procedures used in this study were reviewed and approved by the Institute of Primate Research (IPR) Institutional Review Committee which incorporates Animal Care and Use Committee (IACUC) review (IRC/19/10).

3. Results

3.1. Parasitological Methods. The prepatent period was two to three days. The parasitaemia rose to a peak of 10^7 trypanosomes/mL of blood between 8 and 9 days after infection (dpi). Thereafter, the parasitaemia declined and was characterized by fluctuations to a minimum 2.5×10^5 trypanosomes/mL of blood by 123 dpi. However from 123 to 180 dpi the parasitaemia dropped to undetectable levels (Figure 1).

3.2. PCR. The positive control (*T. b. gambiense*) gave the expected 308-base-pair (bp) band. The negative controls (*T. b. brucei* and *T. b. rhodesiense*) were negative (Figure 2).

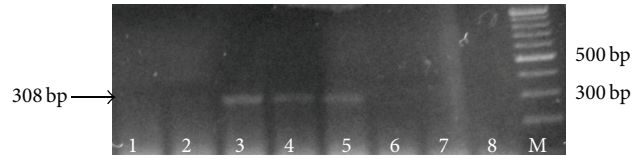

Figure 2: PCR results on gels after electrophoresis. Lane 1 (*Tbb*); Lane 2 (*Tbr*); Lane 3 (positive control *Tbg*); Lane 4 (saliva sample obtained on 14 dpi); Lane 5 (saliva sample obtained 28 dpi); Lane 6 (saliva sample obtained 56 dpi), Lane 7 (saliva sample obtained 70 dpi), Lane 8 (saliva sample obtained 84 dpi), and M (100 bp molecular marker).

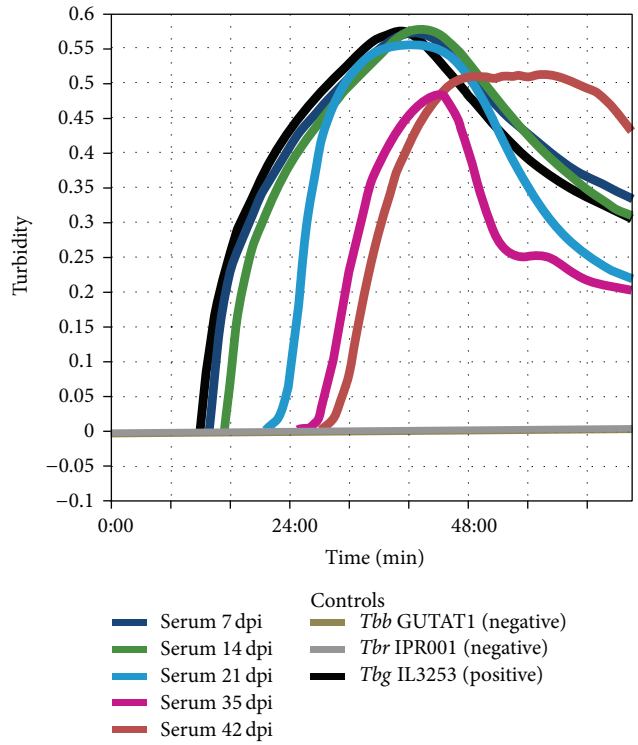

Figure 3: Amplification curves after LAMP reaction in turbidimeter.

3.3. LAMP. Increase in turbidity was noted for the positive control and some samples within 48 minutes of incubation. There was no increase in turbidity in the negative controls as expected (Figure 3). After addition of SYBR green I dye the positive LAMP reactions turned green while the negative ones remained orange (Figure 4).

3.4. Comparison between LAMP, PCR, and Parasitological Methods. Parasitological methods detected parasites in the infected monkeys by day 3 after infection (Figure 1). The detection rate gradually dropped and by 119 dpi only 50% detection was obtained. Thereafter the methods could not detect the parasites. Both PCR and LAMP recorded 100% detection in serum samples starting from 7 dpi. LAMP detected trypanosome DNA until 180 dpi but maintained 100% detection up to 133 dpi. On the other hand, PCR could

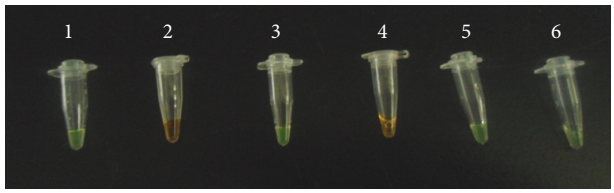

Figure 4: Visual appearance of LAMP results after addition of SYBR green I dye. Green color represents positive reaction while orange represents negative reaction. Tube 1: *Tbg positive* control, Tube 2: negative control (*Tbr*), Tube 3: serum sample obtained 28 dpi, Tube 4: CSF obtained 28 dpi, Tube 5: saliva sample obtained 28 dpi, and Tube 6: urine sample obtained 28 dpi.

Table 1: Detection (%) of parasitological methods, PCR, and LAMP in serum, saliva, and urine determined at weekly time points in vervet monkeys infected with *T. b. gambiense*.

DPI	Parasito.	PCR		LAMP		
		Serum	Saliva	Serum	Saliva	Urine
7	100	100	17	100	33	0
14	83	100	83	100	83	17
21	100	100	100	100	100	33
28	83	100	100	100	100	83
35	67	100	100	100	100	100
42	83	100	83	100	100	100
49	83	100	33	100	100	100
56	83	100	17	100	100	100
63	83	100	17	100	100	83
70	67	100	0	100	100	100
77	83	100	0	100	100	83
84	67	100	0	100	83	83
91	50	83	0	100	83	83
98	67	83	0	100	83	67
105	67	83	0	100	83	67
112	50	83	0	100	83	33
119	50	67	0	100	33	17
126	0	83	0	100	33	17
133	0	67	0	100	17	0
140	0	33	0	83	0	0
147	0	33	0	83	0	0
154	0	17	0	83	0	0
161	0	0	0	83	0	0
168	0	0	0	67	0	0
175	0	0	0	67	0	0
180	0	0	0	33	0	0

Key: Parasito. = parasitological techniques; DPI = days after infection.
*There was no amplification in any CSF sample with either LAMP or PCR. There was also no amplification noted in urine using PCR.

only sustain the 100% detection rate up to 84 dpi. Thereafter the detection dropped and from 161–180 dpi PCR did not detect trypanosome DNA (Table 1).

Table 2: Duration of detection of parasites in blood, serum, saliva, urine, and CSF samples using parasitological methods, PCR, and LAMP in vervet monkeys infected with *T. b. gambiense* for 180 days after infection (dpi).

Sample	Parasitology	PCR	LAMP
Blood	3–123 dpi (18%)	—	—
Serum	—	7–154 dpi (4%)	7–180 dpi (3%)
Saliva	—	7–63 dpi (11%)	7–133 dpi (5%)
Urine	—	—	14–126 dpi (8%)
CSF	—	—	—

*(1) The percentage of negative samples is given in brackets.

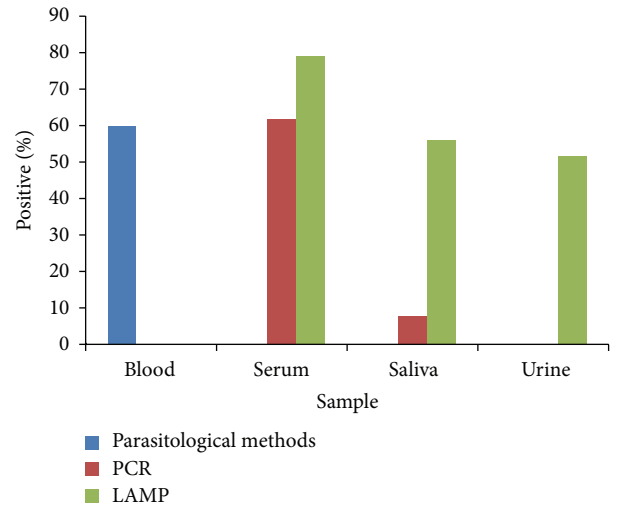

Figure 5: Comparison of LAMP, PCR, and parasitological methods in trypanosome mean detection rate in serum, saliva, and urine samples obtained from monkeys infected with *T. b. gambiense* IL3253 from 3 to 180 days after infection. *There was no amplification in any CSF sample with either LAMP or PCR. There was also no amplification noted in urine using PCR.*

In saliva samples, PCR detected trypanosome DNA from 7 to 63 dpi, thereafter no trypanosome DNA was detected. Between 21 and 77 dpi LAMP recorded 100% detection in the saliva samples. The detection dropped thereafter and from 140 to 180 dpi there was no trypanosome DNA detection. LAMP detected trypanosome DNA in urine samples between days 14 and 126 after infection (Table 1). PCR did not detect trypanosome DNA in the urine samples. Neither PCR nor LAMP detected trypanosome DNA in the CSF samples. Both PCR and LAMP detected parasites beyond the period when conventional parasitological methods did. Trypanosome DNA was detected longest in serum samples followed by saliva and urine (Table 2). The detection rate of LAMP was higher than that of PCR in the serum, saliva, and urine samples (Figure 5).

There was a significant difference in detection between LAMP and PCR ($p < 0.05$) in all the samples. Similarly, the difference in detection between LAMP and parasitological methods was significant ($p < 0.05$). There was good agreement between PCR and parasitological methods in detection

Table 3: Comparative analysis of LAMP, PCR, and parasitological techniques in trypanosome detection in serum, saliva, and urine samples obtained from monkeys infected with *T. b. gambiense* IL3253.

Test	Kappa value	Level of agreement	χ^2 statistic	p value
PCR and parasitology	0.83 (0.36–0.59)	Very good	2.22	0.136
LAMP and parasitology	0.48 (0.36–0.59)	Marginal	26.47	<0.001
LAMP and PCR (serum)	0.59 (0.47–0.72)	Marginal	10.42	0.0012
LAMP and PCR (saliva)	0.14 (0.06–0.21)	Poor	79.79	<0.0001
LAMP and PCR (urine)	0	Poor	6.12	0.0134

of trypanosomes. The difference in detection between both tests was not significant ($p > 0.05$) (Table 3).

4. Discussion

The presence of trypanosome DNA in saliva and urine samples is of great significance given the need for noninvasive samples for diagnosis of HAT. The higher sensitivity of LAMP compared to parasitological methods and PCR is also of significance in regard to the search for new diagnostic tests for sleeping sickness. In the current study the performance of LAMP and PCR was assessed in an early stage HAT model [15].

The high number of trypanosomes positive serum samples suggests that the trypanosomes were circulating in the hemolymphatic system. The lack of detection of trypanosomeDNA in the CSF samples may mean that the trypanosomes did not cross the blood-brain barrier and hence late stage disease did not occur. Infected monkeys without trypanosomes in CSF and having WBC counts of less than 5 cells/mm^3 are regarded as being in early stage of the disease.

PCR performed better than the parasitological methods in monitoring the presence of the infection but, however, it cannot be used as a gold standard in diagnosis of trypanosomiasis because of its challenges to implement in clinical settings. The test was able to detect trypanosome infection for a longer duration compared to the parasitological methods which are regarded as the gold standard. PCR detected trypanosome DNA in the saliva samples during early infection corresponding to the period of high parasitaemia. The lack of detection in urine samples could be due to the presence of inhibitors such as urea and uric acid [19] and elevated acidic conditions that may inactivate the highly sensitive *Taq* DNA polymerase enzyme used in PCR reactions. This enzyme used is also easily inactivated by tissue and blood derived inhibitors [20–22]. Similarly, in a previous study on *T. b. rhodesiense*, trypanosome DNA was not detected in urine samples of infected vervet monkeys [unpublished data]. In this study, all the CSF samples were negative for trypanosome DNA although previous studies have shown that PCR on CSF samples has high sensitivity in staging of HAT [23]. Optimization of reaction conditions is a major setback in development of PCR as a diagnostic tool for HAT. This has especially been noted in samples from serologically positive but aparasitemic patients [9]. In addition, PCR is cost restrictive due to the need for specialized equipment such as automated thermal cyclers and the presence of cold chain to preserve reagents. Thus, recent studies have focused on development of tests such as LAMP which can overcome some of these challenges [10, 24].

LAMP performed better than PCR and parasitological methods as demonstrated by its higher detection rate. In contrast to PCR, LAMP detected trypanosome DNA in urine samples in this study. Indeed, the test detected trypanosome DNA during periods of low parasitaemia when both PCR and parasitology were negative. The significant difference in detection proves that the performance of LAMP was markedly better than that of both parasitological methods and PCR. This is possibly due to the use of *Bst* polymerase that unlike *Taq* polymerase is hardly inhibited by impurities [25]. The detection of trypanosomal DNA in urine and saliva samples is of great value because they are noninvasive samples and are an improvement from the current blood and CSF samples [26].

Trypanosomes have been found to be present in many organs and body fluids. There has been demonstrated localization of *T. b. brucei* in kidney glomeruli of infected rats [27] and *T. lewisi* in kidney capillaries of infected rats [28]. Filtration of the parasite or its DNA may explain the presence of trypanosomal DNA in the urine of the infected monkeys used in this study. However, formation of ammonia in exposed urine causes degradation of DNA and may lower the sensitivity of the tests.

Trypanosome DNA was also detected in the saliva samples of the infected monkeys. It is possible that parasites could have seeped into salivary ducts from either the blood or lymphatic systems. Saliva has a higher pH as compared to urine hence reducing the likelihood of DNA deterioration.

In this study, we targeted TgsGP gene a single gene which encodes a protein specific to the *T. b. gambiense* subspecies and hence ideal for specific detection of *T. b. gambiense* [29]. The LAMP detection rate varied and was 100% in some durations depending on the sample used. The sensitivity appeared to be affected by parasitaemia and hence the amount of DNA in the sample. The concentration of the trypanosomal DNA in the sample was however not assessed. The highest mean detection rate (78.9%) over the entire 180 days experimental period was lower than obtained using a repetitive element (repetitive insertion mobile element, RIME) [30]. A repetitive DNA like RIME means that many copies of DNA are available for amplification and hence greater sensitivity is expected. However, it is important to note that in that study 90% sensitivity was obtained with the samples from parasitological confirmed patients. We recommend that the sensitivity of

LAMP targeting the TgsGP gene be further evaluated in clinical samples and especially the noninvasive samples such as urine and saliva. There is also promising development in serological tests. A number of rapid tests are under clinical evaluation in many countries [31]. Combined use of the rapid serological tests and molecular techniques such as LAMP will enhance early diagnosis of HAT in the rural Africa where the disease is endemic.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

This work was funded by the Institute of Primate Research (IPR) and JKUAT-RPE program. The authors are grateful to the technical assistance provided by Tom Adino and Samson Mutura of IPR.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 135689, 16 pages
http://dx.doi.org/10.1155/2015/135689

Research Article

Comparative Study of the Accuracy of Different Techniques for the Laboratory Diagnosis of Schistosomiasis Mansoni in Areas of Low Endemicity in Barra Mansa City, Rio de Janeiro State, Brazil

Maria Cristina Carvalho Espírito-Santo,[1,2] Mónica Viviana Alvarado-Mora,[3] Pedro Luiz Silva Pinto,[4] Maria Carmen Arroyo Sanchez,[5] Emmanuel Dias-Neto,[6,7] Vera Lúcia Pagliusi Castilho,[8] Elenice Messias do Nascimento Gonçalves,[8] Pedro Paulo Chieffi,[9,10] Expedito José de Albuquerque Luna,[10] João Renato Rebello Pinho,[3] Flair José Carrilho,[1] and Ronaldo Cesar Borges Gryschek[1]

[1] *Department of Infectious and Parasitic Diseases and Laboratory of Immunopathology of Schistosomiasis (LIM-06), School of Medicine, University of São Paulo, 05403-000 São Paulo, SP, Brazil*
[2] *University Center of Volta Redonda, 27240-560 Volta Redonda, RJ, Brazil*
[3] *Department of Gastroenterology and Laboratory of Tropical Gastroenterology and Hepatology, School of Medicine, University of São Paulo, 05403-000 São Paulo, SP, Brazil*
[4] *Department of Enteroparasites at the Parasitology and Mycology Service from the Adolfo Lutz Institute, 01246-902 São Paulo, SP, Brazil*
[5] *Laboratory of Seroepidemiology and Immunobiology, Institute of Tropical Medicine, University of São Paulo, 05403-000 São Paulo, SP, Brazil*
[6] *Laboratory of Medical Genomics, AC Camargo Cancer Center, 01509–010 São Paulo, SP, Brazil*
[7] *Institute of Psychiatry (LIM-27), São Paulo Medical School, University of São Paulo, 01246-903 São Paulo, SP, Brazil*
[8] *Section of Parasitology, Central Laboratory Division of Hospital das Clínicas, School of Medicine, University of São Paulo, 05403-000 São Paulo, SP, Brazil*
[9] *Santa Casa Medical School, 01221-020 São Paulo, SP, Brazil*
[10] *Institute of Tropical Medicine, University of São Paulo, 05403-000 São Paulo, SP, Brazil*

Correspondence should be addressed to Maria Cristina Carvalho Espírito-Santo; cristinasanto@usp.br

Received 2 March 2015; Revised 6 May 2015; Accepted 10 May 2015

Academic Editor: Stephan Karl

Schistosomiasis constitutes a major public health problem, with an estimated 200 million people infected worldwide. Many areas of Brazil show low endemicity of schistosomiasis, and the current standard parasitological techniques are not sufficiently sensitive to detect the low-level helminth infections common in areas of low endemicity (ALEs). This study compared the Kato-Katz (KK); Hoffman, Pons, and Janer (HH); enzyme-linked immunosorbent assay- (ELISA-) IgG and ELISA-IgM; indirect immunofluorescence technique (IFT-IgM); and qPCR techniques for schistosomiasis detection in serum and fecal samples, using the circumoval precipitin test (COPT) as reference. An epidemiological survey was conducted in a randomized sample of residents from five neighborhoods of Barra Mansa, RJ, with 610 fecal and 612 serum samples. ELISA-IgM (21.4%) showed the highest positivity and HH and KK techniques were the least sensitive (0.8%). All techniques except qPCR-serum showed high accuracy (82–95.5%), differed significantly from COPT in positivity ($P < 0.05$), and showed poor agreement with COPT. Medium agreement was seen with ELISA-IgG (Kappa = 0.377) and IFA (Kappa = 0.347). Parasitological techniques showed much lower positivity rates than those by other techniques. We suggest the possibility of using a combination of laboratory tools for the diagnosis of schistosomiasis in ALEs.

1. Introduction

Schistosomiasis is a major public health problem, with 200 million people infected worldwide and 700 million people residing in areas of infection risk [1, 2].

In Brazil, schistosomiasis has been reported to occur in 19 states, and it is estimated that approximately 6 million people are infected and 25 million are at risk of contracting the disease. The national positivity rate is 6.94%, ranging from 0.04% in Piauí State to 11.88% in Pernambuco State. In Rio de Janeiro State, the positivity rate is 1.56% [3].

Brazil has areas of different prevalence rates varying from state to state, as shown in Figure 1 [3].

Of the various known species of Schistosoma, *S. mansoni* has the widest global distribution and is the only species that causes schistosomiasis in Brazil [4].

Although the serious forms of schistosomiasis have become less prevalent, thanks mainly to the implementation of mass chemotherapy, the geographic expansion of schistosomiasis continues apace with the expansion of agricultural zones and irrigated areas [5].

The classification of the individual infection intensity criteria for *S. mansoni* is estimated by the quantity of eggs observed in parasitological examination of feces, using the Kato-Katz (KK) technique [2]. According to WHO, 2002 [6], the infection classification is high parasitic load (≥400 epg), medium parasite load (100–399 epg), and low parasite load (<100 epg) and areas with high, medium, or low endemicity show prevalence of ≥50%, ≥10% <50, or <10%, respectively. In ALEs, approximately 75% of infected individuals are asymptomatic, show few symptoms, and have low parasite load, which hinders diagnosis [6].

The state of Rio de Janeiro presents the lowest number of confirmed cases and deaths due to schistosomiasis in the southeast region of Brazil [3, 7]. The city of Barra Mansa is defined as microregion 2 of the Vale do Médio Paraíba, Rio de Janeiro State [8]. The city of Barra Mansa is located in the southern part of the state of Rio de Janeiro.

Barra Mansa is one of the foci of *S. mansoni* infection in the state of Rio de Janeiro [8]. The average prevalence was estimated to be 1%, from 2001 to 2008, based on the cases reported by the Notifiable Diseases Information System (SINAN) from 2001 to 2008 [9].

The endemic foci lie within the urban perimeter. The neighborhood of Siderlândia shows the highest prevalence, followed by the neighborhoods of Santa Clara, São Luiz, Cantagalo, and Nova Esperança. Isolated cases of infection by *S. mansoni* have been reported in further 30 neighborhoods [9].

Detection of *S. mansoni* eggs in feces has historically been used as the reference for diagnosing schistosomiasis, and *Schistosoma* species are identified by their characteristic morphology showing a lateral spicule. The parasitological methods are highly specific, inexpensive, and relatively simple to execute [2, 10–12]. The Kato-Katz (KK) technique is most commonly used for detecting *S. mansoni* eggs in epidemiological studies, allowing the quantification of eggs in fecal samples. The Hoffman technique (HH) is based on spontaneous sedimentation, and it is effective because

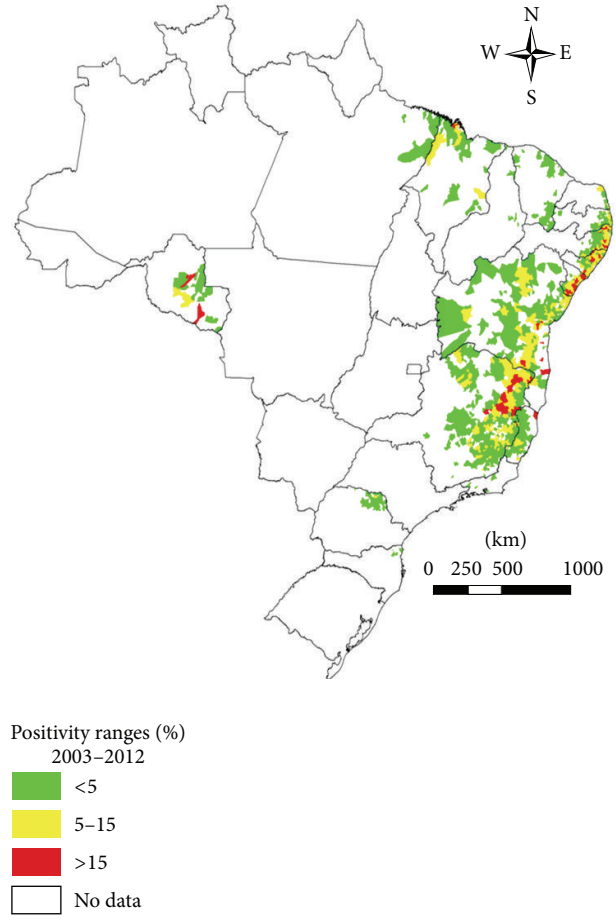


Figure 1: Distribution of positivity ranges for schistosomiasis based on the record of cases on investigated cities, Brazil, 2012. Source: SISPCE-SVS/MS.

embryonated *S. mansoni* eggs are heavy; however, it is not suitable for quantification of eggs in feces.

Although these parasitological methods are inexpensive and simple to perform, they lack sensitivity, especially in ALEs [13–18]. The Secretariat of Health Vigilance in Brazil has proposed the elimination of this form of helminthiasis. Therefore, there is a need to define alternative laboratory diagnostic techniques for detection of *S. mansoni* in ALEs. Thus, the aim of this study was to compare the efficiency of existing parasitological, immunological, and molecular diagnostic methods in areas of low prevalence of *S. mansoni*, using the circumoval precipitin test (COPT) as a reference, due to its high sensitivity and specificity in ALEs [19, 20].

2. Materials and Methods

2.1. Study Design, Population, and Sample Size. *S. mansoni* is endemic in the city of Barra Mansa, Rio de Janeiro State, Brazil, with an estimated prevalence of 1% [9]. Data for 2001–2008 from the Notifiable Diseases Information System (SINAN) showed that the disease is most prevalent in the neighborhoods of Siderlândia, Santa Clara, São Luiz, Nova

Esperança, and Cantagalo, which belong to the Barra Mansa River Basin, a tributary of the Paraíba do Sul River. These five neighborhoods, located on the outskirts of the city of Barra Mansa, were selected for this cross-sectional study. Samples of feces and serum were collected from April to December 2011.

The sample size was calculated assuming a prevalence of 1%, with an addition of 30% to compensate for losses. The estimated sample size required was 650 individuals residing in the above neighborhoods. Households were systematically selected (one in six), and individuals were randomly selected by a draw among those who agreed to participate in the study. Subjects who were older than 5 years of age and had not been treated for *S. mansoni* in the last year were eligible for inclusion.

2.2. Statistical Analysis. Statistical analysis was performed using SPSS (Statistical Package for the Social Sciences) for Windows, version 15.0 (SPSS Inc., Chicago, IL, USA) and Microsoft Excel 2003. Significance levels were fixed by accepting a Type 1 error of 5% ($\alpha = 0.05$). Population characteristics were described using absolute and relative frequencies and calculation of mean ages and standard deviations. All participants were evaluated by each diagnostic technique.

Each *S. mansoni* infection measurement technique was compared and marginal associations were verified using McNemar's test. Pairwise concordance between results was assessed using Cohen's kappa index and 95% confidence intervals. The Kappa index values range interpretation was as follows: poor agreement (<0.20); low agreement (0.20 to 0.40); moderate agreement (0.41 to 0.60); good agreement (0.61 to 0.80); and very good agreement (0.81 to 1.00) [21].

Associations between *S. mansoni* infection and age range, sex, neighborhood, river water use, and history of schistosomiasis were assessed for each technique using the Chi-square test, Fisher's exact, or likelihood ratio tests.

We compared the accuracy (sensitivity, specificity, likelihood ratio, and predictive values) of serological techniques to those of parasitological techniques. We also compared results among techniques to determine which were most effective in diagnosing *S. mansoni* in areas with a similar epidemiological profile to the target area of this study.

2.3. Ethical Aspects. In accordance with the rules governing human subject research, informed consent was obtained to meet the recommendations of Resolution n° 466 from December 12, 2012, of the National Council of Health. This research project was approved by the Research Ethics Committee of the Department of Infectious and Parasitic Diseases of the Faculty of Medicine of the University of São Paulo and the Research Ethics Committee of the Hospital das Clínicas (CAPPesq) of the Faculty of Medicine of the University of São Paulo (Approval number 0405/09).

The experimental research procedures met Laws 6.638/79 and 9605/98, Decree 24.645/34, the Ethical Principles of Animal Experimentation, the Principles for Research Involving Animals [22], and other directives governing animal research. The study began after approval of research project number CEP-IMT 2011/096 by the Animal Ethics Research Committee of the Institute of Tropical Medicine of São Paulo, University of São Paulo, Brazil.

2.4. Methods of Diagnostic Investigation. This was an interdisciplinary study, and the research laboratories developed their activities independently, following the conditions and operationalization timelines that respected the routine of each institute involved. Samples from all individuals in the study were subjected to the diagnostic techniques described and the reference diagnostic technique (COPT) [23]. The professionals who standardized and interpreted the COPT did not know the results for the diagnostic techniques, thus, reducing any interpretation bias.

The participating laboratories and the diagnostic techniques they developed were as follows: Municipal Secretary of Health of Barra Mansa/RJ (feces and serum collections); CentroLab Laboratory, Volta Redonda/RJ (preparing sample preanalysis); Institute Adolfo Lutz (COPT and ITF-IgM); Institute of Tropical Medicine/USP (ELISA); Laboratory of Gastroenterology and Tropical Hepatology at the Department of Gastroenterology/FMUSP (molecular biology), Research Center at A.C. Camargo Hospital (molecular biology), René Rachou Research Center/FIOCRUZ, Belo Horizonte/MG (molecular biology); Parasitological Section of the Central Laboratory, Division, HCFMU/SP (KK and HH).

The standardization and application of the diagnostic techniques were developed from December 2010 to December 2012, when the statistical analyses began.

2.5. Methods for Laboratory Diagnosis. The Family Health Program and health agents of the Municipal Schistosomiasis Control Program (PCE) collected 610 randomized fecal samples and 612 serum samples. Of these, 572 samples were paired, from inhabitants of 5 peripheral neighborhoods of Barra Mansa, Rio de Janeiro. The serum samples were aliquoted and stored at −20°C and then transported to São Paulo in thermal boxes containing dry ice and stored at −20°C in the lab until further testing.

2.6. Parasitological Methods. Stool samples were prepared following the KK (Helm Test, Bio-Manguinhos, Fiocruz, Rio de Janeiro, RJ, Brazil) and HH techniques and stored at 4°C until shipment. For the KK technique, two slides were prepared and stored in boxes fixed with a rigid polypropylene cover lined with cork (Prolab-Prolab, São Paulo, SP, Brazil). For the HH technique, samples were preserved in 10% formalin (Indalabor Indaiá Laboratório Farmacêutico Ltda., Dores do Indaiá, Minas Gerais, MG, Brazil), and two slides were prepared for each participant.

2.7. Laboratory Maintenance of the S. mansoni Experimental Cycle. The *S. mansoni* cycle was maintained through periodic infection of hamsters (*Mesocricetus auratus*) and *Biomphalaria glabrata* mollusks (strain BH). Each week, five animals were subcutaneously infected with 200–300 cercariae and sacrificed after 49 to 56 days to collect adult worms and parasite eggs. *S. mansoni* eggs were collected from liver granulomas. After washing with physiological solution

for complete removal of blood, the worms and eggs were counted and frozen.

2.8. Adult Worm Total Extract. Approximately 10,000 *S. mansoni* adult worms of both sexes were thawed and resuspended at a concentration of approximately 1,000 worms/mL in 10 mM phosphate buffered saline (PBS), at pH 7.2. The protease inhibitor phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, St. Louis, MO, USA) was then added to produce a final concentration of 1 mM. The suspension was ground in a manual tissue homogenizer in an ice bath for 1 h. After thorough mixing, the suspension was subjected to two centrifugations at 10,000 ×g, at 4°C for 45 min. The supernatant was then removed, and the protein content was measured using the DC Protein Assay reagent (Detergent-Compatible Colorimetric Assay Kit, Bio-Rad, Hercules, CA, USA) employing a modified Lowry method [24, 25]. The extract was aliquoted and stored in a freezer at −80°C until further use [26]. This total extract was used for the sensitization of microplates to IgG (ELISA-IgG) antibodies [27, 28].

2.9. TCA-Soluble Fraction. A soluble fraction in trichloroacetic acid (TCA-soluble fraction) was prepared from the crude extract of adult worms of *S. mansoni*, according to a previously described methodology [27], with modifications. An equal volume of 10% TCA was added to the total adult worm extract. After mixing vigorously for three cycles of 1 min, using a vortexer (Vortex Genie-2, Scientific Industries Inc., Bohemia, NY, USA), the suspension was subjected to centrifugation at 10,000 ×g, at 4°C for 45 min. The supernatant (containing the TCA-soluble fraction) was removed and then dialyzed against PBS on a cellulose membrane (Sigma-Aldrich) that retains substances with a molecular weight of 12,000 kDa or greater, with continuous stirring overnight at 4°C. We subsequently determined the protein content of the antigen solution (TCA-soluble fraction), which was then aliquoted and stored in a freezer at −80°C until further use. The TCA-soluble fraction was used for the sensitization of microplates to IgM (ELISA-IgM) antibodies [26, 28].

2.10. Enzyme-Linked Immunosorbent Assays (ELISA). For ELISA-IgM, polystyrene plates (Costar High Binding 3590, Corning, NY, USA) were sensitized with 1 μg/well of the TCA-soluble fraction of the *S. mansoni* total extract diluted in 0.1 M carbonate/bicarbonate buffer (pH 9.6), incubating for 2 h at 37°C, followed by 18 h at 4°C in a humid chamber. After washing three times with PBS containing 0.05% Tween-20 (PBS-T), the plates were blocked for nonspecific sites with 200 μL of 5% skim milk in PBS-T (PBS-TM 5%), incubating for 2 h at 37°C in a humid chamber. The plates were then washed again with PBS-T. Serum samples were diluted (1 : 100) in PBS-T with 2% skim milk (PBS-TM 2%) and tested in duplicate (50 μL/well). After incubation, for 30 min at 37°C in a humid chamber, the plates were washed three times with PBS-T, and 50 μL of a μ-chain-specific anti-human IgM peroxidase conjugate (Sigma-Aldrich) diluted 1 : 5000 in PBS-TM 2% was added. After 30 min of incubation in a humid chamber, the plates were washed with PBS-T, and 100 μL of a chromogenic mixture consisting of tetramethylbenzidine (TMB) and H_2O_2 was added. After 10 min of incubation in the dark, the reaction was quenched with 25 μL of 2 N H_2SO_4. The absorbance at 450 nm was measured using an ELISA plate reader (Titertek Multiskan MCC/340, Lab Systems, Helsinki, Finland).

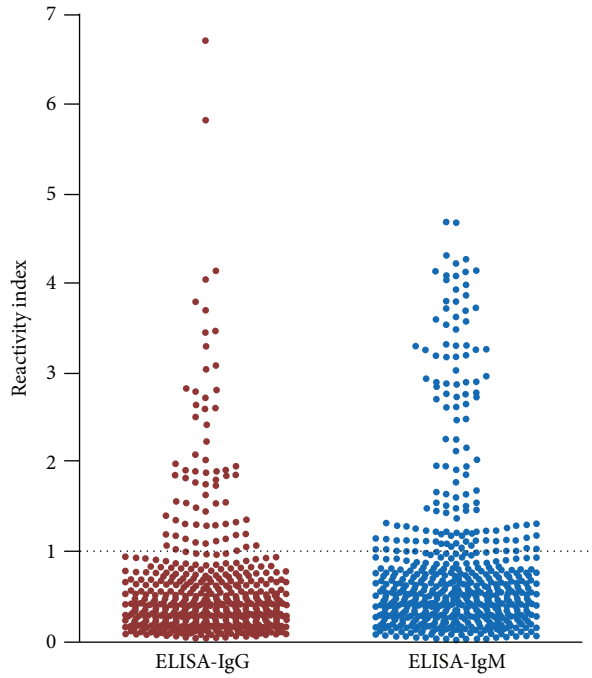


Figure 2: Reactivity index of 612 sera obtained by ELISA-IgG and IgM-ELISA technique of the individuals in the city of Barra Mansa, RJ, Brazil, 2011.

The reaction conditions described above for performing ELISA-IgM testing were also used for ELISA-IgG testing, except that polystyrene plates (Nunc PolySorp, Roskilde, Denmark), a 500 ng/well total antigen extract of *S. mansoni*, and an Fc-specific anti-human IgG peroxidase conjugate (Sigma-Aldrich) diluted 1 : 20,000 were used.

To determine the threshold of reactivity (cut-off) for ELISA-IgG and ELISA-IgM, receiver operating characteristic (ROC) curves were constructed. We used 13 and 29 samples from patients who tested positive and negative, respectively, for *S. mansoni* in the parasitological examination and IFT, and 13 samples from patients who tested positive for other helminth parasites in the parasitological examination and negative for *S. mansoni* in IFT.

The reactivity index (RI) of the samples was calculated using the equation IR = sample absorbance/cut-off. Serum samples with IR ≥ 1.00 were considered reactive, Figure 2.

2.11. Detection of IgM Antibodies against Antigens of the S. mansoni Digestive Tract. IFT-IgM was used to detect anti-antigen polysaccharide IgM antibodies in the digestive tract of adult *S. mansoni* worms on paraffin sections, according to the technique described by Silva et al. [29] and Nash, 1974 [30] and 1978 [31].

2.12. Obtaining Adult Worms. The infected hamsters were anesthetized with an intramuscular injection of 100 mg/kg of ketamine and xylazine and sacrificed, following the animal sacrifice guidelines of our institution. After sectioning the portal vein, 50 mL of 0.85% saline solution with ethylenediaminetetraacetic acid (EDTA) was infused in the left ventricle using a 20 mL syringe, through successive infusions, by not having an infusion pump for maintaining a continuous flow. Adult worms were obtained after perfusion of the portal system, removed from the abdominal cavity, and numbered and stored at −20°C. Adult male worms were separated for "particulate" antigen processing in paraffin sections for IFT-IgM analysis.

2.13. Slide Preparation for IFT-IgM. Approximately 60 adult male worms were placed on an end-jointed 200 μL mesh screen (Tecmolin, PA-6-212/XX, São Paulo, SP, Brazil), immersed in Rossman's solution fixative for 2 h at room temperature and then immersed in 90% ethanol three times for 2 h. The samples were then immersed in absolute alcohol for 15 h and incubated in methyl benzoate for 4 h, xylol at 60°C for 15 min, 50% xylene/paraplast for 15 min, and 100% paraplast (Monjet Scient.) at 60°C for 30 min. The material was embedded in an L-shaped aluminum frame, placed at room temperature for 12 h, and subsequently stored at the same temperature for processing of histological sections. Serial sections of 5 μm thickness were cut using a microtome, at ten sections per slide.

The slides containing paraffin sections were subjected to dewaxing and rehydration, using successive baths of xylene and ethanol at different concentrations, with a final bath in PBS, pH = 7.2. The slides were stored at room temperature until use.

2.14. IFT-IgM. Serum samples were diluted 1 : 10 in PBS solution (pH 7.2) and deposited on the paraffined sections of adult worms. After incubation at 37°C for 50 min in a humid chamber, slides were washed in 0,01 M PBS (pH 7.2) baths for 10 min. Anti-human IgM fluorescent conjugate (goat anti-human IgG γ-chain-specific fluorescein isothiocyanate antibody) was added (Sigma-Aldrich; St. Louis, MO, USA) in accordance with its optimal use titer (1/320) in PBS, pH = 7.2 containing 1% Evans blue solution (Bio-Rad Laboratories, Washington, DC, USA). After further incubation and washes, slides were dried and mounted with glycerol and coverslips. Positive standard serum was used for 1/10, 1/40, and 1/160 dilutions. Negative standard serum was used for 1/10 dilutions.

The IFT-IgM reading was performed using an Olympus BX-FLA fluorescence microscope (Olympus Corporation, Tokyo, Japan) equipped with an epi-illumination system, with 100x and/or 200x magnification.

A sample was considered positive when fluorescence was present only in structures related to the parasite digestive tract (Figure 3). Fluorescence detected in membranes or parasite parenchyma was considered nonspecific. The results were expressed as reactive (presence of fluorescence in the digestive tract) and nonreactive (absence of fluorescence) sera.

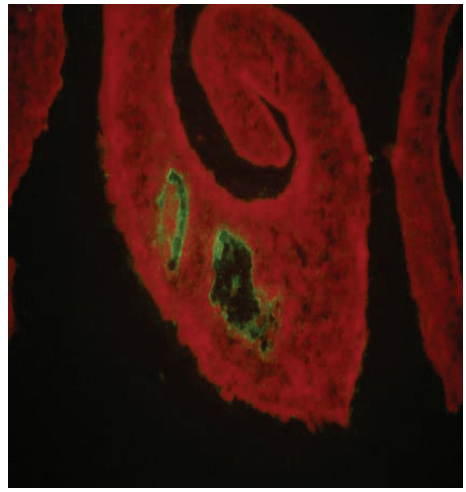


Figure 3: Reactivity of the inner lining of the digestive tube: positive human serum IgM antibodies.

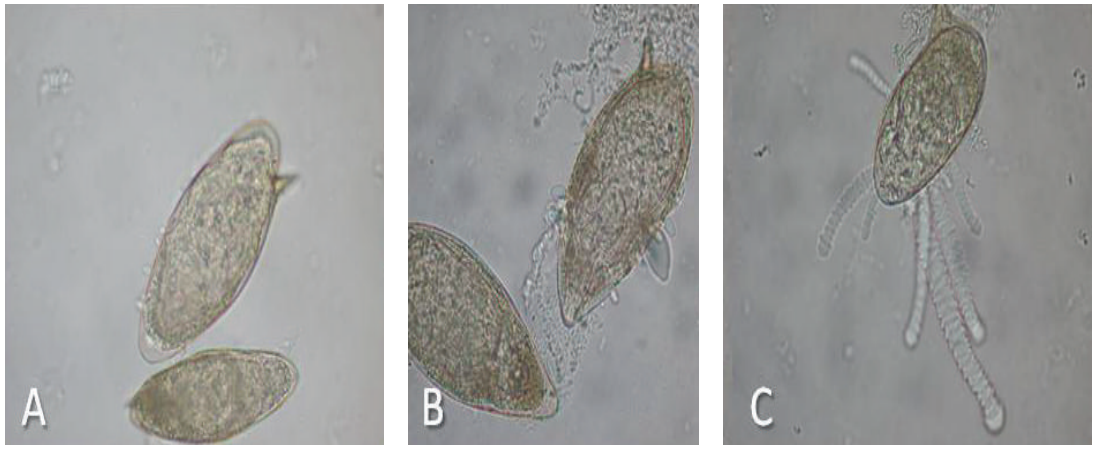
The classification of the sera was based on the fluorescence intensity in the internal portion of the worms digestive tract (only male worms were used for embedment), according to a targeted fluorescence intensity scale. The attribution of 1+ to 4+ was obtained comparing the results with a photographic standard built with serum: nonreactive (0), low (1+), medium (2+), strong (3+), and very strong (4+).

2.15. Antibody Detection of S. mansoni Egg Antigens. Circumoval precipitin test (COPT) was used to detect antibody reactions against excretion and secretion products of *S. mansoni* eggs, using previously described techniques [32].

2.16. Isolation and Purification of S. mansoni Eggs. *S. mansoni* eggs were isolated and purified as described by Dresden and Payne [33] and Pinto et al. [34] with some modifications.

Livers from three infected hamsters were cut into small pieces (3 mm) and incubated in a water bath at 37°C for 20 min, in a solution containing 0.004% pepsin and 0.7% hydrochloric acid. After incubation, the peptic solution was discarded and the tissue fragments were added to 150 mL of 0.9% ice-cold saline solution containing 50 μL of Triton X-100. The material was homogenized using several drive pulses in a domestic blender (Walita Philips, Amsterdam, Netherlands) until the fragments were completely ruptured. The material was then filtered in fourfold gauze and screen processed under negative pressure through a series of metallic sieves with mesh numbers 100 (0.150 mm), 200 (0.075 mm), and 400 (0.038 mm) (Granutest, Telastem, Peneiras Para Análise Ltda., São Paulo, SP, Brazil).

Eggs retained on the last sieve were removed by successive washing with ice-cold 0.9% saline solution and concentrated to 1 mL volume by centrifugation at 154 ×g for 15 sec. From this concentrated suspension, a 10 μL aliquot was placed between the blade and the coverslip and evaluated under a microscope (Olympus Corporation, Tokyo, Japan) at 100x magnification. The number of eggs under the entire area



(a) (b) (c)

Figure 4: Intensity patterns of RPO: (a) RPO pattern 1; (b) RPO pattern 2; (c) RPO pattern 3.

of the coverslip was counted. Suspensions exceeding 20% cell debris in relation to the number of eggs found were reprocessed through the 200 and 400 mesh sieves. For COPT, the egg suspensions were adjusted to contain 300 viable eggs per 10 μL of 0.9% saline solution and stored at 4°C for 4 h until reaction preparation.

2.17. Circumoval Precipitin Test. Aliquots (10 μL) of each purified egg suspension, adjusted to contain 300 viable eggs in 0.9% saline solution, and 50 μL of each serum sample, were added to 2 mL Eppendorf tubes (Eppendorf do Brasil Ltda., São Paulo, SP, Brazil) and incubated at 37°C for 48 h. Subsequently, a 30 μL aliquot of each mixture (serum + viable egg suspension) was placed on a slide and covered with a coverslip (22 × 22 mm). Assessment was performed on a binocular Olympus-CX41 microscope (Olympus Corporation, Tokyo, Japan), with a 10x or 40x objective lens. The number of reactive eggs per 100 viable eggs and the periovular precipitate morphology were used for assessment. *S. mansoni* eggs were considered reactive if they contained globular precipitates of variable size and form, or if they appeared as small or long septated chains, similar to *Taenia* segments (Figure 4). Sera were considered positive if at least 9% of the mature eggs were reactive in the presence of different precipitates [35].

2.18. TaqMan Real-Time PCR Assays. Preanalysis preparation of feces samples for qPCR. Fecal samples, without conservatives, were passed through a Nylon Baltex PA-7-200/XX filter (Tecmolin, São Paulo, SP, Brazil) to remove larger impurities. Using a marked stainless steel measuring plate, approximately 500 mg of feces from each sample was aliquoted and stored in a freezer at −20°C.

2.19. DNA Extraction from Serum. DNA was extracted from hamster serum samples and the 0.9% saline solution containing 200 eggs/mL, using the guanidine isothiocyanate-phenol-chloroform (GT) method [36, 37]. DNA was stored at −20°C after extraction.

2.20. DNA from Fecal Samples. DNA extraction from hamster feces was performed in two phases: in the first phase, after resuspending approximately 500 mg of stool in 1 mL 0.1 M PBS, five glass beads were added. This mixture was homogenized for 5 min using a vortex mixer, followed by centrifugation for 8 min at 16.863 ×g at 4°C. An aliquot of 400 μL of the supernatant was mixed with 100 μL of Rapid One-Step Extraction (ROSE) solution: 10 mM Tris-hydroxymethyl amino methane HCl, pH 8; 300 mM EDTA, pH 8.0; 1% sodium lauroyl sarcosinate (Sarkosyl); and 1% polyvinylpolypyrrolidone (PVPP) [38]. Then, 30 μL of Proteinase K (Life Technologies, Carlsbad, CA, USA) was added and homogenized using a vortex mixer. The sample was incubated for 120 min at 65°C. In the second phase, the DNA present in this solution was extracted and stored as described for serum samples.

2.21. Purification of DNA Extracted from Fecal and Serum Samples. DNA extracted from serum and feces, at distinct times after infection, was purified using the InstaGene Matrix in accordance with the manufacturer's instructions. Once purified, extracted DNA was stored at −20°C until use.

2.22. Amplification of DNA from Serum and Fecal Samples

Primers and Probes. A set of primers and probes complementary to a 121 bp tandem repeat sequence from *S. mansoni* strain SM 1-7 (GenBank accession number M61098), described by Hamburger et al. [39], were used for amplification and detection of *S. mansoni* DNA. Primer sequences were as follows: forward F2: 5′-CCG ACC AAC CGT TCT ATG A-3′; reverse R2: 5′-CAC GC TCT CGC AAA TAA TCT AAA-3′; probe PO2: 5′-6[FAM] TCG TTG TAT CTC CGA AAC CAC TGG ACG [(BHQ1])-3′; all probes were synthesized by Sigma Life Sciences (Woodlands, TX, USA).

All samples were evaluated using TaqMan Reagents Exogenous Internal Positive Control (IPC) (Life Technologies), in accordance with the manufacturer's instructions to test for the presence of Taq DNA polymerase inhibitors.

For the positive control of the qPCR-feces, in all assays, we used DNA obtained in the extraction of 200 eggs/mL of saline 0.9%.

2.23. TaqMan Real-Time PCR Conditions for Serum and Fecal Samples. TaqMan Real-Time PCR was performed in a final volume of 20 μL containing 10 μL TaqMan Universal PCR Master Mix 2X, 20 pmol of primers F2 and R2, 5 pmol of the PO2 probe, and 2 μL of purified DNA. For each sample, another reaction was performed in parallel using the TaqMan Reagents Exogenous Internal Positive Control (IPC) in a final volume of 21 μL, containing 10 μL of TaqMan Universal PCR Master Mix 2X, 5 μL 10X Exogenous IPC mix, 1 μL 50X Exo IPC, and 5 μL of purified DNA samples. For each batch of reactions, two additional controls were used: a no amplification control (NAC) and a no template control target (NCT). PCR was performed in an Applied Biosystems 7300 Real-Time PCR System (Life Technologies) using the following cycling conditions: 50°C for 2 min; 95°C for 10 min; and 40 cycles at 95°C for 15 sec and 60°C for 1 min.

To detect *S. mansoni*, the Applied Biosystems 7300 Real-Time PCR System standard cycles were used: 50°C for 2 min; 95°C for 10 min; 40 cycles at 95°C for 15 sec, and 60°C for 1 min.

For the positive control of the qPCR-serum, in all assays, we used DNA obtained in the extraction of 200 eggs/mL of saline 0.9%.

Furthermore, the possibility of contamination was minimized by performing DNA extraction and amplification in separate rooms, by performing all experiments inside a laminar flow cabinet, by frequent use of ultraviolet (UV) (Bio II A-Telstar, Life Science, Vancouver, Canada) irradiation, and by using only disposable, sterile laboratory equipment and pipette tips with filters.

2.24. Criteria Used to Evaluate the Results of qPCR Using Stool and Serum Samples. The following criteria were used to evaluate the qPCR:

Positive qPCR: duplicates of the sample were amplified by qPCR.

Undetermined qPCR: one aliquot of the sample was amplified by qPCR.

qPCR IPC: exogenous control for the testing sample was not amplified (negative) by qPCR.

The undetermined qPCR and qPCR IPC samples were tested in triplicate. Duplicate amplifications of the samples obtained by qPCR were included in the positive results [38].

Table 1: Sociodemographic characteristics of the study population, individuals from the city of Barra Mansa, RJ, 2011.

Characteristic	Frequency	%
Sex		
Female	385	59.2
Male	265	40.8
Age group (years)		
1 to 9	27	4.2
10 to 19	144	22.2
20 to 49	250	38.5
50 or over	229	35.2
Average age (SD)	39.7 (21.1)	
Literacy		
Yes	624	96.0
No	22	3.4
Not reported	4	0.6
Neighborhood		
Cantagalo	47	7.2
Nova Esperança	187	28.8
Santa Clara	35	5.4
São Luiz	102	15.7
Siderlândia	279	42.9
Water supply		
General network	551	84.8
Well or spring	79	12.2
Others	1	0.2
Not reported	19	2.9
Use of river water		
No	473	72.8
Washing clothes	15	2.3
Washing utensils	2	0.3
Baths	5	0.8
Swimming	5	0.8
Sand extraction	7	1.1
Not reported	143	22.0
Destination of feces and urine		
Sewer system	486	74.8
Septic tank	7	1.1
In the open	117	18.0
Not reported	40	6.2
Previous schistosomiasis		
Yes	25	3.8
No	519	79.8
Not reported	106	16.3

3. Results

The characteristics of the sample population (650 freely participating individuals) residing in the five suburbs of the city of Barra Mansa (RJ), used to evaluate the techniques ELISA-IgG, ELISA-IgM, ITF-IgM, COPT, qPCR-serum, qPCR-feces, KK, and HH, are shown in Table 1.

Analysis of Table 1 reveals that the majority of the individuals were female with an average age of 39.7. The greatest number of participants resided in the neighborhood of Sinderlândia (42.9%) and approximately 4% of the participants reported a history of schistosomiasis.

The positivity for infection by *S. mansoni* according to each diagnostic technique used in this study is shown in Table 2.

The technique that presented most evidence of infection by *S. mansoni* was the ELISA-IgM (21.4%), whereas

Table 2: Positivity for infection by *S. mansoni*, according to diagnostic technique, in samples collected from individuals in the city of Barra Mansa, RJ, 2011.

Technique	Positive/total	%
KK-HH	5/610	0.8
ELISA-IgG	71/612	11.6
ELISA-IgM	131/612	21.4
COPT	33/612	5.4
ITF-IgM	97/612	15.8
qPCR-feces	60/610	9.8
qPCR-serum	9/612	1.5

Table 3: Prevalence of schistosomiasis mansoni and other enteroparasites, as determined using the KK and HH techniques in individuals from the city of Barra Mansa, RJ, 2011.

Parasitosis HH/KK	Frequency	%
Schistosoma mansoni	5	0.8
Endolimax nana	106	17.4
Entamoeba coli	28	4.6
Entamoeba hartmanni	1	0.2
Entamoeba histolytica/dispar	5	0.8
Blastocystis spp./*hominis*	66	10.8
Giardia lamblia/intestinalis	11	1.8
Iodamoeba butschlii	1	0.2
Enterobius vermicularis	6	1.0
Strongyloides stercoralis	9	1.5
Ascaris lumbricoides	4	0.7
Trichuris trichiura	3	0.5
Taenia sp.	2	0.3

the parasitological techniques (KK and HH) showed the lowest rates of infection (0.8%).

In the KK technique, the quantity of eggs detected per gram of feces varied from 0 to 456 eggs. One of the five positive cases was diagnosed only with the HH technique.

The prevalence of infection with *S. mansoni* and various enteroparasites diagnosed by the KK and HH techniques in individuals from the city of Barra Mansa/RJ, 2011, is shown in Table 3.

Of the individuals tested, 27.9% were found to be infected with at least one parasite.

The results of the KK technique showed low positivity for any diagnosed parasitosis, and the positivity rate for infection by *S. mansoni* was 0.7% ($n = 4$).

The ELISA-IgG reactivity index showed an average of 2.12, with an SD of 1.10 (median = 1.85; minimum = 1.01; maximum = 6.72; $n = 71$). The ELISA-IgM reactivity index showed an average of 2.24, with an SD of 1.09 (median = 1.91; minimum = 1.02; maximum = 4.67; $n = 131$). The IFT-IgM positivity showed an average of 1.56 crosses, with an SD of 0.69 (median = 1; minimum = 1; maximum = 3; $n = 97$). COPT reactivity was observed in an average of 21% of viable eggs, with an SD of 9% (median 21% viable eggs; minimum = 9% viable eggs; maximum 38% viable eggs; $n = 33$).

Table 4 shows that the results of the qPCR technique that were positive (Figure 6) and undetermined occurred with a frequency of 9.8% and 8.9% and a median of 34.8 and 37.1, respectively.

In this study, we found that in 16.2% ($n = 99$) of the fecal samples amplification of the exogenous control (IPC) and *S. mansoni* DNA was unsuccessful.

In serum samples (Table 5), the Ct values of the qPCR positive (Figure 6) and undetermined samples were 1.5% ($n = 9$) and 5.1% ($n = 31$) and the median was 36.3 and 37.0, respectively.

Table 6 shows that the positivity rate of the COPT technique differed significantly from all other techniques; it showed a lower positivity rate than the ELISA-IgG, ELISA-IgM, IFT-IgM ($P < 0.001$), and qPCR-feces ($P = 0.001$) techniques and a higher positivity rate than the KK, HH, and qPCR-serum techniques ($P < 0.001$). The results of the COPT technique presented greater concordance with those from ELISA-IgG (Kappa = 0.377), IFT-IgM (Kappa = 0.347), and qPCR-feces (Kappa = 0.311) techniques.

When comparing COPT with the other diagnostic techniques, ELISA-IgM assay presented the highest sensitivity (Table 7).

4. Discussion

Diagnosis of *S. mansoni* is challenging, and determining the prevalence of infection is therefore difficult. Schistosomiasis is a chronic infection that may not progress to a severe form but that can trigger debilitating sequelae that are secondary to parasitism. This is particularly true in children, who may experience delayed growth and develop anemia and chronic malnutrition [2]. Moreover, asymptomatic carriers can potentially transmit schistosomiasis in areas that lack adequate sanitation [40].

Because of the present state of endemism of schistosomiasis in Brazil, the current proposal is the interruption (elimination) of its transmission. Thus, there is a need to develop new techniques that are more sensitive and specific, to permit early diagnosis and treatment of infected individuals, necessary for the control of this helminthiasis [3].

In this study, we compared the performance of the KK and HH techniques, the ELISA-IgG, ELISA-IgM, and the ITF-IgM techniques, as well as qPCR of stool and serum samples, using the COPT technique as a reference [19, 20, 41].

The positivity rate for schistosomiasis in our study population, as determined by immunological techniques, ranged from 21.4% (131/612) for ELISA-IgM to 15.8% (97/612) for ITF-IgM and 11.6% (71/612) for ELISA-IgG. These rates are above those for parasitological techniques to diagnose *S. mansoni* infection. The low sensitivity of the parasitological method in ALEs had previously been described [13–18, 28, 42, 43].

The high positivity rates observed in this study for the ELISA-IgG, ELISA-IgM, and ITF-IgM techniques could have been caused by inclusion of individuals previously infected by *S. mansoni*, but properly treated and cured, or inclusion of those that were exposed to very small loads of cercariae

Table 4: Results from the positive and undetermined qPCRs, with the threshold cycle (Ct) values, in fecal samples from the population of the city of Barra Mansa, RJ, 2011.

Technique	Cases/total	%	Ct values (LOG) qPCR				
			Average	SD	Median	Minimum	Maximum
qPCR positive	60/610	9.8	33.6	4.9	34.8	14.7	38.8
qPCR undetermined	54/610	8.9	32.3	8.5	37.1	11.5	40.0

Table 5: Description of the threshold cycle (Ct) for the results from the positive and undetermined qPCR in serum samples from the individuals sampled from the city of Barra Mansa, RJ, 2011.

Sample	Technique	Cases/total	%	Ct values (LOG) qPCR				
				Average	SD	Median	Minimum	Maximum
Serum	qPCR positive	9/612	1.5	36.9	1.3	36.3	38.8	35.2
	qPCR undetermined	31/612	5.1	35.5	4.4	37	39.7	20.3

Table 6: Concordance between the positive results obtained using the COPT technique and those obtained using the other techniques, in fecal and serum samples collected from the sample population in the city of Barra Mansa, RJ, 2011.

Techniques	COPT		Total	*P* McNemar	Kappa	IC (95%)	
	Negative	Positive					
	n (%)	*n* (%)	*n* (%)			Lower	Higher
KK-HH							
Negative	542 (94.8)	25 (4.4)	567 (99.1)				
Positive	1 (0.2)	4 (0.7)	5 (0.9)	**<0.001**	0.224	0.038	0.409
Total	543 (94.9)	29 (5.1)	572 (100.0)				
ELISA-IgG							
Negative	530 (86.6)	11 (1.8)	541 (88.4)				
Positive	49 (8.0)	22 (3.6)	71 (11.6)	**<0.001**	0.377	0.255	0.500
Total	579 (94.6)	33 (5.4)	612 (100.0)				
ELISA-IgM							
Negative	475 (77.6)	6 (1)	481 (78.6)				
Positive	104 (17.0)	27 (4.4)	131 (21.4)	**<0.001**	0.266	0.178	0.354
Total	579 (94.6)	33 (5.4)	612 (100.0)				
IFT-IgM							
Negative	508 (83.0)	7 (1.1)	515 (84.2)				
Positive	71 (11.6)	26 (4.2)	97 (15.8)	**<0.001**	0.347	0.241	0.454
Total	579 (94.6)	33 (5.4)	612 (100.0)				
qPCR-feces							
Negative	503 (87.9)	14 (2.4)	517 (90.4)				
Positive	40 (7.0)	15 (2.6)	55 (9.6)	**0.001**	0.311	0.176	0.446
Total	543 (94.9)	29 (5.1)	572 (100.0)				
qPCR-serum							
Negative	574 (93.8)	29 (4.7)	603 (98.5)				
Positive	5 (0.8)	4 (0.7)	9 (1.5)	**<0.001**	0.171	0.013	0.330
Total	579 (94.6)	33 (5.4)	612 (100.0)				

or unisexual infections [16, 44, 45]. The possibility of cross-reactivity with cercariae antigens from parasites that infect other animal species and cross-reactivity with other parasites must also be considered [28, 46–48].

The positivity rate of the COPT technique also differed significantly from the positivity rates of the parasitological techniques ($P < 0.001$). The level of concordance ($k = 0.224$) for these techniques was higher than that observed for the other immunodiagnostic techniques (ELISA-IgG, ELISA-IgM, and ITF-IgM).

This higher positivity rate observed for the COPT could be due to the fact that oviposition occurs with egg deposition in the deeper layers of the intestine, even when eggs are absent from the feces, mainly in chronic infections in patients over 40 years of age and in schistosomiasis infections with low parasite load [49, 50].

Table 7: Description of the sensitivity, specificity, the positive likelihood ratio, the negative likelihood ratio, the positive predictive value, and the negative predictive value of all diagnostic techniques compared to the COPT technique in individuals from the city of Barra Mansa, RJ, 2011.

Techniques	Parameters	Estimate	IC (95%)	
			Lower	Higher
KK-HH	Sensitivity (%)	13.8	3.9	31.7
	Specificity (%)	99.8	99.0	100.0
	Likelihood ratio (+)	74.9	8.6	649.0
	Likelihood ratio (−)	0.9	0.7	1.0
	Positive predictive value (PPV) (%)	80.0	28.4	99.5
	Negative predictive value (NPV) (%)	95.6	93.6	97.1
	Accuracy (%)	95.5	95.1	95.8
ELISA-IgG	Sensitivity (%)	66.7	48.2	82.0
	Specificity (%)	91.5	89.0	93.7
	Likelihood ratio (+)	7.9	5.5	11.3
	Likelihood ratio (−)	0.4	0.2	0.6
	Positive predictive value (PPV) (%)	31.0	20.5	43.1
	Negative predictive value (NPV) (%)	98.0	96.4	99.0
	Accuracy (%)	90.2	89.5	90.9
ELISA-IgM	Sensitivity (%)	81.8	64.5	93.0
	Specificity (%)	82.0	78.7	85.1
	Likelihood ratio (+)	4.6	3.6	5.8
	Likelihood ratio (−)	0.2	0.1	0.4
	Positive predictive value (PPV) (%)	20.6	14.0	28.6
	Negative predictive value (NPV) (%)	98.8	97.3	99.5
	Accuracy (%)	82.0	80.9	83.2
IFT-IgM	Sensitivity (%)	78.8	61.1	91.0
	Specificity (%)	87.7	84.8	90.3
	Likelihood ratio (+)	6.4	4.9	8.5
	Likelihood ratio (−)	0.2	0.1	0.5
	Positive predictive value (PPV) (%)	26.8	18.3	36.8
	Negative predictive value (NPV) (%)	98.6	97.2	99.5
	Accuracy (%)	87.3	86.4	88.1
qPCR-feces	Sensitivity (%)	51.7	32.5	70.6
	Specificity (%)	92.6	90.1	94.7
	Likelihood ratio (+)	7.0	4.4	11.1
	Likelihood ratio (−)	0.5	0.3	0.8
	Positive predictive value (PPV) (%)	27.3	16.1	41.0
	Negative predictive value (NPV) (%)	97.3	95.5	98.5
	Accuracy (%)	90.6	89.9	91.3
qPCR-serum	Sensitivity (%)	12.1	3.4	28.2
	Specificity (%)	99.1	98	99.7
	Likelihood ratio (+)	14.0	4.0	49.8
	Likelihood ratio (−)	0.9	0.8	1.0
	Positive predictive value (PPV) (%)	44.4	13.7	78.8
	Negative predictive value (NPV) (%)	95.2	93.2	96.8
	Accuracy (%)	94.4	94	94.9

The COPT positivity was lower than that for the ELISA-IgG and ELISA-IgM and ITF-IgM tests, probably as a result of the different nature of the antigens. The COPT detects antibodies against excretion products and secretions of miracidia or antibodies against antigens present in the fluid surrounding *S. mansoni* eggs, whereas the ELISA-IgG, ELISA-IgM, and ITF-IgM techniques detect antibodies against antigens in the internal coating of the digestive tube of adult worms [32, 50].

The appearance of the antibodies detected by the COPT is precocious and coincides with the beginning of the elimination of the eggs in the feces [49, 51, 52]. Thus, the antibodies detected by the COPT are only produced when worms of both sexes cause the infection; the ELISA-IgG, ELISA-IgM, and ITF-IgM techniques can also detect unisexual infections [44, 52, 53].

To the best of our knowledge, this is the first time that qPCR-feces and qPCR-serum were used in a population study in low endemicity area for *S. mansoni* infection.

The qPCR-feces technique exhibited 12-fold higher positivity than the KK and HH techniques, at 9.8% ($n = 60$) compared to 0.8% ($n = 5$) for the parasitological techniques. The qPCR-feces positivity rate was only higher than the rates for the COPT (5.4%) and the qPCR-serum (1.5%) techniques; its results differed significantly from all techniques ($P < 0.05$) except the ELISA-IgG technique.

The positivity rate of the qPCR-serum technique was higher than those of the parasitological techniques but lower than those of the other techniques, including the qPCR-feces technique. A study by Pontes et al. [23] also indicated that the qPCR-serum technique has lesser sensitivity than the conventional PCR-feces technique for detecting *S. mansoni* infection. However, Wichmann et al. [54] and Zhou et al. [55] correlated the positivity in the acute phase of schistosomiasis infection with inflammatory alterations. The acute phase would cause increased circulation of schistosomiasis degradation products, both adult worms and eggs, thus increasing the quantity of circulating DNA. This phase would usually continue until the eighth week after infection, and these data would explain the higher rate of amplification using the qPCR-serum technique.

The median of the Ct values of the logarithmic curves for the qPCR-feces and serum techniques was 34.8 and 36.3, respectively, indicating low amplification of the DNA template, found in egg fragments (0.02 eggs at a dilution of 1/10,000), based on the standardization of the technique. The low parasite load in *S. mansoni* infections observed in ALEs could explain this result [56].

Wichmann et al. [57] reported a higher positivity rate for the qPCR-serum technique in patients with acute schistosomiasis, using the presence of *S. mansoni* DNA in duplicate samples as a criterion for positivity, with a limit of $Ct < 45$.

In spite of the presence of factors that inhibit the qPCR-feces technique and the low parasite load in the sampled population, the qPCR-feces technique showed a 12-fold higher positivity rate (60/610) than the KK and HH (5/610) techniques; the qPCR-serum technique presented a positivity (9/612) of, approximately, twofold higher than those of the parasitological techniques.

According to Cnops et al. [58], all PCR-based techniques for genomic DNA of *S. mansoni* were able to detect the DNA from all phases of the life cycle of this parasite: eggs containing miracidia, cercariae, schistosomulae, and adult worms. In summary, a positive PCR assay indicates the presence of the parasite but does not provide information regarding its life cycle phase or its viability, including the presence of mature, male, and female worms capable of egg deposition. This is likely to explain why we have not found a positive correlation between the Ct value and the number of eggs.

The technique developed by Kato and Miura [10], modified by Katz et al. [59], became the international reference technique for diagnosing schistosomiasis infection [60]. However, a decline in the sensitivity of this technique was observed in individuals with low parasite load infections residing in ALEs, following specific treatment, which compromises the evaluation of new techniques for diagnosing infection by *S. mansoni*, when it is used as a reference [61, 62].

Various studies have been performed to identify alternative methods of diagnosis applicable on a large scale that could be used to aid the programs for epidemiological vigilance in areas where, despite control actions, new cases of confirmed autochthony still occur [63].

Immunodiagnostic techniques are still used in schistosomiasis control programs in countries such as China and Venezuela [43, 50, 64, 65]. In Brazil, various techniques have been tested in regions considered to have low endemicity, such as in the states of São Paulo and Rio de Janeiro [28, 40, 48, 66–68].

The COPT is considered by some researchers to be the gold-standard technique for diagnosing schistosomiasis in ALEs [69, 70].

Taking into account the possibility of employing parasitological and immunological techniques for diagnosing schistosomiasis infection in ALEs, Alarcón de Noya et al. [50] proposed three laboratory criteria for case definition in these areas:

(I) individuals eliminating *S. mansoni* eggs in feces. In this group, COPT and serological positivity reactions are common;

(II) individuals without *S. mansoni* eggs in their feces, with positive COPT who have not received specific treatment in the previous 12 months. Usually, this group shows positivity for one and/or two serological techniques;

(III) individuals without *S. mansoni* eggs in their feces with negative COPT who have not received specific treatment in the previous 12 months but who have shown positivity for two serological techniques.

Use of criteria (I) and (II) presented above would yield 34 positive cases in our study population, and the prevalence would be, approximately, 5.6% (34/612) greater than the prevalence detected by the parasitological techniques 0.8% (5/612) prevalence detected by the parasitological techniques used in the program for schistosomiasis vigilance.

As we verified in this study, various techniques could be used for diagnosis of schistosomiasis; however all present

problems of sensitivity or specificity. Thus, we could not identify in this study the ideal technique that combines high rates of sensitivity and specificity and low cost and easy applicability, even in the field, probably due to the low prevalence of infection (approximately 1%) in the area studied. All assays employed showed low sensitivity and specificity when applied to the study population. Low parasite load, very recent infections, and the low number of positive parasitological exams may explain this.

One limitation of this study is the collection of a single fecal sample per individual as recommended by the guidelines of the National Program for Schistosomiasis, since the sensitivity of the KK test could have been improved by testing samples collected on two or three successive days to compensate for daily variation in egg production in an infected individual.

The present study indicates that the use of combined tools can improve the diagnosis of low parasite load *S. mansoni* infections, in both clinical and epidemiological studies. The benefits of parasitological techniques for the control of *S. mansoni* are widely recognized, particularly the KK technique. We consider the presence of ALEs to be an indicator of the positive outcome of schistosomiasis control programs worldwide. However, in order to eliminate the risk of schistosomiasis in populations of low endemicity areas, the pursuit of novel techniques must be considered. Potentially effective methods may include novel immunodiagnostic techniques, molecular biology tools, and parasitological techniques, such as saline gradient, and magnetic bead-based isolation of eggs [26, 37, 71–75]. The development of these tools and the subsequent elimination of schistosomiasis will be a great challenge that will require the combined efforts of governments, health professionals, and researchers.

To advance this agenda, we suggest a change to the third criterion proposed by Alarcón de Noya et al. [50], considering cases of schistosomiasis in which individuals present positive qPCR-serum and/or qPCR-feces results. We withdrew the criterion regarding positivity in two serological techniques and negative results in the parasitological and the COPT techniques (Table 8).

With regard to field activities, we propose serological and parasitological survey of ALEs. We recommend the use of the ELISA-IgM and ELISA-IgG techniques for an initial triage of the target population, due to their greater sensitivity, to the possibility of automation, and to their ability to provide quantitative results. We suggest the use of the KK and HH parasitological techniques for evaluating the intensity of *S. mansoni* infections and identifying the presence of other parasitic infections, respectively.

Following the triage, we recommend that actions be determined according to laboratory classification criteria. Thus, individuals classified under criteria (I), (II), and (III) would be referred for clinical evaluation, specific chemotherapy, and health education. For individuals with positive results for one and/or two serological techniques (ELISA-IgG or ELISA-IgM), we propose performing the COPT. For the patients who present a positive COPT, we recommend a clinical evaluation, treatment, and health education. If the result of the COPT is negative, we recommend performing qPCR-feces and qPCR-serum; if any of these molecular techniques yield a positive result, we suggest clinical evaluation, specific treatment, and health education (Figure 5).

Table 8: Proposal for laboratory criteria for defining cases of schistosomiasis in ALEs.

Criterion I	Criterion II	Criterion III
Individuals eliminating *S. mansoni* eggs in feces	Positive COPT	Individuals with no *S. mansoni* eggs in feces
With/without positive COPT	Individuals with no *S. mansoni* eggs in feces	Negative COPT
With/without positive ELISA-IgM and/or ELISA-IgG techniques	Without specific treatment in the last 12 months	
	With/without positive ELISA-IgM and/or ELISA-IgG techniques	Positive qPCR-serum and/or positive qPCR-feces

We propose the combination of the ELISA-IgM and IgG techniques in the triage phase due to the possibility of automation, and to their higher levels of sensitivity, thus enabling the identification of a greater number of cases. In addition, the ELISA-IgM technique is more sensitive and the ELISA-IgG technique is more specific; hence, their combination will improve diagnosis.

According to our proposal, positivity of one or two immunological techniques indicates the need for confirmation by the COPT, which, despite being more difficult to perform, can identify active infections, or by the molecular reactions, which can identify the presence of the parasite at any stage of infection.

The possibility of the use of rapid tests with recombinant antigens [76] or with Circulating Anodic Antigen (CAA) in urine, feces, or serum [77–80] might be an interesting alternative for field works in places where health workers do not have the proper training and equipment to perform sophisticated serological assays.

However, continued use of the parasitological techniques will be important, in particular the HH technique, as it permits the evaluation of the parasite load in positive cases and allows observation of different levels of prevalence as well. Besides the evaluation and treatment, the infection risk factors must be taken into account.

We believe that maintaining this level of surveillance in ALEs will present a great challenge and will require evaluation of costs, interactions with other studies, and finally a broad discussion involving the various actors in the context of public health, research, and society.

In Brazil, recent epidemiological data on *S. mansoni* point to an increase in ALEs, and the current proposal aims to interrupt the transmission of this helminth (Ordinance number 2,556 of 28 October 2011). In this context, diagnosis of *S. mansoni* becomes a strategic hurdle we must overcome

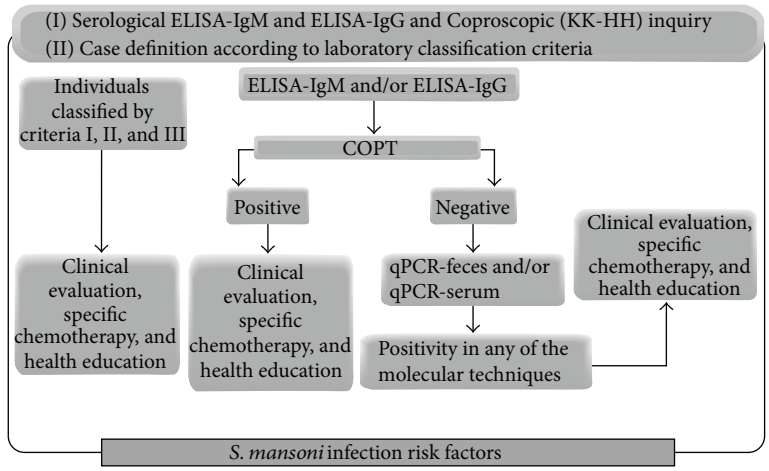


Figure 5: Proposal for epidemiological vigilance in ALEs.

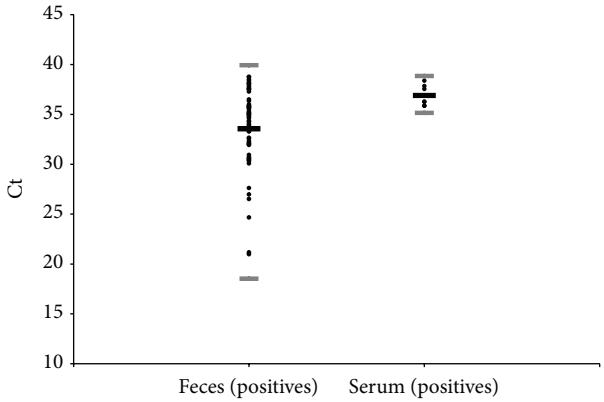


Figure 6: Description of the threshold cycle (Ct) for the results from the positive and qPCR in serum and feces samples from the individuals sampled from the city of Barra Mansa/RJ, 2011.

in order to reach the desired result indicators in the process of eliminating this parasitic infection. In addition, the development of new diagnostic laboratory techniques for detecting low parasite load infections represents an important strategy to overcome this need, in clinical contexts as well as in public health.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contribution

Maria Cristina Carvalho do Espírito-Santo, Mónica Viviana Alvarado-Mora, Pedro Luiz Silva Pinto, Expedito José de Albuquerque Luna, and Ronaldo Cesar Borges Gryschek conceived and designed the experiments. Maria Cristina Carvalho do Espírito-Santo, Mónica Viviana Alvarado-Mora, Emmanuel Dias-Neto, Pedro Luiz Silva Pinto, Maria Carmen Arroyo Sanchez, João Renato Rebello Pinho, Vera Lúcia Pagliusi Castilho, and Elenice Messias do Nascimento Gonçalves performed the experiments. Maria Cristina Carvalho do Espírito-Santo, João Renato Rebello Pinho, Flair José Carrilho, Expedito José de Albuquerque Luna, Emmanuel Dias-Neto, and Ronaldo Cesar Borges Gryschek contributed with reagents/material/analysis tools. Maria Cristina Carvalho do Espírito-Santo, Mónica Viviana Alvarado-Mora, Expedito José de Albuquerque Luna, Maria Carmen Arroyo Sanchez, Pedro Luiz Silva Pinto, João Renato Rebello Pinho, Emmanuel Dias-Neto, and Ronaldo Cesar Borges Gryschek wrote/edited the paper. All authors read and approved the final paper.

Acknowledgments

This study was supported by the São Paulo Research Foundation (Grants 07/53457-7, 08/50461-6, and 10/52615-0). The authors would also like to thank Ms. Maria Cristina Conceição de Mello (LIM-06-FMUSP) for her excellent technical assistance concerning the maintenance of the *S. mansoni* biological cycle. Emmanuel Dias-Neto and João Renato Rebello Pinho are research fellows of the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). Finally, they would like to thank Dr. Wilton Neri and staff of the Municipal Health Department of Barra Mansa, Rio de Janeiro, Brazil.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 428205, 9 pages
http://dx.doi.org/10.1155/2015/428205

Review Article

Serological Diagnosis and Follow-Up of Human Cystic Echinococcosis: A New Hope for the Future?

Raúl Manzano-Román,[1] Carlos Sánchez-Ovejero,[1] Ana Hernández-González,[2] Adriano Casulli,[3] and Mar Siles-Lucas[1]

[1]*Instituto de Recursos Naturales y Agrobiología de Salamanca (IRNASA-CSIC), Cordel de Merinas 40-52, 37008 Salamanca, Spain*
[2]*Instituto de Salud Carlos III, Centro Nacional de Microbiología, Majadahonda, 28220 Madrid, Spain*
[3]*Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy*

Correspondence should be addressed to Adriano Casulli; adriano.casulli@iss.it

Received 13 April 2015; Accepted 30 August 2015

Academic Editor: Malcolm Jones

Cystic echinococcosis (CE) is an important helminthic zoonotic disease caused by the *Echinococcus granulosus* complex. In humans, CE is a chronic disease driven by the growth of echinococcal cysts in different organs. Prognosis of this disease depends on multiple factors, including location, number, size, and stage of the cysts, making CE a disease of complex management. CE is usually asymptomatic for years and attracts limited attention from funding organizations and health authorities. For this reason, only experts' recommendations are available but no evidence-based conclusions have been drawn for CE clinical management. One of those pitfalls refers to the lack of evidence to support the use of serological tools for the diagnosis and follow-up of CE patients. In this respect, crude antigens are used to detect specific antibodies in patients, giving rise to false positive results. The advent of molecular techniques allowing the production of recombinant proteins has provided a number of candidate antigens that could overcome the problems associated with the use of crude parasite extracts in the serological assays. In this review, we present the last advances in this field, proposing the use of serology to support cyst stage-specific diagnosis and follow-up.

1. Introduction

Cystic echinococcosis (CE) is a parasitic disease caused by the larval stage (metacestode) of *Echinococcus granulosus* complex which affects livestock, wildlife, and humans. CE has a worldwide geographic distribution, remaining highly endemic in many pastoral communities, including several European countries [1]. CE global prevalence is estimated at 2-3 million human cases and a burden of 1 million DALYs accounting for underreporting [2]. In humans, CE is a chronic disease characterized by the long term growth of hydatid cysts in internal organs, mainly liver and lungs, with a complex clinical management. CE results in severe and life-threatening complications, with estimated mortality rates of 2–4% per 100.000 inhabitants [3, 4]. Many CE cases are asymptomatic for years and its diagnosis is still challenging due to the absence of pathognomonic signs. For this reason CE is frequently underdiagnosed and detected only when complications arise or by chance. Additionally, the clinical management of CE (i.e., surgery, percutaneous treatment, and/or chemotherapy) has many associated risks for relapses, pointing out the importance of the follow-up of patients.

CE diagnosis and monitoring firstly rely on imaging techniques. Ultrasonography (US) standardized classification of stage-specific cystic images has been issued by the WHO Informal Working Group on Echinococcosis (WHO-IWGE) for the diagnosis and the clinical management of CE [5]. Effective serological tests for CE diagnosis would be of great help to define and support cyst status and their evolution (active: CE1, CE2, and CE3b, transitional: CE3a, or inactive: CE4 and CE5) [5, 6]. The main serological methods used for human CE diagnosis and follow-up are based on the detection of specific IgG antibodies. In this context, a number of drawbacks have been detected, including low sensitivity/specificity (Se/Sp) and a poor prognostic value for follow-up due to the long-lasting persistence of antibodies

against hydatid fluid (HF) [7]. These pitfalls lead clinicians to consider serology against HF as an approach of little value, with doubtful benefit for the clinical management of CE. Alternative methods based on the detection of other antibody isotypes and IgG subisotypes against HF have been published [8]. Additionally, many authors have focused their research both on recombinant proteins and on synthetic peptides, to develop more sensitive and specific tests. Numerous recombinant proteins (Rec) and related peptides, mainly derived from the antigen B and antigen 5, have been tested for the detection and follow-up of antibodies in correlation with US findings. Unfortunately, available data were generated from small and underpowered clinical studies that have showed dissimilar Se and Sp for the same recombinant antigen [9]. Nevertheless, there are hints showing that some antigens are differentially expressed in different cyst stages, and thus antibody levels against these antigens could be associated with cyst activity and posttreatment outcome (i.e., surgery or chemotherapy) and could be applied for diagnosis and follow-up of CE patients [9, 10]. In this context, a better characterization and standardization of each antigen should be performed to clearly define its role within CE serology.

In this paper we summarize the current knowledge on the use of HF for human CE diagnosis. Additionally, results obtained from different purified fractions of parasite antigens, recombinant antigens, and synthetic peptides are also revised. A comprehensive review of the different available antigens and their performance in the diagnosis of CE was published by Carmena and colleagues [11]. In this review, we also update the findings about the available serological tools from 2006 to date.

2. Hydatid Fluid

HF is a complex mixture of parasite-derived proteins, mainly produced by the germinal layer of the cyst. Some of the HF components have been characterized as highly immunogenic, reaching the host environment and triggering antibody responses. The HF is the main antigenic component in the majority of commercially and in-house serological assays. This antigen mixture is used in several techniques such as the enzyme linked immunosorbent assay (ELISA), the indirect haemagglutination test (IHA), and the immunoblotting (IB). Both the ELISA and the IHA are usually the first line tests for CE patients, while the IB is used as confirmatory test. As mentioned, the use of HF for the detection of CE specific antibodies is limited by several drawbacks. First, a percentage of CE patients are serological negative against HF. Specifically, the use of HF for the detection of total IgG in ELISA test leads to variable results regarding Se and Sp. In Table 1, a number of recent studies that used IgG-ELISA tests for CE diagnosis are shown. Se reported in these studies varied from 64.8% to 100%. Reasons for false negative results depend on several factors comprising cyst location other than the liver [12, 13], early (CE1) and inactive (CE4 and CE5) cyst stages [14–16], serum collection before treatment [15, 17], single and small cysts [17], and HF antigenic source variability [18]. Additionally, *E. granulosus* complex comprises several genotypes that potentially express different antigenic sets. For instance, it has been shown that the G1 and G2 genotypes from Europe, contrary to those from China, express high quantities of antigen B2 in HF [19]. This may lead to different diagnostic performance of a specific HF. The differential expression of antigens is not only a qualitative matter, but also quantitative, depending on cyst stages. Recently, a proteomic and immunoproteomic study has shown that CE1 and CE2 cyst stages differ in the expression of their immunodominant antigens (antigen B and antigen 5). Antigen 5 is predominant and recognized by antibodies from patients with early cyst stages, while antigen B is the most scarce in CE1 cyst stage and mainly detected in patients with CE2 and CE3 cyst stages [20]. These differences might be useful in clinical practice to correctly define cyst stages and their viability by using the most indicated antigen for a stage-specific diagnosis. A second problem using HF is the percentage of false positive results detected. For instance, IgG-ELISA tests based on HF as antigen give rise to low false positive results in healthy donors (e.g., Sp from 87.5% in Indian donors to 100% in Italian donors) [15, 21]. Cross-reactivity is quite high in patients with other parasitic diseases, such as alveolar echinococcosis (AE) and cysticercosis, but also schistosomiasis and fascioliasis [22, 23]. For instance, the cross-reactivity of *E. granulosus* HF with antibodies from AE patients can reach more than 50% [17]. The HF has also been shown not to be a good antigen for patients' follow-up during the clinical management of CE. During the follow-up, ELISA-IgG test is difficult to interpret [24], and anti-HF IgG antibody reactivity may remain high many years after successful cyst removal [25].

The detection of antibodies other than IgG has shown some promising results in relation to cyst activity, relapses, and follow-up. It has been shown that both IgG2 and IgG4 could be related to cyst stages, disease evolution, and relapses [9, 26, 27]. Remarkably, it is known that the subisotype responses against CE1, CE2, and CE3 cyst stages are mainly IgG4, while IgG1, IgG2, and IgG3 responses predominate against CE4 and CE5 cysts, although this is still a question of debate [11, 22]. Antibody isotypes different than IgG can be also detected against HF in CE patients. IgE and IgM antibodies have been considered as better markers than IgG after chemotherapy and surgery [28]. Nevertheless, these isotypes are more frequently underdetected in CE patients, similar to different IgG subisotypes [29–31].

3. Antigens Derived from Hydatid Fluid

3.1. Antigen B. In an attempt to overcome the problems related to the use of HF for the detection of antibodies in CE patients, many authors have described the production and the use in serological tests of partially purified native antigens, recombinant antigens, and synthetic peptides. These are mainly represented by the two most immunogenic antigens in HF: antigen B (EgAgB) and antigen 5 (EgAg5).

EgAgB is a polymeric protein of 120–160 kDa that dissociates under reducing conditions in 8, 16, and 20–24 kDa subunits. Its biological role includes the protease inhibitor activity, neutrophil chemotaxis inhibition, triggering of non-protective Th2 responses, induction of apoptosis of immune cells, and sequestration of xenobiotics [22]. EgAgB is codified

Table 1: Performance of the hydatid cyst fluid in ELISA test for the detection of total IgG in CE patients (articles published from 2006).

Number of CE patients	Confirmatory test	Sensitivity (%)	Negative serology more frequent when	Reference
23	Histopathology	100	Not specified	[40]
5	Imaging techniques plus serology	80	Not specified	
13	Serology	100	Not specified	
41	Surgery	95.1	Not specified	[21]
6	Imaging techniques	66.7	Cyst location other than liver	[12]
144	Imaging techniques	92.4	CE1, CE4, and CE5 cyst stages	[14]
123	Imaging techniques	64.8*	CE4 and CE5 cyst stages, no pretreatment	[15]
59	Surgery	95.1	Not specified	[20]
10	Surgery	100	Not specified	[52]
54	Surgery	81.5	Single cyst, no pretreatment	[17]
186	Surgery	83.3		
32	Imaging techniques	93.8	CE4 and CE5 cyst stages	[16]
155	Surgery	90.3*	Not specified	[29]
40	Surgery	92.5	Not specified	[30]
47	Surgery	95.7[1] 93.6[2] 91.4[3] 97.8[4] 93.6[5] 78.5[6] 72.2[7]	HF collected from cysts of different hosts or of different anatomical locations from the same host	[18]
63	Surgery	90.5 82.4*	Not specified	[48]
68	Imaging techniques	92.6	Lung cysts	[13]

*Commercial test; HF of cysts from [1]sheep liver, [2]sheep lungs, [3]goat liver, [4]human liver, [5]camel lungs, [6]cow lungs, and [7]cocktail.

by a multigenic family, with at least five genetic groups: EgAgB1, EgAgB2, EgAgB3, EgAgB4, and EgAgB5 [32–35]. These different subunits share from 44% to 81% of sequence identity. Each isoform has more than 90% homology with *Echinococcus granulosus* and *E. multilocularis* species, and although lower, similar antigens are also found in the genus *Taenia* [11]. These homologies can give rise to cross-reactivity with other helminthic parasites. EgAgB has been obtained as a native purified antigen, in its recombinant form. The purified native protein has a Se ranging from 60% to 85% in ELISA test and from 60% to 92% in immunoblot [11]. From 2006 up to date, several publications showed a high variability in the Se of this purified antigen, ranging from 54% to 100% (Table 2). Different cyst stages and their follow-up in US may reflect different profiles of antigens produced and released by the host immune system [20]. For instance, EgAgB recombinant antigen, in ELISA test, showed a Se of 74%, 96%, 90%, and 56% in patients with CE1, CE2, CE3, and CE4/CE5 cyst stages, respectively [36]. Additional reasons for false negative results have been pointed out by several authors, including cyst localization other than liver and small size of cysts (see Table 2). These results are similar to those found for false negative reactions against HF. The heterogeneity and thus the limited value of native antigens are also found when the purified EgAgB is used for the serodiagnosis of CE patients. In this respect, antigens purified from different sources of HF result in a variable Se in the same patients' cohort (from 82.1% to 96.9%; [37]; Table 2).

In its recombinant form (Rec), mainly two isoforms have been assayed: RecEgAgB1 and RecEgAgB2. RecEgAgB1 gives rise to variable sensitivities, ranging from 55% to 84% [11] and from 71% to 94.6% in subsequent studies [38] (Table 2). The lowest Se (71%; [38]) was attributed to false negative results due to patients with *Echinococcus* genotypes other than G1. Nevertheless, additional factors such as the presence of CE4 and CE5 cyst stages and serum sampling collection with respect to treatment are probably contributing to false negative results against this recombinant antigen [15]. Some of these factors have been also pointed out for RecEgAgB2, for which false negatives have been related to single cysts and to sera collected before treatment with benzimidazoles [17]. RecEgAgB2 has given rise to very variable sensitivities as well ([11]; Table 2). A modified version of the RecEgAgB2, consisting of twofold tandem repeat of the original recombinant protein, showed a Se similar to the single antigen unit [17]. Additional isoforms of EgAgB have been tested on few occasions (Table 2). RecEgAgB3 and B5 did not improve the Se of other antigenic isoforms [19], but RecEgAgB4 has shown promising results (Se from 75.8% to 91.7%) [19, 39]. Remarkably, EgAgB3 and B5 were not found after a proteomic

Table 2: Use of antigen B and derivatives for the diagnosis of CE patients (articles published from 2006).

Antigen	Number of CE patients	Confirmatory test	Technique, antibody	Sensitivity (%)	Negative serology more frequent when	Reference
Purified	21	Surgery	Immunochromatography, IgG Immunochromatography, IgG4	100 95	Not specified	[42]
	23	Surgery		100		
	5	Imaging techniques and serology*	ELISA, IgG	80	Not specified	[46]
	13	Serology*		0		
	32	Imaging techniques	ELISA, IgG	93.8	CE4 and CE5 cyst stages	[16]
	40	Surgery	ELISA, IgG ELISA, IgG4	87.5 80	Not specified	[30]
	108	Surgery	DIGFA, IgG	89.8	Cyst location other than liver; CE1, CE4, and CE5 cyst stages; small cysts	[43]
	113	Imaging techniques	DIGFA, IgG	92.9	Not specified	[44]
	35	Imaging techniques	ELISA, IgG	54	CE1, CE4, and CE5 cyst stages	[63]
	56	Surgery	ELISA, all (Protein G)	96.9[1] 82.1[2]	Not specified	[37]
Recombinant B1	31	Surgery	ELISA, all (Protein G)	71	Parasite genotype other than G1	[38]
	124	Surgery[$]	ELISA, IgG	83	Not specified	[19]
	246	Imaging techniques	ELISA, all (Protein G)	77.6	Not specified	[64]
	113	Imaging techniques	DIGFA, IgG	77.9	Not specified	[44]
	56	Surgery	ELISA, all (Protein G)	94.6	Not specified	[37]
	123	Imaging techniques	ELISA, IgG	73.9	CE4 and CE5 cyst stages, no pretreatment	[15]
Recombinant B2	54[3] 186[4]	Surgery	ELISA, IgG	77.8 79	Single cyst, no pretreatment	[17]
	124	Surgery[$]	ELISA, IgG	62.9	Not specified	[19]
Recombinant 2B2	54[3] 186[4]	Surgery	ELISA, IgG	92.6 87.6	Single cyst, no pretreatment	[17]
Recombinant B3	124	Surgery[$]	ELISA, IgG	29	Not specified	[19]
Recombinant B4	124	Surgery[$]	ELISA, IgG	75.8	Not specified	[19]
	36	Surgery	ELISA, IgG	91.7	Not specified	[39]
Recombinant B5	124	Surgery[$]	ELISA, IgG	41.3	Not specified	[19]
P176 peptide	61	Surgery	ELISA, IgG	78.7	Lung cysts, no complications, single cyst, and small cysts	[48]
	63	Surgery	ELISA, IgG	23.8	Not specified	[50]
Long D8-9 peptide	35	Imaging techniques	ELISA IgG	74.3	Not specified	[41]

*Positive serology against HF in ELISA IgG, [1]EgAgB purified from a Chinese sheep isolate, [2]EgAgB purified from a Iranian sheep isolate, [$]patients selected by their positivity in ELISA IgG against HF, [3]Spanish patients, and [4]Peruvian patients.

study on the composition of the HF in CE1 and CE2 cyst stages from sheep [40].

As mentioned, several synthetic peptides derived from EgAgB1 have been also tested in ELISA for the detection of specific antibodies. From those tests, the most promising results have been obtained with the p176 peptide ([11]; Table 2). Nevertheless, the diagnostic performance of synthetic peptides has been worse than that of the whole original molecule, either in its purified native or in recombinant forms. In addition, pooling three peptides has not resulted in the improvement of the test Se [41]. In any case, the use of bioinformatics for peptides selection and their screening in high-throughput platforms have been stated as a proof of principle and this approach could be used in the future for the identification of relevant diagnostic epitopes. As mentioned, a dominant IgG4 response is usually associated with active and transitional cysts, whereas inactive cysts mainly trigger IgG1, IgG2, and IgG3 responses. This has important implications in the interpretation of the usefulness of the detection of different IgG isotypes, since the level of reactivity of each subisotype could be driven by the cyst stages found in patients. This is the case for IgG4, since the Se obtained when detecting this subisotype is lower than that detected when total specific IgG levels are evaluated [42]. Whether this lower Se is associated with inactive cysts, it should be further evaluated to ascertain the usefulness of IgG4 instead of total IgG, both for the diagnosis and for the follow-up of CE patients. The Sp of EgAgB in its different isoforms is also variable, although the main cross-reactivity has been reported with patients affected by AE and cysticercosis [11]. Additionally, antibodies from patients with other parasitic diseases including onchocerciasis, schistosomiasis, and toxocariasis have given rise to false positive reactions when tested against EgAgB [11]. It is worth mentioning the efforts of several authors to test new affordable and easy to use serological techniques instead of ELISA or the IB tests, making those immunoassays stand out based on particles such as DIGFA (dot immunogold filtration assay [43, 44]; Table 2).

In the light of the results obtained by several authors using EgAgB, this antigenic family looks promising for the serodiagnosis of CE patients and potentially useful in the follow-up. Anyway, testing of this antigen family obtained in a standardized format, with a well-defined and wide panel of CE samples, including the clinical information influencing the interpretation of the test (the parasite genotype, location, number, size, and type of cysts, and presence of complications and coinfections), should be further performed.

3.2. Antigen 5. Antigen 5 (EgAg5) is also an abundant highly immunogenic component of HF. EgAg5 is a thermolabile protein of around 400 kDa, composed of two subunits of 57 and 67 kDa. Studies on the N-terminal sequence of the 38 kDa subunit have shown that several isoforms exist similar to EgAgB, and thus EgAg5 could be also coded by a multigenic family [45]. The biological function of EgAg5 is largely unknown, and although the 38 kDa subunit shows homologies with trypsin peptidases, the enzymatic activity is lacking [46]. Additionally, the smallest subunit of 22 kDa contains proteoglycans of the heparan sulfate group and calcium-binding motifs, suggesting that this subunit could interact with the extracellular matrix and the cell surface [47].

Molecules very similar to EgAg5 (more than 90% identity) are also expressed by *E. multilocularis* and the genus *Taenia*; thus cross-reactivity of this antigen with antibodies from patients affected by AE and cysticercosis is expected. As we explain in the following paragraphs, the diagnostic use of EgAg5 has shown mainly drawbacks regarding Se and Sp. The purified native EgAg5 has been obtained using different methodologies, including size exclusion chromatography and immunoaffinity. Lately, an easy and efficient method for the enrichment of this antigen from HF has been described [48]. Variability of its performance in serodiagnosis could be also attributed to differences in the clinical status of the patients. For instance, EgAg5 seems to play a role in the induction of specific antibodies mainly when CE1 and CE5 cyst stages are present [40]. This variable Se has been reported by several authors, ranging from 50% to 87.5% [11, 49]. As recombinant proteins, EgAg5 has been tested in its full format (RecEgAg5) and in its 38 kDa version (RecEgAg53.38) by Auer and colleagues [50]. Although the 38 kDa subunit has been described as the most antigenic component detected by IB, the Se of the corresponding recombinant antigen was very low (21%) [50, 51]. Similarly, low and variable sensitivities (from 16% to 85%) have been detected using the p89-122 EgAg5 peptide [11]. Due to the low Se of EgAg5 and derivatives, this antigen has been less frequently tested than EgAgB. Nevertheless, reasons for this low Se could be due to cyst stage; thus the usefulness of this antigen for the detection of early active and late inactive cyst lesions should be further evaluated.

3.3. Other Antigens. The most frequently used crude extract of *E. granulosus* for the serodiagnosis of CE has been the somatic extract of protoscoleces (EgPpsSom). This extract has a Se ranging from 69.4% to 96.9% [11, 16, 43, 44, 52–54]. When compared with HF, the EgPpsSom performs worse. Less frequently, other somatic extracts have been applied in the serodiagnosis of CE patients such as those derived from the cyst wall (96.7% Se; [52]), the tegument of protoscoleces (81.3% Se; [16]), and the adult worm (82% Se; [55]). Cross-reactivity of those extracts is similar to that detected in HF. Drawbacks associated with the heterogeneity of somatic extracts could be attributed to these antigenic preparations. In addition, reasons for the presence of false negative results are similar to those found when the HF is used as antigenic source (cyst location other than liver, small size cysts, and CE1, CE4, and CE5 cyst stages).

Additionally, several recombinant antigens different from EgAgB and EgAg5 have been obtained by several authors and tested for the diagnosis of CE. These include, among others, the malate dehydrogenase (RecEgMDH), the calcium binding protein (RecEgCaBP), the actin filament fragmenting protein (RecEgAFFP), the RecEgEpC1, the thioredoxin peroxidase (RecEGTPx), and the RecEg19 [11, 56, 57]. RecEGTPx and RecEg19 showed low Se (<45%; [56–58]). The rest of the above-mentioned antigens showed variable Se (even for the same recombinant) ranging from 45% (MDH) to 90%

(MDH, EpC1) and variable Sp (even for the same antigen) ranging from 83% to 95% [11]. For this reason these antigens could be considered as good alternatives for serological tests, although further characterization is needed to evaluate their diagnostic potential.

4. Antigens for Clinical Management

The clinical management of CE should be based on recommendations done by experts and driven by cystic stages identification [54]. In this context, the WHO-IWGE has proposed a cyst classification based on active, transitional, and inactive cysts.

Some antigens could be mainly expressed by defined cyst stages, showing that serology could be potentially useful for the definition of cyst activity and thus for the clinical management of CE patients. As mentioned, the HF has been also evaluated for the follow-up of patients treated by surgery and/or chemotherapy, but its usefulness is hampered by the long persistence of antibodies in patients with nonactive cysts [59]. A better correlation of specific antibodies against defined active cyst stages has been reported in patients after surgical treatment, including EgAgB [59, 60], EgAgP29 [60], and the heat-shock protein 20 [61]. Several authors have also found a correlation between a low level of specific antibodies against RecEgAgB2, RecEgAgHSP20, and RecEgAgB1 and the presence of inactive cysts [36, 59, 61]. Nevertheless, some of those antigens are not recognized by a percentage of CE patients [60]. Additionally, it has been shown that the banding pattern in IB from CE patients changes depending on the cyst stage [62].

Similarly, other authors have detected the loss of defined bands in IB after cure. The identification and characterization of the above-mentioned antigens could support the clinical decision making actually based on imaging techniques. Similarly, the shift of defined subisotypes of IgG antibodies during cyst evolution could be used for the follow-up of CE patients. As mentioned, IgG4 levels could be correlated with cyst activity, and declining of the levels of isotypes other than IgG seems to be useful to define the success of treatment [26, 27]. Nevertheless, detectable levels of specific IgG4 and especially of IgE and IgM are only found in a relative small percentage of CE patients.

5. Conclusions: A New Hope for the Future?

Diagnosis and follow-up of CE patients are mainly based on imaging techniques. These can be used for the identification of cystic stages, leading to a stage-specific approach in CE clinical management. Serological tools supporting imaging techniques would be desirable. However the available tests are based on antibodies against crude antigens and thus marred by poor Sp and Se, with low or no usefulness for the follow-up of patients during the treatment. Specific recombinant antigens have good potential as diagnostic and follow-up tools for CE, but progress in this field is hampered by lack of standardization. Thus, a challenge still exists to develop a reliable world standard based on serology for the diagnosis and monitoring of CE patients. In this respect, a multicentre study with a wide panel of sera from CE patients, including relevant clinical data to properly define the usefulness of the available recombinant antigens, should be performed. Along this path, a project focusing on CE has been recently funded by the European commission under the Seventh Framework Programme (HERACLES; http://www.heracles-fp7.eu/). In the framework of HERACLES, six recombinant antigens (RecEgAgB1, RecEgAgB2, RecEgAg5, RecEgAgMDH, RecEgAgCaBP, and RecEgAgAFFP) will be tested for the serodiagnosis and follow-up of CE on a wide panel of samples obtained from extended ultrasound surveys in Eastern Europe. These recombinant antigens have been already produced in a standardized way and preliminary tested in ELISA for the detection of specific IgG in sera. Additionally, two databases for the collection of clinical data from retrospective/prospective patients providing serum samples have been developed (the database behind the European Register of Cystic Echinococcosis ERCE, http://www.heracles-fp7.eu/erce.html, and CYSTRACK database, http://cystrack.irnasa.csic.es). Hosting of samples and clinical data has been organized in a dedicated biobank (EchinoBiobank). This strategy will hopefully pave the way to improve the diagnosis and follow-up of CE patients, providing evidence-based data on the usefulness of serology in CE clinical management.

Disclosure

The funding sources had no involvement in the preparation, ideas, writing, interpretation, or the decision to submit this paper.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The research that will lead to these results has received funding from the European Community's Seventh Framework Programme under the Grant agreement 602051 (Project HERACLES: Human cystic Echinococcosis ReseArch in CentraL and Eastern Societies; http://www.Heracles-fp7.eu/). The authors are grateful to Belgees Boufana for reviewing the text.

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